Makesh M.
Rajendran K. V. *Editors*

# Fish immune system and vaccines

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# Preface

Aquaculture, being the fastest growing food producing sector, has grown leaps and bounds in the last few decades. The world aquaculture production reached 82 million tonnes, valued at USD 250 billion in the year 2018. However, intensification and diversification of aquaculture have resulted in disease outbreaks, leading to significant production losses globally. To counter the disease problems in aquaculture, as the use of chemotherapeutants is discouraged due to its possible impact on the environment, vaccination has emerged as a promising prophylactic measure. Although vaccination is a very common practice in human and animals, fish vaccination is being practised only for a few species in some of the countries. Nevertheless, to develop new prophylactic measures to prevent or manage diseases in aquaculture, a better understanding of the immune system of farmed aquatic species and their response to pathogens and vaccination is essential.

Over the past few decades, there has been tremendous progress in the understanding of the fish and shellfish immune system and their mechanisms. Further, considerable progress has been made in understanding their response to infection and immunization, and several vaccines have been developed and used successfully during the past 50 years to curtail bacterial and viral diseases of finfish. Use of vaccines against bacterial diseases has reduced the use of chemotherapeutants considerably. Fish vaccination strategies are continuously evolving with the development of alternative routes for easy administration and development of improved adjuvants for increased vaccine efficacy. Considering the importance of a comprehensive resource material on fish immune system and vaccines especially for postgraduate students specializing in this area, a 'writeshop' was organized by ICAR–Central Institute of Fisheries Education, Mumbai, during 24–25 April 2015 to bring out study materials, especially review articles, contributed by established researchers on various aspects of fish immune system and vaccines. This book primarily focuses on finfish immune system and vaccines against bacteria, viruses and parasites infecting farmed finfish. However, two major chapters are included, which provide detailed information on crustaceans with a focus on shrimp, which contributes ~22% in terms of value in the international trade of fish commodities. The book also provides comprehensive information on different types of vaccines used in aquaculture, methods of vaccine delivery, use of biofilm as oral vaccine, application of RNA interference in fish vaccination, role of pattern recognition

receptors and interferons in fish vaccination, methods for evaluating the efficacy of vaccines, biosafety and adverse effects of fish vaccines. We hope that this book will be a useful resource material for students, academicians, researchers, aquaculture professionals and other stakeholders.

We would like to express our gratitude to the Director, ICAR–Central Institute of Fisheries Education for the financial support to conduct the 'writeshop'. We are also immensely grateful to all the contributors for their unstinted support in bringing out this book.

Finally, we, on behalf of the contributors, thank the publisher Springer Nature for their highly professional support in publishing this book.

Chennai, India Makesh M.
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# About the Editors

**Makesh M.** obtained his doctorate from Madras Veterinary College, Chennai, India on crustacean virology. He joined ICAR–Central Institute of Fisheries Education, Mumbai in 1999 and worked on fish and shrimp diseases and their diagnosis. He was awarded the Department of Biotechnology, Cutting-Edge Research Enhancement and Scientific Training (CREST) Award. He did his postdoctoral research at University of Idaho, Moscow, USA on fish mucosal immunity during 2013–2014. Later, he moved to ICAR–Central Institute of Brackishwater Aquaculture, Chennai in 2015. Currently, Dr. Makesh is a Principal Scientist at Fish Culture Division of Central Institute of Brackishwater Aquaculture. He has more than 70 peer-reviewed research publications, three edited books, and several conference presentations to his credit. He has developed an injectable recombinant viral nervous necrosis vaccine and is currently working on technologies for mucosal delivery of fish vaccines.

**Rajendran K. V.** graduated from the University of Calicut. He joined ICAR–Central Institute of Brackishwater Aquaculture, Chennai, as Scientist in 1993. In 1998, he moved to ICAR–Central Institute of Fisheries Education, Mumbai as Senior Scientist. He was awarded the Korea Science and Engineering Foundation (KOSEF) postdoctoral fellowship and worked at Yeosu National University, South Korea (2000–2001). In 2003, he worked as Fish Pathologist at CSIRO, Australia (2003–2006). He was awarded Fulbright-Nehru Senior Research Fellowship in 2010 and worked at Auburn University, USA. Currently, Dr. Rajendran is a Principal Scientist and Head, Division of Aquatic Environment and Health Management, ICAR-CIFE, Mumbai. He has published more than 100 research papers in peer-reviewed journals, apart from various other publications and contributions to various national and international seminars and conferences. He is one of the Editors of the journal *Virus Disease* (Springer) and Editorial Board Member of *Fish and Shellfish Immunology Reports* (Elsevier).

# Overview of Fish Immune System

Makesh M., Megha K. Bedekar, and Rajendran K. V.

**Abstract**

Fish immune system can be categorized into innate and adaptive immune systems. The innate immune system comprises physical barriers such as mucus, skin, gill, gut and nostril epithelium, effector molecules such as antimicrobial peptides, interferons, complement proteins, natural antibodies, growth inhibitors, protease inhibitors and cells such as macrophages, monocytes, non-specific cytotoxic cells (NCC) and neutrophils. The innate immune molecules are generally heat labile and are more active at lower environmental temperature ranges. The onset of innate immune response occurs early in the life of teleosts than the adaptive immune response. The adaptive immune system comprises lymphoid organs such as head kidney, thymus, spleen and mucosa-associated lymphoid tissue and, the effector molecules comprise IgM, IgD and IgT, while the cells involved in the adaptive immunity are B and T lymphocytes. The age at maturation of the adaptive immune system of fish varies from species to species. Although the innate immunity is more diverse in fish, protection offered by adaptive immune system is also significant, especially when stimulated by vaccination. The adaptive immune response is modulated by various factors such as temperature, age, stress and route of vaccine administration and, hence, a better understanding of the immune system will help to formulate prophylactic measures such as vaccination to prevent disease outbreaks in aquaculture farms.

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**Keywords**

Innate and adaptive immune system · IgM · IgD · IgT · Humoral immunity · Cell-mediated immunity

## 1 Introduction

Adaptive immunity, characterized by lymphocytes bearing antigen receptors generated by somatic recombination, developed first in jawed fish about 500 million years ago [1]. Fish immune system has been studied, often, in comparison with the mammalian counterpart, as they share many similarities. However, the fish immune system differs from the mammalian system in having limited immunoglobulin diversity, predominant and diverse innate immune components, absence of isotype switching etc. Further, fish lack lymph nodes and bone marrow, and head kidney takes the role of bone marrow in producing lymphocytes and erythrocytes. Moreover, the immune system varies widely among fishes, unlike mammals, and, hence, it is very difficult to generalize the structure and function of the immune system of fish.

The immune system of teleosts, as in other vertebrates, possesses two arms, innate and adaptive. The innate immune system is inborn and the response to pathogen invasion is very rapid and often happens in minutes. The response is, often, not specific to the pathogen and is a generalized action against all foreign objects. On the contrary, the acquired immune system responds specifically against the pathogen which triggered the immune response. The response is mostly mediated through immunoglobulins and cytotoxic T cells and takes a few to several weeks before it can be detected. Recognition of the pathogen/antigen and development of memory cells that can proliferate rapidly upon subsequent exposure to the pathogen are the hallmarks of the acquired immune system [2].

## 2 Ontogeny of Fish Immune System

Proper understanding of the ontogeny of immune system is essential to ascertain the immunological capacities of different life-stages of an organism. This includes not only the morphological features but also the first lympho-haematopoietic sites and the origin and distribution of T- and B-lymphocytes. However, information on the ontogeny of immune system of teleosts is limited to a few species such as rainbow trout, zebrafish, European seabass, catfish, etc. In vertebrates, blood cells and haematopoietic organs develop from ventral and dorsal mesoderm. In teleosts, initial haematopoiesis occurs in the yolk sac and intraembryonic intermediate cell mass [3]. By 12 h post-fertilization (hpf), haematopoietic progenitors can be seen in the intraembryonic region. In hatched-out larvae, the major haematopoietic organs are the anterior kidney, spleen and thymus. The haematopoietic tissue appears in the kidney 4 days post-fertilization (dpf) and mature eosinophils appear 6 dpf [3].

In freshwater teleosts, thymus is the first lymphoid organ to possess lymphocytes, though haematopoietic progenitors can be present in kidney earlier than this. However, in marine teleosts, lymphocytes develop in kidney and spleen before thymus [3]. In all the teleosts, the thymic epithelium is continuous with the pharyngeal epithelium and, in zebrafish, the thymic primordial cells appear 60 hpf lining the pharyngeal cavity. Lymphoblasts colonize thymus 65–75 hpf and the thymus is fully developed by the 4th day. By 3 weeks post-fertilization, the thymus develops and differentiates into cortex and medulla [3]. Although involution of thymus is observed at the time of sexual maturity of zebrafish (around 15 weeks), thymus is reported to be active till 2 years of age [4].

In teleosts, pronephros (head kidney) is the first organ to produce B-lymphocytes. Transcripts of recombination activating gene 1 (RAG-1), which are responsible for the V(D)J recombination in B and T lymphocytes, appear in a few cells of pronephros by 2 weeks post-fertilization and, by 3–4 weeks, cluster of cells become positive for RAG-1 transcripts [3]. Immunoglobulin (Ig)M, the major immunoglobulin of teleosts, and complement proteins appear first in yolk sac suggesting maternal transfer of immunity. However, IgM and B cells that secrete IgM appear several days (12–14) post-hatch and vary with species.

## 3 Innate Immune System

### 3.1 Organs of the Innate Immune System

#### 3.1.1 Skin

Skin is considered as the largest immunologically active organ. The skin along with the scales and mucus form the primary barrier preventing the entry of pathogens and parasites. The mucus secretion increases in conditions of stress, infection and chemical irritants. The mucus is periodically sloughed off removing majority of the parasites and pathogens. The mucus also contains many proteins and peptides viz., lysozyme, proteases, antimicrobial peptides, lectins, lactoferrin and immunoglobulins which have antimicrobial and antiparasitic activity [5].

Unlike mammals, where the skin is keratinized, fish skin is composed of living non-keratinized cells. The integrity of the skin is essential for the homeostasis and prevention of entry of pathogenic organisms. The skin physically protects the fish from microbial pathogens, parasites and other toxic compounds and helps in osmotic regulation. The mucosa-associated lymphoid tissue (MALT) is one of the secondary lymphoid organs which comprises skin-associated lymphoid tissue (SALT), gut-associated lymphoid tissue (GALT), gill-associated lymphoid tissue (GIALT) and nasopharynx-associated lymphoid tissue (NALT) [6]. The ontogeny of the immune cells of the skin and mucus, the sites of antigen capture and presentation and the site of antibody secretion within the skin are still not known [5].

The skin epithelial cells are the major portal of entry of pathogens and, hence, the mucosal immunity plays an important role in preventing infections caused by pathogens. A greater understanding of the mucosal immunity including

pathogen-induced signalling pathways, the effector cells and effector molecules is necessary to develop new immersion vaccines to control infectious diseases [5].

### 3.1.2 Gill

The gill of teleosts is a multifunctional organ which helps in respiration, osmoregulation, pH regulation, nitrogenous waste excretion and hormone production [7]. The gill comprises a large surface area of about 0.1–0.4 $m^2$ $kg^{-1}$ body weight and the interface between the blood and the water comprises 2–3 layers of cells measuring about 6 μm in thickness. This vast surface area and thin lining is ideal for exchange of gas and other molecules and also for the entry of pathogens [7]. The gill surface is one of the major routes of pathogen entry. It is also the site for many parasitic infestations such as gill flukes and other ectoparasites. The gill surface is also covered by mucus thus providing protection to the underlying cells to some extent. In addition, the gill also functions as a secondary lymphoid organ in which specific immune response is reported. Both B and T lymphocytes present in the mucosal surfaces respond to pathogens or vaccination [6]. The GIALT comprises primarily T lymphocytes, which constitute 10–20% of all lymphoid cells [8]. Increased level of antibodies in gill mucus compared to serum, when challenged with *Flavobacterium branchiophilium,* and a lack of correlation between serum and mucus antibodies suggest that a separate gill immune system exists [9]. Similar results have been reported by many workers [10, 11]. Secretory IgD and IgT mRNA expression levels were significantly upregulated in the gill of rainbow trout immunized by immersion route [12] suggesting that IgD and IgT are mucosal immunoglobulins similar to IgA of mammals. However, report on the secretion of IgT and IgD antibodies in gill and skin mucus in response to antigens is lacking.

### 3.1.3 Gut

The entire gastrointestinal tract of fish can be divided into three segments, the first segment or the anterior gut comprises 60–75% of the total length of the gut where the enterocytes have an absorptive role in the uptake of proteins. The second segment or midgut comprises 15–30% of gut length and is involved in the uptake of macromolecules. The third segment or hindgut comprises 5–15% of gut length and has osmoregulatory function [13]. The entire gastrointestinal tract is lined with mucus membrane which in turn is covered with gut mucus. The anterior gut, i.e. the stomach, which secretes digestive enzymes that are highly proteolytic in nature, is extremely hostile to pathogens due to its acidic nature. About 15% of the fish (like cyprinidae) do not have a proper stomach and lack the low pH (acidic) region.

The hindgut houses the GALT while fishes lack the Peyer's patches found in mammals. IgT is the predominant isotype found in gut [6]. The lymphoid tissues have a greater IgT to IgM ratios compared to plasma which is a common feature observed in all the four MALTs [14]. Increased secretion of IgT in the gut of fish immunized through oral or immersion routes has been reported in several species.

### 3.1.4 Olfactory Organ

Fish derive information about its environment through its olfactory system. It is a paired organ present on the dorsal surface of the head. Water enters into the olfactory cavity through the anterior opening or anterior nostril and leaves through the posterior olfactory opening or posterior nostril. These openings are separated by a nasal bridge. In most fishes, the nasopharynx is not linked to the oral cavity [15]. Like other mucosal sites, the olfactory organ also has diffused lymphoid cells called NALT. Similar to gut and skin, $IgT^+$ cells make up 51.5% of the B lymphocytes while the rest are $IgM^+$ cells. The ratio of IgT/IgM in nasal mucus is 20 times that of serum [14]. Fish immunized through intranasal routes provide protection comparable to intramuscular injection [14].

## 3.2 Cells of Fish Innate Immunity

A wide variety of cell types are involved in the innate immunity of teleosts, including macrophages, monocytes, natural killer (NK)-like cells, non-specific cytotoxic cells (NCC) and granulocytes such as neutrophils [16]. Their role is more or less similar to that of mammalian counterparts.

Monocytes and macrophages are important phagocytic cells; the former being more commonly found in the kidney while the latter is found widely distributed in tissues including gill, kidney, spleen and heart. Both monocytes and macrophages are capable of engulfing a wide variety of pathogens and particles. Monocytes are considered to be the precursors of macrophages which migrate to the sites of inflammation and differentiate into macrophages. After differentiation at the site of inflammation, the macrophages become more phagocytic with increased antimicrobial activity [17]. Macrophages, in addition to phagocytosis, release hundreds of bioactive molecules that control inflammation which is crucial for the survival of an organism [18]. Macrophages containing melanin in its lysosome are called melanomacrophages. Melanomacrophage centres (MMCs) are found in kidney and spleen and are reported to have a role in bactericidal activity [19]. Macrophages also play a role in antigen presentation to T cells thus bridging the innate and adaptive immune system [20].

Neutrophils of teleosts are structurally and biochemically similar to mammalian counterparts and are characterized by the presence of myeloperoxidase in its cytoplasmic granules [19]. Neutrophils are phagocytic and bactericidal and are found in kidney, spleen and in the circulatory system. They traverse the blood vessels and reach the site of inflammation through chemotaxis and help in the removal of pathogens and toxins. Neutrophils are the first cells to migrate to the site of inflammation. NCC and NK cells are involved in non-specific cell-mediated cytotoxicity in fish [16]. NCCs kill virus-infected cells, tumorigenic cells and protozoan parasites.

## 3.3 Humoral Factors of Innate Immune System

### 3.3.1 Peptides

Antimicrobial peptides (AMPs) and polypeptides are found in a wide variety of organisms ranging from plants, microbes to human beings. These molecules are one of the important components of the mucosal defence mechanisms of fish. Many AMPs are reported in fish, although all are not present in all the species studied. The major classes of AMPs found in fishes are piscidins, defensins, hepcidins, cathelicidins and histone-derived [21].

The piscidin family consists of several evolutionarily conserved groups of peptides which are unique to fishes. Some of the piscidin families of AMPs identified in fishes are pleurocidins, misgurin, moronecidin, epinecidin and dicentracin [21]. The gene structure and the predicted secondary structure of these piscidins have similarities. Piscidins have wide antimicrobial activities both in vivo and in vitro against Gram-positive and Gram-negative bacteria, fungi, parasites and viruses [21]. Piscidins are expressed commonly in skin, gill and intestine and to a lesser extent in head kidney and spleen. Piscidins are salt- and thermal-tolerant and have low cytotoxicity to mammalian cells and, hence, have the potential to be used as therapeutants in mammals.

Three types of defensins have been identified in mammals viz. α, β and θ while fish defensins belong to the type β [22]. The fish β defensins act against both Gram-positive and Gram-negative bacteria and also against specific fish viruses. However, there are no reports of its activity against parasites and fungi [21]. Fish defensins are constitutively expressed in a wide range of mucosal and systemic tissues and they are induced in gill, skin and gut following bacterial challenge in rainbow trout [23].

Hepcidins are cysteine-rich peptides first discovered in humans [24] and subsequently in amphibians, reptiles and fish. The first hepcidin identified in fish was bass hepcidin from the gill of hybrid striped bass [25]. Since then, hepcidins have been discovered in several species. Hepcidins are significantly expressed in liver, although other organs such as gill, skin, muscle, spleen, blood, head kidney, heart and intestine can express it. The hepcidins of different species have antimicrobial activity against different pathogens; for example, Japanese flounder hepcidin (JF2) has antibacterial activities against Gram-negative bacterium, *Escherichia coli* and Gram-positive bacteria such as *Staphylococcus aureus* and *Lactococcus garvieae* and tilapia hepcidin (TH1–5) has antiviral activity against nervous necrosis virus [26].

Cathelicidins have a conserved N-terminal sequence and a C-terminal domain of varying sequence and length. They have antimicrobial activity against a wide-range of microbes. Cathelicidins can be induced by bacterial pathogens indicating their role in innate immunity of fish. The mature ayu cathelicidin has antimicrobial activity against a range of bacteria with very strong activity against *Aeromonas hydrophila* [27]. The histone-derived peptides have a sequence similar to the histone proteins and have broad-spectrum antimicrobial activity against human and fish pathogens and have been reported in a number of fish species [21].

AMPs are constitutively expressed in the mucosal tissues, and their production increases on exposure to pathogens and parasites. In addition to mucosal tissues, AMPs are found in immune cells such as mast cells and in the liver. AMPs are also found in eggs and newly hatched larvae [28] indicating its maternal transfer to offspring to offset the pathogenic challenge at early larval stages when the adaptive immune system is not functional. Although fish rely more on the innate immune mechanisms, compared to mammals, the information available on fish AMPs are relatively less. Fish AMPs are a potential source of antimicrobial molecules for the pharmaceutical industry.

### 3.3.2 Complement System

Complement system, an important effector system of innate humoral immunity, is evolutionarily conserved and is present in all vertebrates and in some invertebrates [29]. It consists of a group of 35 soluble and membrane-bound proteins having properties such as pathogen recognition and destruction, and removal of apoptotic cells [30, 31]. Teleosts have a well-developed complement system with diversified complement proteins and increased complement activity, which compensates for the not so well-developed adaptive immunity. Although many similarities exist between the mammalian and teleost complement systems, the complement system of fish is more diverse with many isotypes of complement proteins that can bind with many types of pathogens, compared to the mammalian counterparts. Fish complement proteins are more heat-labile, have lower optimal reaction temperature (10–27 °C) and have high alternative complement pathway titre [32–34], probably because of poikilothermic nature of fish. The complement system helps in the destruction of pathogens and apoptotic cells in addition to the mediation of inflammation and modulation of the adaptive immune mechanism. All three pathways of complement activation viz., classical, lectin and alternative complement pathways reported for mammals are also found in teleost. All the three pathways result in the formation of membrane attack complex resulting in lysis of the target cell.

### 3.3.3 Growth Inhibitors

Growth inhibitors inhibit the growth of pathogens, especially bacterial pathogens by depriving them of essential nutrients or by inhibiting their metabolism. Growth inhibitors commonly found in fish serum are transferrins and interferons. Transferrin, a protein with a high affinity for iron, is found in the serum of all vertebrates. It acts by chelating iron and, thereby, preventing the availability of iron for the bacteria, which is essential for its growth and metabolism, exerting bacteriostatic and fungistatic effects [19]. Interferons are a large family of cytokines produced by most cells upon viral induction. Based on the gene sequence and the receptors they interact with, interferons are classified into type-I and type-II. Interferons are constitutively expressed in spleen, gill, liver and kidney, with the highest expression observed in spleen [35, 36]. Interferons, along with other antiviral proteins stimulated downstream, exerts its antiviral activity by degrading the viral RNA or by inhibiting RNA synthesis [37].

### 3.3.4 Protease Inhibitor

Bacteria produce enzymes to enter the host and infect them. The host also produces enzyme inhibitors to counter the pathogens. Fish tissue fluids and serum contain many enzyme inhibitors which play an important role in neutralizing the enzymes of bacterial pathogens, thereby protecting the host against the infection. α2-macroglobulin is an important broad-spectrum protease inhibitor found in the plasma of vertebrates and invertebrates. α2-macroglobulin is reported in all the fish studied and it acts by entrapping the protease of the pathogen [38].

### 3.3.5 Natural Antibodies

Although antibodies are secreted in response to antigenic stimulation and, hence, a component of adaptive immune response, natural antibodies are found naturally in serum without any antigenic stimulation. Natural antibodies are found in all vertebrates. In mammals, natural antibodies are polyreactive acting against nonself-antigens and have low affinity for self-antigens [39]. In fish, natural antibodies play a role in the defence against bacterial and viral pathogens [40] and offer protection against infections. The quantity of natural antibodies varies as much as 20-folds between individuals and are directly involved in the first line of defence against pathogens. They also probably influence the level of specific antibodies upon subsequent exposure to the antigen [41].

## 4 Adaptive Immune System

### 4.1 Lymphoid Organs of Fish

The primary lymphoid organs of fish are thymus and head kidney. The secondary lymphoid organs consist of kidney, spleen and mucosa associated lymphoid tissue. The relative positions of the lymphoid organs are marked in Fig. 1.

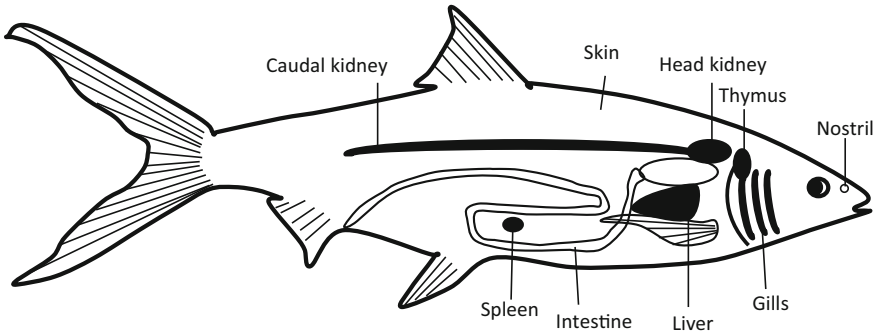


**Fig. 1** Relative positions of fish immune organs

### 4.1.1 Thymus

Thymus is an important primary lymphoid organ of teleosts. The structure and functions of the fish thymus are very similar to that of mammals. The fish thymus is a paired organ located on the dorsal side of the branchial cavity and is closely associated with the pharyngeal epithelium [3]. The thymus is organized into lighter inner medulla and darker outer cortex, although this zonation is not marked in many species [16]. The thymus consists of T lymphocytes in various stages of maturation. T lymphocytes mature in thymus with a high mitotic rate and migrate to other tissues. Two types of T lymphocytes are seen in thymus, the cytotoxic T cells (CTLs) and T helper (Th) cells. The CTLs express CD8 and interact with MHC class I, while Th cells express CD4 and interact with MHC class II molecules. All the T cells possess T cell receptors (TCR) through which they recognize peptides when presented by MHC along with other molecules [42]. The size of the thymus is maximum during the fingerling stage when they are about 2–3 months old and start regressing at the time of onset of sexual maturity [19].

### 4.1.2 Kidney

Bone marrow is a primary lymphoid organ in mammals which has the haematopoietic stem cells. These cells differentiate into different haematopoietic cells and are capable of self-renewal. However, fish lack bone marrow and the pronephros or head kidney is the major haematopoietic organ equivalent to bone marrow of mammals. The trunk kidney or mesonephros also has haematopoietic function in addition to renal function. The kidney is a Y-shaped organ found along the axis of the body embedded on the ventral side of the vertebral column with the head kidney starting from the gill region. The macrophages found in the kidney aggregate into MMCs and contain primordial germ cells. The head kidney also functions as an endocrine organ and has functions similar to mammalian adrenal glands secreting corticosteroids and other hormones that control osmoregulation, stress and immune response [16]. The head kidney is a major antibody-producing lymphoid organ and has a large number of B lymphocytes in different developmental stages equivalent to the lymph nodes in mammals. The time of first antibody production varies widely between species and is usually seen about a week after hatching.

### 4.1.3 Spleen

The spleen is the only lymph node-like organ and a major secondary lymphoid organ in fish. Usually, spleen occurs as a single organ, although in some species it may be found in two or more lobes. The spleen consists of red and white pulp, blood vessels and ellipsoids. Germinal centres are absent in spleen but it possesses MMCs, where mature B lymphocytes are activated by antigens resulting in the development of antibody-secreting plasma cells and memory cells [16]. The spleen contains a large number of erythrocytes and lymphocytes and has the capacity to trap particulate material in circulation. The structure of splenic pulp is very similar to the head kidney, and upon antigenic stimulation, a large number of antibody-secreting B lymphocytes are observed, as seen in kidney.

## 4.2 Humoral Immunity

In teleosts, the adaptive humoral immunity is mediated by immunoglobulins (Ig) as in mammals. The functions of immunoglobulins include neutralization of virus, toxins and bacterial adhesins, activation of the complement system and opsonization of pathogens [19]. Immunoglobulins, in addition to their role in systemic immunity, also play an important role in mucosal immunity. The structure of teleost Igs are similar to that of mammalian Ig comprising of two identical heavy chains and two identical light chains. The heavy and light chains are held together by disulfide bonds (Fig. 2a). Teleosts have three types of immunoglobulins viz. IgM, IgD and IgT. IgM, a tetramer, is the major immunoglobulin found in high concentrations in serum and mucus (Fig. 2b). Its concentration varies widely between species and is influenced by age, temperature and season [43, 44]. IgM is found in gill, skin, intestine and bile, in addition to serum. IgD is the second immunoglobulin to be reported in teleosts, named because of its sequence similarity to IgD of mammals and is an evolutionarily conserved molecule [45]. IgD has a significant role in skin and gill mucosal immunity. The immunoglobulin concentration increases on exposure to pathogens and parasites and this plays a significant role in protecting the fish against

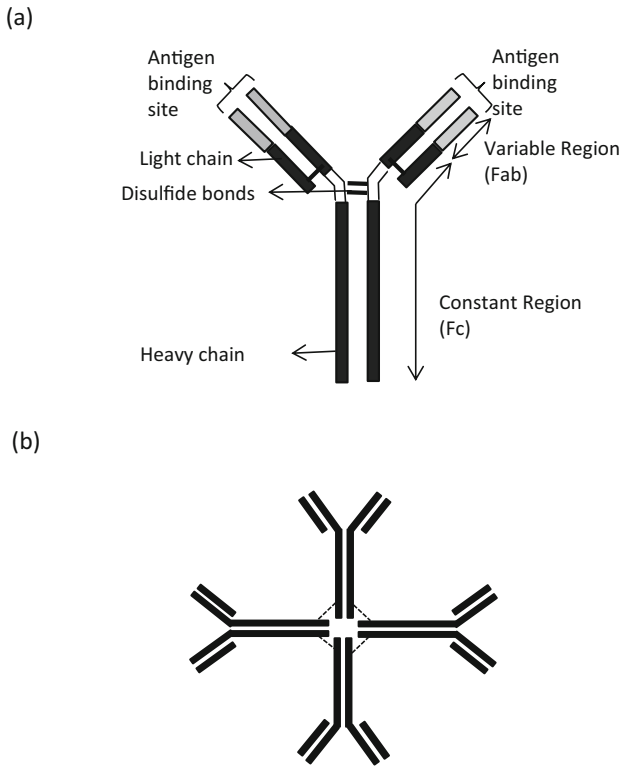


**Fig. 2** Structure of vertebrate immunoglobulin (**a**) and the structure of a teleost IgM (**b**)

the invading pathogens. IgT is reported to play a major role in gut mucosal immunity where its role is similar to IgA of mammals. The subset of B lymphocytes secreting IgM, IgD and IgT are different [46]. The teleost humoral immunity lacks the IgG, IgA and IgE isotypes of immunoglobulins found in mammals. Further, there is no isotype switching in teleosts as in mammals wherein the primary immunoglobulin switches its isotype from IgM to IgG with increased affinity while retaining the specificity. Although there is evidence of affinity maturation of antibodies in fish, the levels of maturation observed in fish are poor compared to mammals. The anamnestic response observed in teleosts is also not as marked as in mammals. The presence of IgM and the transcript in eggs and newly hatched larvae indicate the maternal transfer of immunity to offspring.

Oral or immersion vaccination of fish results in the secretion of Ig at the mucosal surfaces such as gill, skin and gut without eliciting systemic immune response. The ratio of IgT to IgM is much higher in the mucosal surfaces than in blood. Increased mRNA transcripts of IgD and IgT are observed in the mucosal tissues when fish are immunized via immersion routes indicating two compartments of humoral immunity, systemic and mucosal [12].

### 4.3 Cell-Mediated Immunity

Fishes are the most primitive species to possess major histocompatibility complex (MHC), a set of antigens present on cell surfaces responsible for cell-mediated immunity and graft rejection. Fish possess MHC class I, class II and class III antigens. Class I antigens are present on all nucleated cells. The class I antigens, when bound to endogenous foreign antigens such as virus-infected cells, stimulate the $CD8^+$ cytotoxic T cells for the destruction of the infected cell thus, playing an important role in the cell-mediated immunity. The cytotoxic T cell is also involved in graft-versus-host reaction and cell-mediated lympholysis [47]. CD8 and MHC class I molecules are expressed even in larval stages of fish indicating that cell-mediated immunity can be induced in larval stages by vaccination [48]. However, information on cell-mediated immunity in fish is not extensive due to lack of markers to study the cells involved in cell-mediated immunity.

## 5 Factors Affecting the Immune Response

The immune response in fish is influenced by extrinsic factors such as temperature, nature of antigen, dose, adjuvants and intrinsic factors such as the immunocompetency of the fish, age and physiological state, and the immunoregulatory mechanisms in the fish.

Temperature outside the natural range of environmental temperature of a species adversely affects the immune response to pathogens and the outcome of the infection [20]. Rainbow trout maintained at 15 °C had higher relative percent survival compared to fish maintained at 5 and 25 °C following vaccination and challenge

with *Yersinia ruckeri* [49]. Increased incidence of diseases and higher mortality of fish at lower temperatures is partially due to the immunosuppressive effect of suboptimal temperature [20] and the increased virulence of the pathogen at lower temperatures [50]. The amount of antibody produced in fish is proportional to the temperature. In carp immunized with dinitrophenol (DNP) conjugated to keyhole limpet hemocyanin (KLH), a T-dependent antigen, the antibody levels were significantly lower at 12 °C compared to fish maintained at 20 and 28 °C [51]. The natural antibody titre in fish serum is also temperature-dependent. The basal serum antibody level was higher in summer compared to winter [43]. A delay in the production of antibody was observed at lower temperatures and the anamnestic response seen on subsequent immunization was not seen when the temperature was suboptimal [52]. Innate immune parameters are active at lower temperatures, while adaptive immune parameters are suppressed at lower temperatures [44]. In carps, the lytic activity of non-specific cytotoxic cells is increased at lower environmental temperature. In cyprinids, the classical complement pathway, which is antibody-dependent, and the alternate pathway, which is antibody-independent, are suppressed and predominant, respectively at lower temperatures. The adaptive immune responses of teleosts are suppressed during winter when the water temperature is low. However, the fish are not fully immunocompromised during winter due to the predominant innate immune factors at lower temperature [53].

The immune response in fish increases with age. In carp immunized intramuscularly against *Vibrio anguillarum* at 85, 99 and 128 days of age, the serum antibody increased significantly with age [54].

The immune response to primary immunization is dependent on the antigen dose; higher the dose, greater the immune response. The immune memory is also dose-dependent similar to the immune response. The immune response is also dependent on the type of antigen such as particulate or soluble, size of the peptide and its chemical nature. In general, small peptides (<1000 Da) do not stimulate the immune system while larger molecules are highly immunogenic. Soluble antigens when administered along with adjuvants, elicit higher immune response and the immunological memory is also prolonged.

The routes of immunization also determine the serum antibody levels. Injection of antigens, either intramuscularly or intraperitoneally, stimulates good serum antibody response. Immersion or oral routes stimulate mucosal immune response rather than systemic response.

In summary, immune system of fish can be categorized into innate and adaptive immune systems (Table 1). The innate immunity comprises physical barriers such as mucus, skin, gill, gut and nostril epithelium and effector molecules such as AMPs, interferons, complement proteins, natural antibodies, growth inhibitors, protease inhibitors and cells such as macrophages, monocytes, NCC and neutrophils. The innate immune molecules are generally heat labile and are more active at lower environmental temperature range naturally experienced by a species. The onset of innate immune response occurs early in the life of teleosts than the adaptive immune response. The adaptive immune system comprises lymphoid organs such as head kidney, thymus, spleen and MALT and, the effector molecules comprise IgM, IgD

**Table 1** Characteristics of innate and adaptive immunity of teleosts

| Sl no | Parameter | Innate immunity | Adaptive immunity |
|---|---|---|---|
| 1 | Origin | Present before exposure to antigens | Develop subsequent to exposure to antigens |
| 2 | Organs involved | Skin, gill, intestine, olfactory organ, liver and most other organs | Head kidney, thymus, spleen, MALT |
| 3 | Cells involved | Monocytes, macrophages, non-specific cytotoxic cells, natural killer cells, neutrophils | B cells, CTL, Th cells, antigen-presenting cells |
| 4 | Effector molecules | AMPs, complement proteins, cytokines, growth inhibitors, protease inhibitors, natural antibodies | IgM, IgD and IgT |
| 5 | Target pathogens | All pathogens | Specific to the pathogen which induced the immune response |
| 6 | Memory | Absent | Present: mediated by memory cells |
| 7 | Specificity | Non-specific | Specific to the antigens which induced the adaptive immunity |
| 8 | Host cell receptors involved | Pattern recognition receptors | B cell receptor, T cell receptor |
| 9 | Response time | Within minutes | Takes 2–4 weeks |

and IgT, while the cells involved in the adaptive immunity are B and T lymphocytes. The age at maturation of the adaptive immune system of fish varies from species to species. Although the innate immunity is more diverse in fish, protection offered by adaptive immune system is also significant, especially when stimulated by vaccination. The adaptive immune response is modulated by various factors such as temperature, age, stress and route of vaccine administration and, hence, a thorough understanding of the immune system is essential to formulate prophylactic measures such as vaccination to offset the occurrence of diseases.

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# Shrimp Immune System and Immune Responses

Rajendran K. V., Sreedharan K., A. Deepika, and Amod Kulkarni

**Abstract**

Shrimp, being an invertebrate, lack a vertebrate-like adaptive immune system. However, an efficient innate immune system, consisting of physical barriers and cellular and humoral components, exists in shrimp. The innate immunity is activated by the recognition of different microbial cellular components (pathogen-associated molecular patterns, PAMPs) by the host-associated pattern recognition receptors (PRRs), which triggers different signalling pathways and subsequently leads to different cellular and humoral immune responses. Cellular defence involves various processes (phagocytosis, encapsulation, nodule formation, coagulation, apoptosis etc.) directly mediated by haemocytes. However, humoral components include activation of different cascade systems and release of molecules accumulated within the haemocytes (prophenoloxidase (proPO) activating system, antioxidant system, agglutinins, protease-inhibitors, antimicrobial peptides, phosphatase, lysozyme etc.). Apart from this, RNA interference (RNAi), microRNA and complement system have been found to play crucial roles in the protective immunity in shrimp. Moreover, existence of immunological memory and adaptive immunity in shrimp has been suggested, and molecules such as Down syndrome cell adhesion molecules (Dscam), fibrinogen-related proteins (FREPs) and hemolin have been reported to be involved in this. The existence of adaptive immunity in shrimp suggests the possibility of vaccinating shrimp against specific pathogens.

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**Keywords**

Immune system · Immune responses · Cellular immunity · Humoral immunity · Crustaceans · Shrimp

## 1 Introduction

Farming of penaeid shrimp represents one of the most economically important sectors of the rapidly growing aquaculture food production system worldwide. However, sustainable production has been hampered by the emergence of various diseases of infectious (opportunistic and obligate pathogens) and non-infectious (stressful farm conditions) origin resulting in considerable economic losses in many countries [1, 2]. Further, no effective control strategies are available to deal with the infectious disease outbreaks in shrimp farming owing to the limited understanding of the host–pathogen interaction. The susceptibility/resistance of shrimp to invading pathogens is greatly influenced by the immune status of the host and, therefore, understanding shrimp immune system is of utmost importance in designing the strategies for the control and management of diseases in shrimp aquaculture. There has been significant advancement in the understanding of the immune mechanisms of crustaceans, as large repertoire of receptors, signalling pathways and defence mechanisms have been elucidated in the past several years. Crustacean immunology, especially the study of shrimp immune system, has gained prominence not only because of the commercial importance of some of the species but also from the standpoint of being invertebrates which occupy a diverse ecological niche and face a diverse pathogen challenge. Immune mechanism of an animal involves recognition of non-self-molecules, and consists of a series of coordinated events mediated by various cells and molecules that may be specific or non-specific in function, which ultimately aids in resisting the invasion of pathogens.

## 2 Nature of Immune Responses

The fundamental attribute of immune system is its capacity to recognize and respond to no-self. Traditionally, immune mechanisms have been categorized into two, innate and adaptive. According to Medzhitov [3], the major distinction between innate and adaptive immunity is in the types of receptors used in the recognition of pathogens. Further, in the general features, there are many differences between these two arms of the immune system. According to Kvell et al. [4], innate immunity is considered to be natural, non-specific, non-anticipatory and non-clonal but germ-line encoded. Adaptive immunity, on the other hand, is specific, anticipatory, clonal and somatic. It is generally considered that invertebrates possess only innate immunity and vertebrates have innate and adaptive defence mechanisms. Shrimp, being invertebrates, do not possess a vertebrate-like adaptive immune response; however, they have well-developed innate defence mechanisms. Though the innate defence is

non-specific, it provides a highly effective defence response against invading pathogens [5, 6]. Nevertheless, there are compelling evidences which show that shrimp possess a specific or quasi-specific immune defence [7] and many adaptive immune molecules in the immune repertoire [8–15]. The innate defence mechanisms in shrimp consist of physical barriers, cellular and humoral components which work in a coordinated way to deal with all invading pathogens [16]. Cellular responses include phagocytosis, encapsulation, nodule formation etc. and are mediated directly by haemocytes. However, prophenoloxidase (proPO) activating system, agglutinins, protease-inhibitors, anti-microbial peptides, phosphatase, lysozyme etc. are the major components of the humoral responses [16–20].

## 2.1 Physical/Structural Barriers

In shrimp, a hard cuticular exoskeleton provides the physical barrier to the easy entry of microbes into the body. The structure also possesses anti-microbial humoral factors such as polyphenoloxidase and this physical and chemical barrier provides the primary defence against invading pathogens. The cuticle consists of different layers: an outermost epicuticle, followed by inner exocuticle and endocuticle (Fig. 1). The waxy epicuticle is devoid of the polysaccharide, chitin, whereas the sclerotized exo- and endocuticle contain variable levels of chitin and calcium-containing minerals. Below the cuticular layers lies the underlying epidermis and beneath which the basement membrane. The tegumental glands located beneath the epidermis helps in the phenolic tanning of the cuticle by producing polyphenoloxidase. Cuticle covers not only the entire outer body surface of the animal including gills but also the anterior and posterior portions of the gastrointestinal tract [21]. However, the cuticular lining of gills and gut is very thin compared to that of the cuticular lining of the external surface. Moreover, the gastrointestinal tract is equipped to digest the pathogens through the gut enzymes and acids. These features protect the intestinal epithelium from direct access to pathogens that can enter via oral route [22]. The disease-causing agents that need to get entry to the underlying tissues have to penetrate these physical and chemical barriers. However, it is possible that pathogens can break the first line of defence in the event of a physical damage to the overlying structures and/or during moulting when the cuticular structures are vulnerable.

## 2.2 Cellular and Humoral Immune Responses

Shrimp innate immune system possesses both cellular and humoral components that are activated upon pathogen challenge. The immune activation in response to pathogens involves release of soluble molecules and cellular and humoral components mediated by haemocytes [23]. As mentioned, constituents of the cellular immune responses include all the reactions that are controlled directly by haemocytes such as phagocytosis, encapsulation, nodule formation etc. Humoral

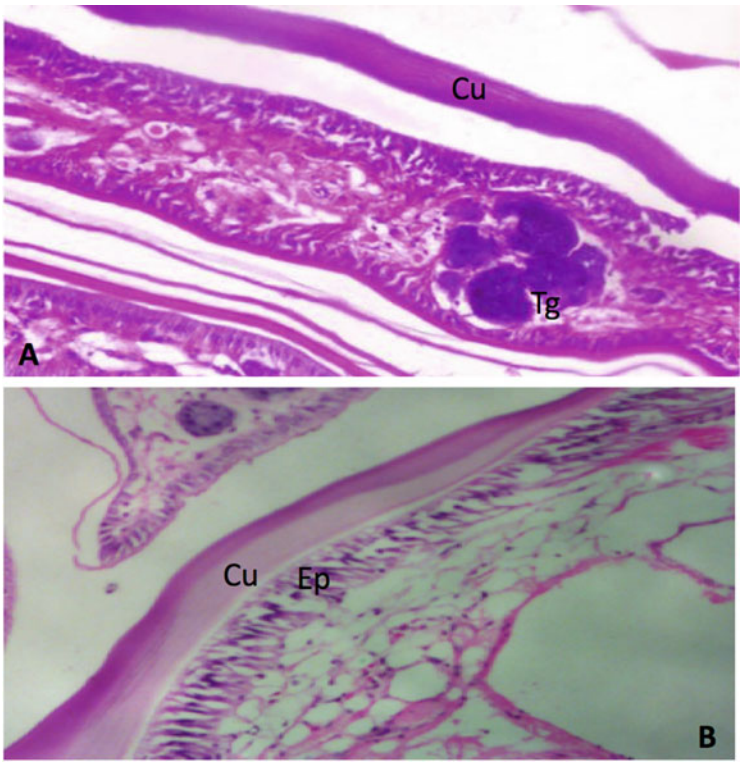


**Fig. 1** Histological section showing cuticular layers of penaeid shrimp. (**a**) cuticular lining of the outer exoskeleton; (**b**) cuticular lining of the gastrointestinal tract. *Cu* cuticular layers, *Ep* epidermal layer, *Tg* tegumental gland

components, on the other hand, include cell-free components of the haemolymph. These include a variety of molecules and cascades such as proPO activating system, clotting cascade agglutinins, protease-inhibitors, anti-microbial peptides, phosphatase, lysozyme, reactive oxygen and nitrogen intermediates etc. [16–20, 23]. Though there is a clear categorization of the components such as cellular and humoral, it is evident that these responses overlap and the two components are interdependent and work in synergy to protect the animal from pathogen challenge (Fig. 2).

### 2.2.1 Cellular Immune Response

Once the invading pathogen escapes the primary barrier, it gets entry into the body cavity (haemocoel) where it will be responded by circulating blood cells and fixed cells in gills, lymphoid organ or hepatopancreas. Unlike vertebrates, crustaceans possess an 'incompletely closed' or 'partly open' circulatory system [24] characterized by blood vessels and channels which permeate various tissues. The circulating blood cells in crustaceans (as they circulate in the haemocoel) are called as haemocytes and are mainly responsible for cellular immunity and play a crucial

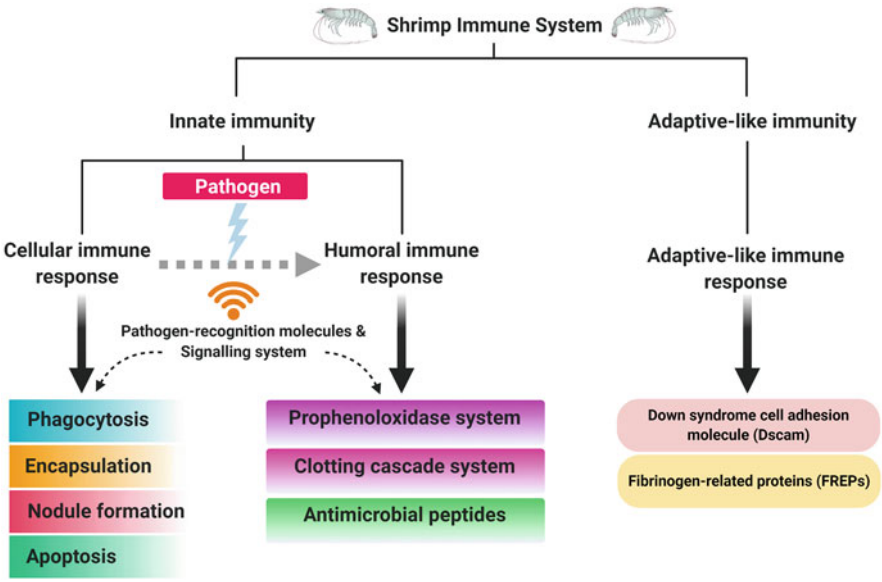


**Fig. 2** Components of shrimp immune system

role in defence against microbial invasion. The haemocytes comprise of three main types: hyaline cells, semi-granular cells (SGCs) and granular cells (GCs); and this classification is mainly based on the cell size, nuclear/cytoplasmic (N/C) ratio and the number of intracellular granules (GCs) [24, 25]. Hyaline/agranular cells (5–15% of the circulating haemocytes), as the name implies, are without or with very limited number of granules in the cytoplasm. These are the smallest among the three types of haemocytes and with the highest N/C ratios. The major role of these cells involves clotting, encapsulation and phagocytosis [21, 26]. Semigranulocytes are the most abundant haemocytes (75%), which possess many small granules and are responsible for phagocytosis, nodule formation, encapsulation, proPO activation and clotting [21, 26, 27]. SGCs also possess peroxinectin, one of the cell adhesion proteins [28]. The GCs contain large number of granules possessing a low nucleus-to-cytoplasm ratio and comprises of 10–20% of the total haemocytes [29]. Immune functions of GCs include nodule formation, encapsulation, proPO activation, clotting and production of anti-microbial peptides (AMPs) [21, 29]. Usually, microbial encapsulation is followed by melanization due to proPO which is stored in an inactive form within the granules of the GCs. Apart from proPO, GCs harbour AMPs, protease inhibitors and cell adhesion/degranulating factor called peroxinectin [24].

## Phagocytosis

In all animal phyla, phagocytosis is considered as the fundamental process for the removal of microorganisms or other small particles (both biotic and abiotic).

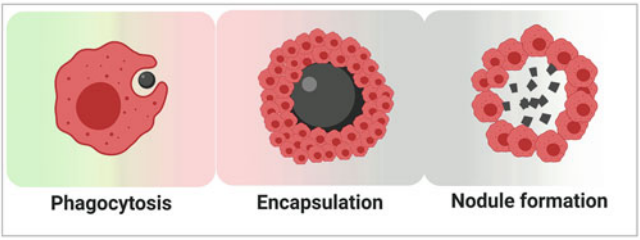


**Fig. 3** Diagrammatic representation of phagocytosis, encapsulation and nodule formation

Phagocytosis in crustaceans is primarily carried out by haemocytes (phagocytes) [16] and the process involves recognition and binding of endogenous foreign materials or pathogen surface receptors on the phagocytic cells (Fig. 3). This is followed by their uptake and engulfment into the cell and subsequent formation of a digestive vacuole called the phagosome. This process initiates a signalling cascade that results in the formation of phagolysosome by fusion of phagosome with lysosomes. Subsequently, several degradative enzymes are released into the phagolysosome and reactive oxygen intermediates (ROIs) are produced, which destroy the engulfed particles [30, 31]. This process, termed as respiratory burst, results in the formation of various ROIs such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), hydroxyl radicals ($OH^-$) and singlet oxygen ($^1O_2$). In crustaceans, phagocytes can be found free in the haemocoel and/or associated with the vascular system of organs such as lymphoid organ, hepatopancreas, gills etc. [32]. The phagocytic process is regulated by many components including pattern recognition receptors (PRRs) [33, 34] such as lectins, scavenger receptors, immunoglobulin-related protein and fibrinogen-related protein [35, 36].

## Encapsulation

When the size of pathogen such as fungal spores, helminths etc. is too large which cannot be phagocytosed by a single cell, several haemocytes cooperate and adhere together to form a capsule around the foreign particles so that the particles can be blocked from getting into circulation. This process is called encapsulation and is a significant cellular immune defence mechanism in invertebrates against pathogens [37] (Fig. 3). Encapsulation is a coordinated response involving multiple cells to eliminate foreign particles which cannot be phagocytosed or cannot be destroyed by humoral factors [38]. Encapsulation involves adherence of several haemocytes to each other and across the surface of intruding particles with the help of adhesion molecules, forming multi-layered cellular sheaths [39]. The cell adhesion molecule peroxinectin plays an important role in enhancing the encapsulation process [28]. A typical capsule is characterized by 5–30 compact layers of haemocytes without intercellular spaces [38]. Some of the humoral substances are responsible for the final destruction of the encapsulated material. Encapsulation can result in significant decline in the number of circulating haemocytes; however, the haemocyte number gets restored to the normal levels in few days [40].

### Nodulation

Nodule formation occurs when the numbers of microorganisms are more than what can be removed by phagocytosis. Similar to encapsulation, the function of nodule formation is to wall off invading microbes and microparasites. However, nodule formation and encapsulation are different in which nodule formation involves the entrapment of aggregated microorganisms by haemocyte cluster whereas encapsulation involves the formation of multi-layered haemocyte capsule around larger eukaryotic parasites (Fig. 3). Nodulation process is activated either through induction or enhanced expression of cell adhesion molecules such as peroxinectins and integrins [37]. The microbes entrapped in the nodule are immediately eliminated from circulation, which could be noticed in the gills and in sinuses between the hepatopancreatic tubules. Encapsulation and nodulation activate proPO activity and melanization, production of free radicals and AMPs, and subsequently result in the destruction of the microbes [16]. These processes also play vital roles in the sclerotization and wound healing process [41].

### Apoptosis

Apoptosis is an evolutionarily conserved form of programmed cell death carried out by specialized cellular machinery [42]. Apoptosis performs different functions such as removal of unwanted or potentially harmful cells, besides playing critical role in development [43]. The apoptotic events are carried out by a family of cysteine proteases called caspases. During viral infection, apoptosis is regulated as a critical innate cellular response to limit the replication of virus and prevent the spread of infection in the host [44]. Apoptosis plays an important role in anti-viral response in shrimp, as many apoptosis-related genes such as caspases, inhibitor of apoptosis protein (IAP), apoptosis inducing factor (AIF) etc. have been identified from shrimp [45–48]. Various caspases have been identified in shrimp and the caspases characterized from *Penaeus vannamei* are designated as initiator or effector caspases on the basis of their domain structure and their localization in cytoplasm near the membrane or within the nucleus [47]. Further, apoptosis-mediated thwarting of virus multiplication has been recorded in shrimp against WSSV and Taura syndrome virus [49, 50]. Role of caspase as the key effector of apoptosis has also been investigated in shrimp (*Marsupenaeus japonicus*) and was shown that silencing of *Pj*caspase gene resulted in the inhibition of WSSV-induced apoptosis resulting in increased viral copy [45]. However, knocking down of cap-3, a homolog of human caspase-3 in *P. vannamei* showed contrasting results when challenged with low and high dose of WSSV leading to the observation that in WSSV-challenged shrimp apoptosis may increase mortality rather than decrease [51]. On the other hand, many viruses, including WSSV, possess genes such as anti-apoptotic proteins (AAP-1) that can suppress or delay apoptosis so that sufficient quantities of viral progeny are produced. Consequently, delay in apoptosis helps in spreading the viral progeny from infected to uninfected cells of the host [43, 52, 53].

**Haemolymph Coagulation or Clotting**

Clotting/coagulation, mediated by cellular and humoral components, is an integral part of the invertebrate immune system. Clotting prevents the excessive loss of body fluid due to injury and blocks the entry of microbes into the hemocoel [16, 54–56]. Three different mechanisms (type A, B and C) of haemolymph coagulation have been described [57–59]. According to Tait's categorization, haemolymph coagulation in shrimp and spiny lobsters follows type C (third category) mechanism and it is characterized by rapid lysis of haemocytes followed by immediate clotting of plasma resulting in low cell aggregation. However, Ghidalia et al. [58] suggested that the three different patterns of coagulation are due to different concentrations of clotting protein (CP) and those species (shrimp) which exhibits type C category possess the highest amount of CP. Later, Hose et al. [59] suggested that the basis of the three categories is related to the proportion of hyaline cells present in the haemolymph. Accordingly, species categorized under type C possess a higher number of hyaline cells (50–70% of total haemocytes). Based on this, Hose et al. [59] proposed that the three categories proposed by Tait [57] actually represent the proportional responses to the percentage of hyaline cells rather than three different mechanisms.

The key components of haemolymph coagulation identified are CP, formerly named as fibrinogen or plasma coagulogen, and transglutaminase (TGase). The CP is a lipoglycoprotein and its subunits can be covalently cross-linked to each other by TGase. The CPs in crustaceans have no structural similarities with fibrinogen from vertebrates or clottable proteins from other arthropods and, therefore, represent a separate group of CPs [16, 32, 60]. TGase is a calcium-dependent enzyme stored in the haemocytes, which helps in the formation of stable clots by mediating the polymerization of CP [60]. Though TGase is primarily involved in coagulation, it is responsible for many other biological processes. TGases involved in haemolymph clotting in crustaceans are stored in an inactive form in the cytoplasm of the cell. It gets activated by the plasma calcium upon getting released from the cell [61, 62]. It is found that unlike vertebrates, the clotting process in crustaceans involve only a single step [63]. Based on the clotting process observed in freshwater crayfish, *P. leniusculus,* the current model of haemolymph clotting in crustacean has been suggested and, according to this, clotting is initiated by the release of TGase from haemocytes and other tissues and this in turn promote rapid polymerization of plasmatic CP into long and flexible and often branching chains (formation of clot). This process takes place in the presence of calcium ions [16, 60, 64].

### 2.2.2 Humoral Immune Response

In the humoral immune response, soluble effector molecules synthesized and stored in the haemocytes are secreted to combat pathogens. Major humoral factors consist of proPO, lectins, agglutinins, AMPs etc., and these factors work in coordination with phagocytes to eliminate the microbial pathogens that have overcome the primary barriers of the host [65].

### Prophenoloxidase System (proPO) and Melanization

The proPO system is the best-studied crustacean immune mechanism and this potent humoral component is involved in melanization, cytotoxic reactions, cell adhesion, encapsulation and phagocytosis [66] and, thereby, helps in wound healing and entrapment of parasites, besides killing of microbes [23]. Semi-granular and granular haemocytes are the repository of proPO system which can be activated by the presence of microbes or microbial components [67]. The proPO system acts upon dihydrophenylalanine, a phenolic substrate and converts it into dopaquinone. Subsequently, melanin, a dark-brown pigment responsible for preventing the spread of foreign particles in the host body as well as for healing cuticle damages, is produced by the non-enzymatic polymerization of dopaquinone. Further, intermediate compounds in the melanin pathway involve in immune reactions, as they have bactericidal properties.

Microbial cell wall components, even in very low quantities, such as lipopolysaccharides (LPS), β-1,3-glucans or peptidoglycans (PGN) etc. can activate the proPO system leading to the formation of melanin [68]. Phenoloxidase (PO) is the main enzyme involved in the cascade and is synthesized as the inactive zymogen (proPO) in the haemocytes and released outside during degranulation. Proteolytic cleavage of inactive proPO can be triggered by the specific recognition of microorganisms by pattern-recognition proteins (PRPs) resulting in the production of active PO. This conversion is mediated by a serine proteinase, prophenoloxidase activating enzyme (ppA). Further, ppA is synthesized and maintained as a zymogen (pro-ppA) and gets activated by proteolytic cleavage in the presence of $Ca^{2+}$. Activation of PO leads to the production of melanin and toxic reactive intermediates against intruding pathogens. Among crustaceans, the proPO gene was first characterized in crayfish, *P. leniusculus* [69, 70] and, subsequently, in shrimp, *P. monodon* [71].

In shrimp (*P. monodon)*, cDNAs of two proPOs, four serine protienase (SP)], three serine proteinase homologues (SPHs) and one PRP have been characterized. In *P. monodon*, LPS-ß-1,3-glucan binding protein (LGBP) acts as the PRP for the activation of proPO [23]. Similarly, two prophenoloxidase activating enzyme (ppA) genes have also been identified in this species. Based on the available information, a model of the proPO cascade in *P. monodon* has been suggested by Amparyup et al. [72]. According to this model, *Pm*LGBP acts as a functional PRP for the activation of proPO system as it can bind to LPS and ß-1,3-glucan and, therefore, can play a role in recognizing microbes. The binding of the PRP with the pathogen-associated molecular pattern (PAMP) will activate the clip-SP cascade and results in the conversion of *Pm*PPAE1 and *Pm*PPAE2 into their active forms. This active form of the enzymes will in turn activate the *Pm*proPO1 and *Pm*proPO2 to their active forms, PO1 and PO2, which will finally lead to the production of melanin and reactive oxygen compounds [72] (Fig. 4). Nevertheless, though melanization and activation of proPO system are essential for the immune defence, these cascades are tightly regulated at multiple levels by different inhibitors to prevent the production of excess melanin and other toxic intermediates which can lead to the damage and death of host cells. These include proteinase inhibitors, PO inhibitors,

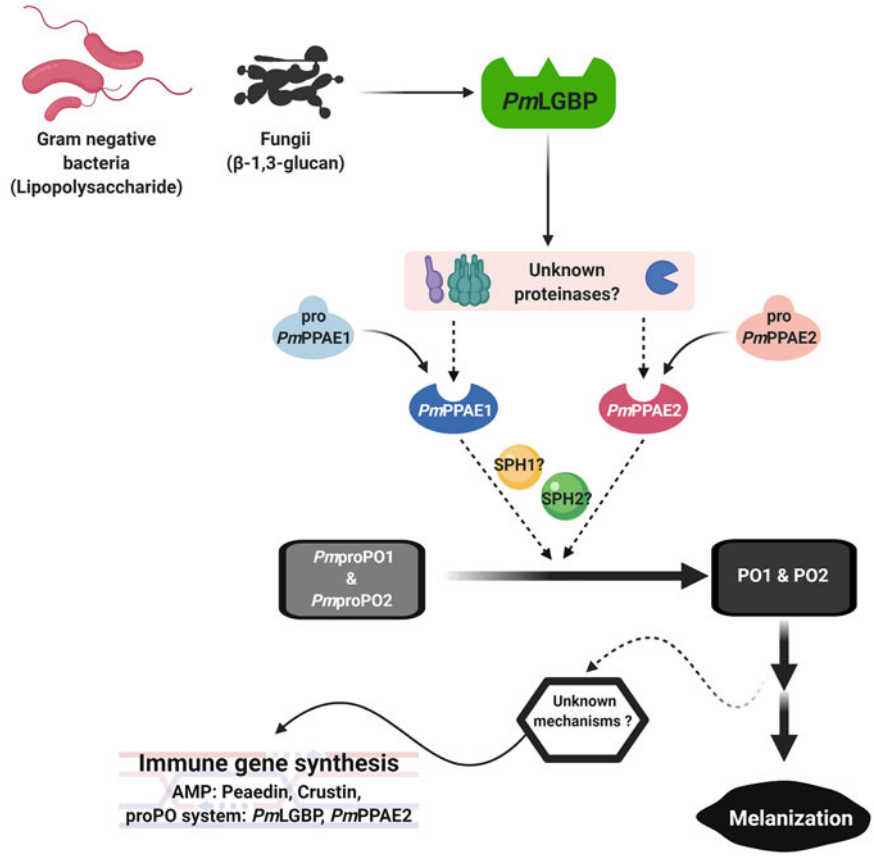


**Fig. 4** Prophenoloxidase system (proPO) and melanization

melanization-inhibiting protein (MIP) etc. Although these inhibitors or the negative regulators of proPO system are reported in insects and some crustaceans, information on the inhibitors in penaeid shrimp is lacking [72].

## Antioxidant System

An important defence reaction of haemocytes is the production of antioxidant factors with powerful microbicidal activity. ROIs ($O_2^-$, $OH^-$ and $H_2O_2$) and reactive nitrogen intermediates (RNIs; nitric oxide and peroxynitrite), produced in phagocytic vacuoles, have the potential to cross the cell barrier and destroy the adjoining cells [73]. To mitigate the harmful effects of different ROIs, cells have efficient antioxidant defence strategies consisting of both the enzymatic (catalase, glutathione peroxidase (GPx), superoxide dismutase (SOD), peroxiredoxin (Prx)) and non-enzymatic components (ascorbate, beta-carotene, flavonoids, alpha-tocopherol and vitamin E) [73]. SOD can scavenge the $O_2^-$ and convert them to $H_2O_2$, which is

subsequently detoxified to water and oxygen by the action of other antioxidant enzymes [74].

### Proteinases and Proteinase-Inhibitors

Proteinases and proteinase-inhibitors, which are distributed ubiquitously in all living organisms, play a major role in the innate immune system of shrimp as the important constituents of proPO cascade and apoptosis. On the other hand, proteinase inhibitors regulate these pathways by inhibiting specific proteinases and, thereby, preventing excessive activation of the pathway and subsequent injury to host tissue. Different serine protease-inhibitors such as the Kazal-type serine proteinase-inhibitors (KPIs), Kunitz-type protease-inhibitor (KUPIs), serpins and alpha-2-macroglobulins (A2Ms), have been characterized and their role in the innate immune system of shrimp has been reported [75, 76].

### Cytokines

The roles of cytokines in the regulation of haematopoiesis and immune responses in vertebrates have been demonstrated; however, only limited information is available on the cytokines of invertebrates [77, 78]. A cytokine-like factor, astakine, containing a prokineticin (PK) domain has been reported for the first time in crayfish, *Pacifastacus leniusculus,* and shrimp, *P. monodon* [77] followed by an astakine in *L. vannamei* (*Lv*AST) [79]. Further, astakine has been demonstrated to play a vital role in promoting haematopoiesis in crustaceans [78, 79]. They are involved in cell proliferation, differentiation and release of mature haemocytes into circulation [77]. In crustaceans, two different astakines (Ast1 and Ast2) have been reported and, while both the variants are present in crayfish, Ast2 shows more similarity with shrimp astakine [24]. Ast1 is secreted by SGCs and is found abundantly in these cells. Further, it is involved in the proliferation of hematopoietic tissue (HPT) cells and induces differentiation of the SGCs. However, Ast2 does not induce proliferation of HPT cells but induces GCs differentiation [24]. These molecules also serve as positive regulators in enhancing immune responses in shrimp against viruses. Administration of recombinant AST in *L. vannamei* (*Lv*AST) resulted in significant reduction in mortality and longer survival time in WSSV-infected shrimp. Further, silencing of the gene showed enhanced severity of WSSV infection and short survival time in experimentally-infected shrimp [79]. Moreover, astakines play a crucial role in clotting cascade by reducing extracellular TGase activity [80].

### Antimicrobial Peptides (AMPs)

AMPs are small molecular weight proteins either cationic or anionic, and possess broad-spectrum activity against a diverse range of organisms including viruses, bacteria, protozoa and fungi. The AMPs have been reported in vertebrates as well as invertebrates and these are present in the epithelial cells and liver/fat body [81]. Many AMPs have been reported from shrimp. These include penaeidins, lysozymes, crustins, stylicins and anti-lipopolysaccharide factor (ALF) which are formed and accumulated in haemocytes and their expression is controlled by Toll and IMD pathways [75, 82].

*Penaedins* are cationic molecules and are divided into four distinct subgroups: PEN2, PEN3, PEN4 and PEN5 [83]. Penaeidin-4 is the most powerful bactericide among the penaeidins, and *Pm*PEN5 serves as antiviral molecule [84]. Crustins, possessing a single whey acidic protein (WAP) domain at the C-terminus [85], are divided into four main types (Types I–IV) based on their domain arrangement [86]. Most crustins found in shrimp belong to Type-II crustins. Type I crustins are mainly found in crabs, lobsters, crayfish and rarely in shrimp. Type III and IV crustins have also been reported from shrimp [87–89]. ALF, with a functional LPS-binding domain (LBD), has been reported to display activity against diverse types of pathogens. The ALFs from penaeid shrimp were divided into seven groups (groups A, B, C, D, E, F and G), based on sequence similarities and phylogenetic analysis [90]. ALF*Pm*3 was found to be involved in anti-WSSV immune response by binding to the envelope proteins [91, 92]. Lysozyme is an enzyme that cleaves the peptidoglycan (PG) in the cell wall of both Gram-positive and Gram-negative bacteria resulting in cell lysis. Of the different types of lysozymes reported, only the c-type and i-type lysozymes have been reported in shrimp so far. Stylicins, the first anionic AMP identified in penaeid shrimps, was first reported from *L. stylirostris* (*Ls*Stylicin1) [93]. *Ls*Stylicin1 exhibits a strong anti-fungal activity against *Fusarium oxysporum*. Stylicins from other shrimp species are reported to respond to WSSV [94] and *Vibrio* [95] infection. Lectins play a key role in immune responses by serving as opsonins and mediating phagocytosis against bacterial pathogens [96]. Their carbohydrate recognition domain (CRD) recognizes and binds with different sugar moieties on the microbial cell surface that leads to their agglutination [97]. *Pm*AV lectin from *P. monodon* and a mannose-binding C-type lectin from *L. vannamei* have been discovered and reported to have antiviral activity [74]. Some AMPs such as *Mj*Pen-II and *Mj*Cru I-1 have been reported to involve in the phagocytosis during microbial infection in shrimp [98, 99].

The relation between clotting system and production of AMPs has been demonstrated, which together form major constituent of humoral innate immune system. In shrimp, activation of clotting cascade mediated by TGase activates the expression of AMP gene and the release of AMPs [99], and the depletion TGase and CP leads to inhibition of AMP expression [100, 101].

### Double-Stranded RNAs (dsRNA)

dsRNAs are produced during the replication of positive-sense RNA, dsRNA or DNA viruses. It consists of gene fragments of greater than 30 bp and are central component of post-transcriptional gene silencing, which is also termed as RNA interference (RNAi). The inhibition of viral replication through RNAi has been reported in diverse types of invertebrates such as *Caenorhabditis elegans*, *Drosophila*, planaria, hydra and trypanosomes [75, 102]. In RNAi, dsRNA is cleaved by the type III endonuclease Dicer into shorter 21–25 bp small interfering RNAs (siRNA), which get incorporated with the RNA-induced silencing complex (RISC) and, that in turn, targets the binding of the siRNA to the homologous mRNA for destruction. Major components of RNAi pathway in shrimp include Dicer1, Dicer-2, HIV-1

transactivating response RNA-binding protein, eukaryotic initiation factor 6 and members of the argonaute family [103, 104].

It has been reported that the administration of artificial dsRNA/siRNA to shrimp can offer effective protection against virus invasion, suggesting that a functional RNAi pathway is an effective strategy for combating viral infection in crustacean [105, 106]. dsRNA injection to shrimp results in specific down-regulation of target gene [107, 108]. Moreover, marked reduction in virus replication could be noticed following injection of sequence-specific dsRNA/siRNA targeting different viral proteins in shrimp [105, 109]. Enhanced expression of immune genes following the administration of dsRNA injection has also been described [110, 111].

### microRNA (miRNA)

miRNA are small, non-coding RNA molecules, which play important roles in the regulation of immune response by post-transcriptionally targeting the immunity or pathogen-associated genes. The role of different miRNA in virus–host interactions in shrimp has been studied recently, and alterations have been found in the transcript levels of both host and virus miRNA during virus infection. Generally, an individual miRNA possesses multiple target genes. Prevention of virus propagation and subsequent infection by shrimp miR-7 and miR-965 which inhibit the expression of WSSV early genes, wsv477 and wsv240, has been reported [112, 113]. Additionally, the role of miR-965 in promoting the antiviral phagocytosis of shrimp has been reported, which acts on shrimp ATG5 (autophagy-related gene 5). Moreover, shrimp miR-12 (inhibit WSSV replication by targeting the viral *wsv024* gene), miR-34 and miR-1000 (targeting *wsv*191 and *wsv*407) play crucial roles in antiviral immunity [114–116]. The role of miR-1000 in the regulation of apoptotic activity of shrimp against WSSV by targeting the shrimp p53 gene has also been reported [117]. On the other hand, some viral miRNA interact with host genes or other viral genes for promoting viral replication or virus latency. Regulation of Dorsal by two viral miRNAs (WSSV-miR-N13 and WSSV-miR-N23) during WSSV invasion has been reported, thus suppressing the signalling pathway [118]. A viral miRNA (WSSV-miR-22) has been reported to promote WSSV infection by interacting with shrimp STAT [119]. Another viral-encoded miRNA (WSSV-miR-N12) interacts with *wsv*399 gene, inducing virus latency [120], which is an efficient strategy for virus to escape its host immune responses.

### Complement System

The complement system, a group of plasma proteins, plays a key role in immune defence against microbial pathogens. Activation of complement leads to a series of events, which culminate in the opsonization and destruction of the pathogen as well as in the generation of the classical inflammatory response through the formation of potent proinflammatory molecules [121]. Three major pathways have been reported to activate the complement system: classical, lectin, and alternative [122]. However, the presence of complement system in shrimps has only been recently reported where a novel complement C3-like gene (*Lv*-C3L) from *L. vannamei* has been

identified and its transcript level showed significant up-regulation following bacterial and WSSV infection [123].

## 3 Pathogen Recognition and Signalling Pathways

The recognition of microbial pathogens is achieved through germline-encoded PRRs that are expressed in a variety of cells, which recognize invariable molecular signatures of pathogens known as PAMPs, and certain danger signals associated with cellular stress, known as damage-associated molecular patterns (DAMPs) [124].

LPS from Gram-negative bacteria, PGN from Gram-positive bacteria, double-stranded RNA (dsRNA) from viruses and β-glucans (GLU) from fungi are the major PAMPs. In vertebrates, binding of PRRs with their PAMPs elicits the expression and up-regulation of respective pro-inflammatory cytokines and anti-microbial molecules during the initial stages of infection that minimize the spread of pathogens [125]. Eleven PRR families, such as LPS and β-1,3-glucan binding proteins (LGBPs), C-type lectins (CTLs), galectins, thioester-containing proteins (TEPs), fibrinogen-related proteins (FREPs), scavenger receptors (SRs), Down syndrome cell adhesion molecules (Dscam), Toll-like receptors (TLRs), β-1,3-glucan binding proteins (BGBPs), serine protease homolog (SPHs) and trans-activation response RNA binding protein (TRBPs) have been identified in shrimp [126]. The tissue-level expression of different PRRs and their response to various ligands and microbial pathogens in several crustaceans have been investigated by many researchers, and found ubiquitous expression of most of these PRRs in many tissues and their differential response against potential pathogens including WSSV and bacteria [127–129].

Activation of PRRs in invertebrates triggers a sequence of cellular or humoral responses which are controlled by signal transduction pathways and are triggered by the binding of PRRs with specific PAMPs [130]. The triggered cellular and humoral responses include proPO, clotting mechanism, phagocytosis and the release of NF-kB-dependent AMPs. Signalling pathways in shrimp have been categorized into three: Toll pathway, Immune deficiency (IMD) pathway and JAK/STAT pathway. The Toll pathway is responsible for defence against fungi, Gram-positive bacteria, Gram-negative bacteria and viruses. The IMD pathway performs essential role in managing Gram-negative bacterial and viral infections and the JAK/STAT pathway in antiviral immunity [33].

### 3.1 Toll Pathway

TLR is the most widely studied signalling molecule. While TLRs of vertebrates can directly recognize PAMPs, TLRs of invertebrates, including shrimp, recognize pathogens through the binding with spätzle, a cytokine-like ligand [33, 131]. Following microbial invasions, the spätzle is cleaved by a proteolytic cascade to form a

mature form which in turn binds to the Toll receptor, initiating the signalling cascade. TLR signalling pathways are broadly categorized into two classes: Myeloid differentiation factor 88 (MyD88)-dependent signalling and MyD88-independent/TRIF-dependent signalling. Upon activation by distinct PAMPs, TLRs homo- or heterodimerize and this in turn induces recruitment MyD88 (adaptor molecule), via the cytoplasmic TIR (Toll-IL-1R) domain. MyD88 then recruits Tube (Mammalian IL-1R-associated kinases-4 (IRAK-4) homolog) and Pelle (Mammalian IRAK-1 homolog), which phosphorylates Pelle. Tube subsequently activates Pelle by phosphorylation, which in turn gets associated with tumor necrosis factor receptor-associated factor 6 (TRAF6), followed by the phosphorylation and degradation of Cactus (homolog of Ikβ) and freeing of Dorsal (homolog of NF-kβ). Activated Dorsal is subsequently translocated to the nucleus and regulates the expression of AMPs [131]. The Spätzle/Tolls/MyD88/Tube/Pelle/TRAF6/Dorsal signalling pathway has been elucidated in shrimp [33, 132].

## 3.2 IMD Pathway

The IMD pathway was initially discovered in *Drosophila* by the identification of a mutation named immune deficiency (IMD), which inhibits the expression of AMPs. IMD pathway has been reported from crustaceans and the pathway regulates AMP expression. IMD codes for a death domain which is identical to that of the Receptor Interacting Protein (RIP) of the tumour necrosis factor receptor (TNF-R) pathway [33]. Relish, a Rel/NF-kB transcription factor, is the key molecule in the IMD pathway that activates the production of AMPs, after its induction by the IMD. Different components of the shrimp IMD pathway have been characterized [133–138]. The *Pm*Relish from *P. monodon* regulates the synthesis of AMPs such as PEN5, PEN3, ALFPm3 and ALFPm6 in response to *V. harveyi* or yellow head virus infection [138, 139]. Silencing of members of the IMD pathway by dsRNA indicated the regulation of expression of different AMPs in crustaceans [133, 140].

## 3.3 JAK–STAT Signalling Pathway

The JAK/STAT pathway, consisting of three key cellular components, the transmembrane receptor Domeless, the Janus Kinase (JAK) and the signal transducer and activator of transcription (STAT), involves in the regulation of various immune responses [141]. Although JAK/STAT pathway has been reported to involve in the antiviral response in insects, the first STAT homologue with the typical functional domains from shrimp was reported in *P. monodon* [142]. The different components of JAK/STAT pathway have been identified and characterized in shrimp such as *Fc*STAT, *Pm*STAT, *Mj*STAT, *Lv*STAT, *Lv*JAK, *Lv*DOME etc. [142–146]. Further, it has been reported that transcription of STAT gets modulated after WSSV infection in shrimp [142, 143].

### 3.4 Other Pathways

Mitogen-activated protein kinases (MAPKs), the serine-threonine protein kinases, which are widely conserved across diverse organisms, play critical roles in cellular responses associated with inflammation, environmental stress and microbial attack. MAPKs in mammals have been categorized into three subfamilies: extracellular signal-regulated kinases (ERKs), stress-activated protein kinases/c-Jun N-terminal kinases (SAPK/JNKs) and p38 MAPK [147]. Many components of MAPK pathway have been reported in shrimp [148–152] and their role in the defence against bacterial and viral infections has been investigated.

Besides the pathogen-recognition pathways described above, interferon (IFN) system like antiviral regulatory mechanisms involving the activation of Vago exist in shrimp [153]. Moreover, a cytosolic sensing pathway with the evidence of functionally characterized IFN-activator *Lv*STING [154] has been reported from shrimp, *L. vannaemi*. The antiviral immunity of Toll3 from *L. vannamei* has been elucidated through dsRNA silencing and was found to be involved in the activation of IFN regulatory factor (IRF) expression and its downstream Vago4/5 [155].

## 4 Existence of Adaptive-Like Immunity in Crustaceans

A characteristic feature of vertebrate immune response is the adaptive or acquired immunity with specificity and immunological memory. T and B cells and antibodies are responsible for this type of immunological memory; however, invertebrates are found to be devoid of these cells. Many immunological studies in invertebrates suggest the existence of alternative adaptive immune response in their innate immune system. Enhancement in phagocytic activity of haemocytes upon re-encountering of foreign antigens following previous exposure has been reported in lobster [156]. Moreover, administration of β-1,3-1,6-glucan to spawners of *P. monodon* resulted in the transfer of disease resistance against WSSV infection in larvae. The phenomenon of 'trans-generational immune priming (TIGP)', which involves the transfer of immune memory to the offspring and to the following generations from a primed parent, has been reported in shrimp, *P. monodon* [157]. By causing transmissible changes in gene expression profiles, TIGP permits better protection of offspring when encountered with the pathogens inhabiting the same parental environment [158]. However, the molecular mechanism involved in these types of memory is not known.

Adaptive immunity in vertebrates is facilitated by the immunoglobulins (Igs) and T-cell receptors. Immunoglobulin superfamily (IgSF) proteins possess a minimum of one Ig domain and are key in recognizing, binding or adhesion processes of cells and/or pathogens [159]. However, several genes possessing Ig-like domains (IGs) which play critical role in host defence have been reported in invertebrates. Dscams have been implicated in alternative adaptive immunity in several arthropods, including crustaceans, which are capable of generating mRNA variants through alternative splicing [8, 9, 160]. Dscam plays a vital role in immunity through specific

recognition of pathogen and formation of pathogen-specific isoforms in response to pathogen challenge [8, 161, 162]. It is a large protein (~220 KDa) having 9 Ig domains followed by 4 fibronectin type (FN) III domains, an Ig domain, 2 FNIII domains, a transmembrane domain and a cytoplasmic tail. However, the tail-less form of Dscam lacking transmembrane domain and cytoplasmic tail also has been reported. Among crustaceans, Dscam was first reported from *L. vannamei* [8] and subsequently from *P. monodon* [9]. Both types of Dscam were reported in shrimp [8, 9]. Dscam-mediated immunity is contributed by the hypervariability of both its extracellular and cytoplasmic tail and generation of several Dscam isoforms through alternative splicing, which mediate recognition of diverse pathogens following exposure to immunostimulants and diverse pathogens [11, 12, 161–163]. Moreover, cytoplasmic tail of membrane-bound Dscam possesses endocytosis motifs that can elicit phagocytosis [163]. Moreover, the variability in C terminal region of Dscam generating various combinations of transmembrane domains and cytoplasmic tail could form the basis for triggering diverse signalling pathways [163]. The tail-less Dscam are produced either by direct exocytosis or can be cleaved off from membrane-bound Dscam mediated by Type III polyadenylation similar to the production of soluble IgM in vertebrates [12]. The tail-less Dscam mRNA expressions were found to be upregulated in shrimp haemocytes when stimulated with PAMPs, including LPS, PGN, β-1,3-glucans and virus [11]. It is presumed that, when invertebrate hosts are exposed to pathogens, a 'cloud' of several isoforms of Dscam with diverse properties and abundance are formed. However, the mechanism of producing specific isoforms of Dscam by host cell after a particular pathogen challenge and the mechanism behind the primed immunity are yet to be understood properly. Functionally, Dscam provides an 'immunological memory' and supports a unique immune mechanism, termed 'innate immunity with specificity' or 'immune priming' which provides adaptive immune characteristics to the innate immune system [161].

Another molecule related to adaptive-like immunity identified is FREPs. These are polymorphic lectin-like molecules involved in various immune responses such as recognition of pathogens, agglutination, lysis of bacteria and defence against parasites [164]. They are characterized by a common C-terminal fibrinogen-related domain (FReD) and variable N-terminal regions [165]. Human ficolin possess N-terminal collagen, whereas molluscan FREPs possess one or two Ig domains in their N-terminus [166]; however, crustacean FREPs lack hypervariable Ig domains. Different categories of FREPs have been reported and named as ficolins, tachylectins and FREPs [167]. The binding abilities of different FREPs towards microbial pathogens vary considerably. Different types of FREPs have been identified in crustaceans that play an important role in the immune response against different pathogens. These proteins showed differential agglutinating properties towards Gram-negative and Gram-positive bacteria and viruses and ligands such as LPS and PGN. They respond differently to bacteria (*Vibrio* sp.) and virus (WSSV) challenges. MIP has been characterized in crayfish (*P. leniusculus*) and shrimp (*P. monodon*) [168, 169], which functions as a regulator of PO-induced melanization. Two ficolin-like proteins reported from crayfish *P. leniusculus*

displayed agglutinating property with Gram-positive and Gram-negative bacteria in the presence of $Ca^{2+}$; however, bacterial clearance was observed only for Gram-negative bacteria [170]. The FREPs from *M. japonicus* (*Mj*FREP1) was reported to be up-regulated by challenge with *V. anguillarum* or WSSV [10] and was able to bind with PGN, LPS and VP28 of WSSV. The two ficolins reported from *Macrobrachium rosenbergii* could bind to Gram-positive and Gram-negative bacteria as well as to PGN and LPS, besides responding to WSSV or *Vibrio* challenge [171]. A FREP has been reported from *L. vannamei* which responded to *Vibrio* only, but not to WSSV [15]. Subsequently, ten transcripts of FREP have been reported from *L. vannmaei* [172] which responded differently to bacterial and virus challenge. Three tachylectin-like genes were reported from *P. monodon* which display agglutinating property with pathogenic vibrios, and RNAi experiment showed marked decline in the survival of the animals following bacterial infection [173, 174].

Hemolin, a specific immune protein belonging to Ig superfamily, has been identified and up-regulation of expression noticed in response to WSSV and *V. parahaemolyticus* in *L. vannamei* [14]. Similar to Dscam, hemolin functions as opsonin and can accelerate the phagocytosis and agglutination in the presence of $Ca^{2+}$. Hemolin consists of seven Ig domains which form a 'horseshoe' tertiary structure, which has been reported to function as the site of attachment to bacterial LPS [175]. However, the role of hemolin in adaptive immune response in shrimp has not been elucidated.

## 5 Summary

In summary, being an invertebrate, shrimps lack a true vertebrate-like adaptive immunity and mainly depend on the well-developed innate immune system consisting of cellular and humoral responses for dealing with the pathogens. The defence mechanisms of shrimp are activated when PAMPs residing on microbial surface are recognized by host-associated PRRs. The binding of PRRs with the specific PAMPs result in the activation of signalling pathways and trigger effector mechanisms (cellular or humoral) leading to the production of AMPs and destruction of invading pathogens. Recent studies on the innate immunity in shrimp suggest the existence of immunological memory and alternative adaptive immunity, though the underlying molecular mechanism is yet to be understood. Nevertheless, these discoveries point to the fact that 'vaccination' can be possible in shrimp which provides protection against pathogens. However, it may not be scientifically correct to expect similar responses and patterns of protection as observed in vertebrates.

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# Types of Vaccines Used in Aquaculture

Megha K. Bedekar, Sajal Kole, and Makesh M.

**Abstract**

Vaccination is the best method for disease prevention. A vaccine is an antigenic preparation intended to produce immunity to disease through stimulation of the production of antibodies and memory cells. An "ideal fish vaccine" should have the potential to generate specific immune response, protection, and memory. There are several methods for vaccine development and application. These methods range from conventional live vaccines to the latest molecular vaccines. Every type of vaccine has its own advantages and disadvantages and the choice of vaccine type depends on the type of target pathogen, immune response, safety of the recipient, and feasibility of the application. Vaccine is classified based on the method of preparation such as live attenuated vaccine, vectored vaccine, inactivated vaccine, and sub-unit vaccine. Live vaccines and killed vaccines are conventional methods of vaccine preparation which has potential for inducing specific immune response in host. However, their applications in aquaculture are limited due to constraint in delivery and uptake. Sub-unit vaccine developed using immunogenic units of pathogen like selected proteins or toxoids hold potential for vaccine development. Recombinant protein vaccine and vectored vaccines such as DNA vaccine, RNA vaccine, edible vaccine, and virus-like

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particles are advantageous because there is no need to culture the pathogen for vaccine production.

**Keywords**

Types of vaccine · Properties · Live vaccine · DNA vaccine · Peptide vaccine

## 1 Introduction

Aquaculture continues to be the fastest-growing food-producing sector in the world [1]. However, infectious diseases of bacterial, viral, mycotic, and parasitic origin still remain a major impediment in the intensification of aquaculture. In view of this, fish health management has become a critical component to disease control and is invaluable for improved harvests and sustainable aquaculture. Since the development of the first fish vaccine in the 1940s, vaccination is regarded as the most efficient and economical remedial measure in protecting the health of farmed finfish from various infectious agents [2]. The importance of vaccination is much higher for aquatic animals than those of terrestrial animals, as they are in continuous contact with the microorganisms in their aquatic environment. However, unlike their terrestrial counterpart, fish vaccine development has faced several challenges viz., limited knowledge of the fish immune system, vast diversity of pathogens and their susceptible host species, difficulties in identification and formulation of antigens, selection of efficient adjuvants and vaccine carriers, challenges related to the mode of delivery, and various laws and restrictions related to food fishes. Nevertheless, over the last four decades, fish immunologists have made profound efforts to understand the immune system and the host-pathogen interactions which in turn help to develop vaccination strategies for control of infectious diseases in commercial fish farming.

## 2 What Is a Vaccine?

A vaccine is an antigenic preparation intended to produce immunity to disease through stimulation of the production of antibodies and memory cells. It works by exposing the immune system of a healthy animal to an antigen and then allowing the host immune system to develop a response and a "memory" to accelerate this response in subsequent infections by the targeted pathogen [3].

## 3 Properties of Vaccine

An "ideal fish vaccine" should have the potential to generate an immune response. From the commercial and practical point of view, the vaccine needs to have long-term immune response, protection, specificity, and memory. While designing a vaccine, it should also be considered that the vaccine candidates should protect

against a broad range of pathogen strains. The vaccine needs to be user-friendly and cost-effective. Further, the vaccines should be safe for the fish, the person (s) vaccinating the fish, and for the fish consumers.

## 4 Types of Vaccine

A vaccine is classified based on the approach used to develop the vaccine. Each approach has its own advantages and specific mechanism of action. Vaccines are designed based on the feasibility of manufacturing and nature of infections. The choices for vaccine design are typically based on fundamental information about the microbe, such as how it infects cells and how the immune system responds to it, as well as practical considerations, such as size and value of the fish species to which it is administered. Broadly, vaccines can be classified based on antigen delivery systems: (1) Replicative antigen delivery system: live-attenuated vaccine, DNA vaccine, vector vaccine, and RNA vaccine; (2) Non-replicative antigen delivery system: whole-cell inactivated vaccine, sub-unit vaccine, toxoid vaccine, peptide vaccine, anti-idiotype vaccine, and edible vaccine (Fig. 1). Individual vaccine types are described as follows:

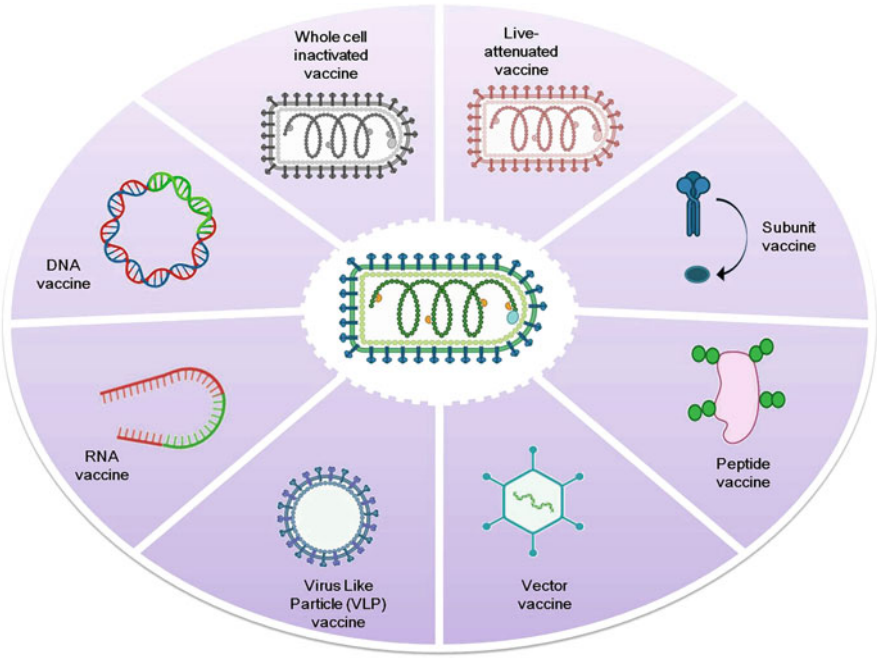


**Fig. 1** Types of vaccines

## 4.1 Live-Attenuated Vaccine

This type of vaccine contains live-attenuated microorganisms which are "weakened" or devoid of disease-causing capacity but still capable of replicating and presenting its immunogenic properties inside the host. These vaccines are prepared by various attenuation methods viz., chemical/heat attenuation, continuous passaging of the pathogen in different heterologous systems (heterologous animals, tissue culture, embryonated eggs), and genetic attenuation (mutation by deletion, disruption, or insertion of the metabolic pathway or virulence gene) [4] (Fig. 2). This vaccine being self-replicating does not need booster immunization and can elicit both humoral and cell-mediated immune responses which in turn help in triggering a high level of long-lasting protective immunity in the host. Live vaccines are the most potent way of active immunization and the results of vaccination are evident in humans and higher vertebrates. Various attenuation strategies have been employed for the development of live vaccines for fish viz., antibiotic mutagenesis for *Flavobacterium* spp., *Vibrio anguillarum*, *Edwardsiella tarda*, and *Aeromonas hydrophila* vaccines [5, 6], mutagenesis using acriflavin dye and novobiocin for attenuation of *Streptococcus agalactiae*, *Streptococcus iniae*, *Edwardsiella ictaluri*, and *A. hydrophila* [7], mutation of koi herpesvirus (KHV) by UV exposure for reducing its virulence and minimizing chances of reversion to pathogenic strain [8], and gene deletion technology used to delete the virulence gene from catfish herpesvirus [9]. Few modified live fish vaccines are licensed in different countries which includes *E. ictaluri* vaccine against enteric septicaemia of catfish (ESC), *Flavobacterium columnare* vaccine against columnaris in catfish [10, 11]; *Arthrobacter* vaccine, licensed in Chile and Canada against bacterial kidney disease (BKD) for use in salmonids having cross-protection against *Rennibacterium salmoninarum* [12]. Among licensed live-attenuated vaccines against viral pathogens, vaccine against viral haemorrhagic septicaemia virus (VHSV) is available in Germany [13], and a live viral vaccine against KHV for carp is available for use in Israel [14].

## 4.2 DNA Vaccine

DNA vaccine comprises a self-replicating extra-chromosomal plasmid containing the immunogenic gene of the pathogen (Fig. 3). DNA vaccination involves the

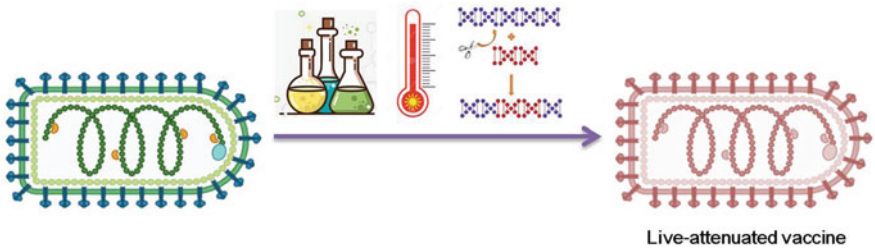


**Fig. 2** Live-attenuated vaccine

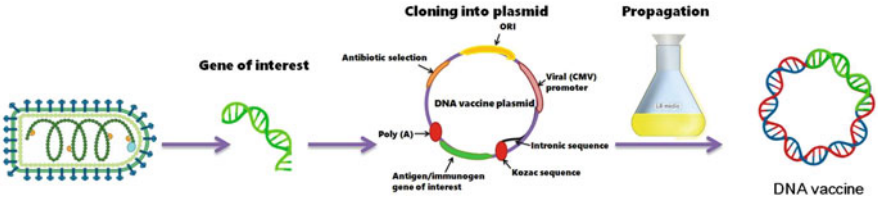


**Fig. 3** DNA vaccine

delivery of plasmid DNA (raised in microorganisms such as bacteria) encoding a vaccine antigen to the host [15]. Under the control of eukaryotic promoters, the plasmid DNA expresses itself inside the recipient, first by transcription into mRNA and then by translation into the protein encoded by the gene. The expressed antigenic proteins are recognized by the host immune system as "foreign", inducing strong and long-lasting humoral and cell-mediated immune responses without the risk of inadvertent infection. DNA vaccines have been experimentally tested against several fish pathogens viz., viral haemorrhagic septicemia virus (VHSV) [16–20], infectious hematopoietic necrosis virus (IHNV) [21–25], hirame rhabdovirus (HIRRV) [26–28], spring viraemia of carp virus (SVCV) [29–31], infectious salmon anaemia virus (ISAV) [32, 33], nervous necrosis virus (NNV) [34–36], salmonid alphavirus 3 (SAV3) [37, 38], grass carp reovirus (GCRV) [39, 40], infectious pancreatic necrosis virus (IPNV) [41–45], Koi herpes virus (KHV) [46–49], Channel catfish virus (CCV) [50], Lymphocystis disease virus (LCDV) [51, 52], *E. tarda* [53–60], *Aeromonas* sp. [34, 61], *Vibrio* sp. [62–69], and *Streptococcus* sp. [70–76]. DNA vaccines have also been effective in the prevention of infection caused by intracellular and difficult-to-culture bacteria, like *Mycobacterium marinum* [77]. Despite its effectiveness, several legal restrictions (primarily related to genome integration) for the use of DNA vaccine in food fishes in most of the countries hamper its licensing and commercialization. Two DNA vaccines have been commercialized for use in aquaculture viz., APEX-IHN (Novartis/Elanco) in 2005, for protecting Atlantic salmon against IHNV in British Colombia and CLYNAV (Elanco) in 2017, a polyprotein-encoding DNA vaccine against salmon pancreas disease virus (SPDV) infection in Atlantic salmon (*Salmo salar*) for use within the European Union (EU).

## 4.3 Vector Vaccine

Vector vaccine utilizes live virus vectors for transferring antigenic genes into the recipient host which in turn express the encoded protein of another pathogenic microorganism, as the vaccine antigen [78] (Fig. 4). The self-assembling ability of viral structural proteins with the resemblance of a native virus has resulted in the development of this class of sub-unit vaccines based on virus-like particles (VLPs) [79]. The baculovirus expression system has proven to be an improved approach for fast expression of plentiful recombinant proteins (VLPs) and is suggested to be an

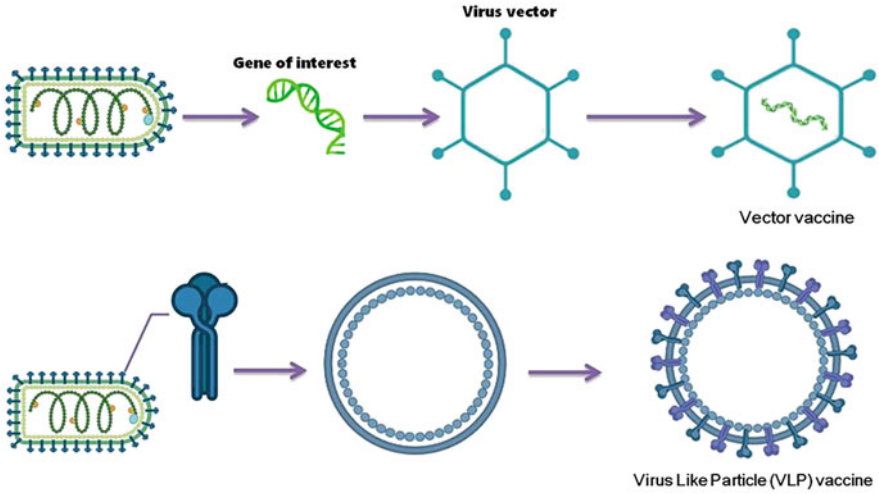


**Fig. 4** Vector vaccine

inexpensive and efficient method for producing heterologous proteins [80–82]. The vaccine antigens are capable of stimulating both humoral and cell-mediated immune responses whereas, the vector has the potential to actively replicate inside the host cells, activating the immune system like an adjuvant. VLPs can be produced in competent hosts such as bacteria, plant, or fungi. VLPs are also produced by genetic recombination of an unrelated virus-producing chimera. Few experimental VLPs-based vaccines have been developed in recent years viz., vaccine against infectious pancreatic necrosis, wherein the IPNV capsid protein VP2 expressed in yeast self-assembles into sub-viral particles (SVPs) and induce immune response in Rainbow trout [83]; vaccine against Atlantic cod NNV (ACNNV) for seabass, wherein the coat protein was expressed in plant, *Nicotiana benthamiana* [84]; vaccines against grouper nervous necrosis [85] and viral nervous necrosis [86] were developed for orange-spotted grouper and European seabass respectively, using self-assembly of VLPs. Salmonid alphavirus (SAV) replicon vectors are also commonly used for developing fish vaccines, as these vectors are functional in cells from a wide range of animal classes and express gene of interest (GOI) in the temperature range of 4 °C–37 °C [87, 88]. The alphavirus-based replicon has the advantage that it does not spread/ re-infect other cells after initial replication [88, 89] and also has the ability to improve mucosal immunity [90].

## 4.4 RNA Vaccine

RNA vaccines are of two types: self-replicating mRNA and non-replicating mRNA. The principle of mRNA vaccine is that the modified mRNA of the target gene is either cloned in a vector or directly injected into the host. This mRNA undergoes

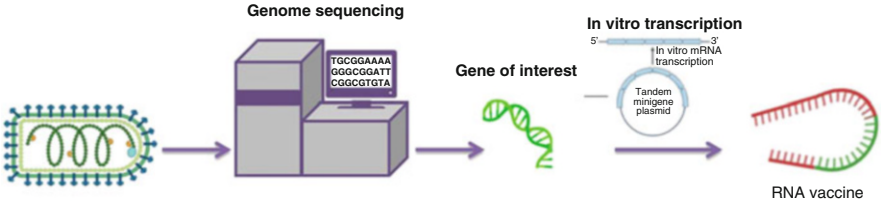


**Fig. 5** RNA vaccine

translation of the target protein. The protein is detected as a foreign substance by the host immune system and specific immunity is generated against the pathogen [47] (Fig. 5). Non-replicating mRNA, also called as NRM, are flanked by 5′ and 3′ untranslated regions (UTRs), a 5′-cap structure, and a 3′-poly-(A) tail [91]. Once the NRM enter the cell cytosol, it is immediately translated to protein. The self-amplifying mRNA, also called as SAM, has the same features as that of NRM. Additionally, the construct encodes replicase components which are able to direct intracellular mRNA amplification. SAM particles once delivered in cytosol, replicate to produce multiple copies of mRNA that are ultimately translated into protein. RNA vaccines are more efficient in stimulating antigen-specific cellular immune responses as compared to the conventional plasmid DNA vaccines [92]. With many advantages over DNA vaccine, mRNA vaccine could be developed against important fish pathogens. SAV-based replicon provided significant protection against SAV3. This SAV3 construct can be a future candidate for mRNA vaccine in fish [93].

## 4.5 Whole-Cell Inactivated Vaccine

Whole-cell inactivated vaccines are based on the principle of Louis Pasteur's "isolate, inactivate, and inject" [94]. These vaccines contain killed microorganisms (virus/bacteria/parasite) that have been inactivated through physical or chemical processes such as heat, formaldehyde, or radiation treatment (Fig. 6). The inactivated pathogens lose their ability to cause disease but remain antigenic or immunogenic to the host. The host in turn recognizes the foreign structure of the killed pathogen, and subsequently activates its immune system (mainly humoral immune system). However, being inactivated, these vaccines induce relatively weaker immune responses than live vaccines so they require suitable adjuvant as well as several booster doses for maintaining adequate level of protective immunity over longer time. Commercial inactivated vaccines have been reported for carps and salmon globally. The first report on vaccine trial in fish was on an inactivated vaccine against *Aeromonas salmonicida*, and an oral vaccine, attempted in cutthroat trout *Oncorhynchus clarkia* [95]. Inactivated vaccine recorded successful immune protection against *Yersinia ruckeri* and this was the first commercially licensed fish vaccine [96]. Following the success of killed vaccine, research on developing killed vaccines increased especially against the infections of high-value fish species such as Atlantic salmon

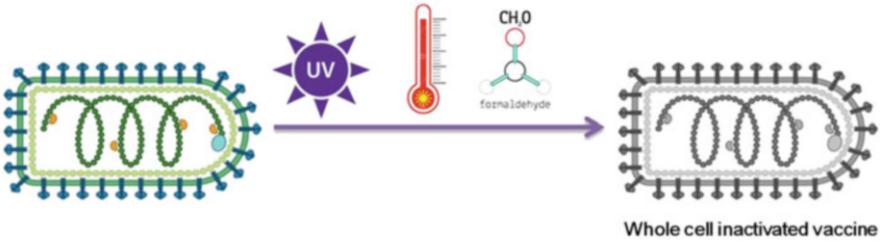


**Fig. 6** Whole-cell inactivated vaccine

[97]. Although this method was effective for developing vaccine against some fish pathogenic bacteria, its utility faced major obstacle for developing vaccine against most other fish pathogens, especially viruses. Nevertheless, the first inactivated viral vaccine for fish, against a carp rhabdovirus, causing spring viremia of carp (SVC) was produced by a Czechoslovakian company (Bioveta) in 1982.

## 4.6 Sub-Unit Vaccine

Sub-unit vaccine uses the recombinant technology where only the immunogenic target regions of a pathogen are expressed in a heterologous host from which the protective antigen is purified and used in vaccine formulation [78] (Fig. 7). Biotechnological tools are used for recognition and designing of the gene sequence of pathogen's protective antigen. After designing, the antigenic genes are inserted into prokaryotic [98] or eukaryotic [99] production hosts and are cultured on a large scale under strictly controlled laboratory conditions by fermentation technology, with the aim to produce the antigenic protein. The production hosts include bacteria [98], cell culture [100], yeast [101], insect cells [99], microalgae as well as transgenic plants [102]. However, in the case of fish vaccines, the administration of the recombinant antigens produced through fermentation was found to be inefficient in inducing protective immunity, which might be due to poor immunogenicity of the antigens [103, 104]. Molecular techniques enabled the expression of highly antigenic proteins of the target pathogen in bulk and subsequent delivery of the purified antigen as a vaccine. Although initial works on sub-unit vaccines in aquaculture were not successful due to the rapid degradation of the protein during production and transport, or in the gut of the animals, improvements were made to stabilize the antigens and many sub-unit vaccines have been developed. Most of the sub-unit vaccines are developed by expressing the sub-unit protein in *Escherichia coli*-based prokaryotic expression system. One of the most successful examples is a sub-unit vaccine against infectious pancreatic necrosis (IPN), comprising of fused IPN-VP2 gene. ISAV vaccine containing recombinant hemagglutinin-esterase protein is available as an oral vaccine in the name of Centrovet in Chile. Baculovirus system and yeast expression system have been used for the vaccine against viral haemorrhagic septicaemia and IHNV [105]. Although there are many reports on sub-unit vaccines for fish, they are not commercially available for use in aquaculture [6]. The major

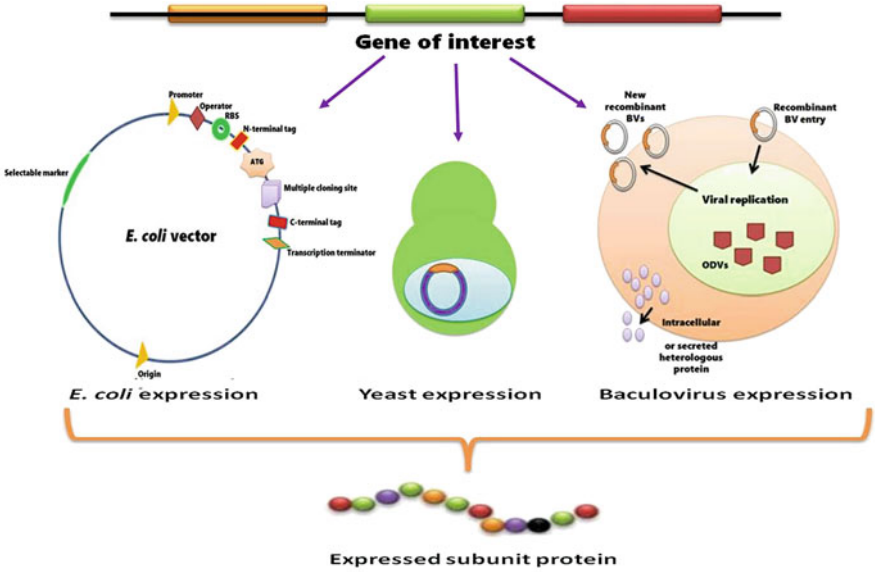


**Fig. 7** Sub-unit vaccine

issue with recombinant vaccines is the environmental safety and regulatory clearance. Thus, recombinant protein-based vaccines need to prove their environmental safety for field testing [106].

## 4.7 Toxoid Vaccine

Toxins (exotoxin and endotoxin) are components that are secreted by bacteria as part of their pathogenic response. Toxoid vaccine is generally developed from exotoxin. When toxicity of the toxin is inactivated or reduced by chemical or heat treatment, while maintaining its immunogenicity, it is called a toxoid (Fig. 8). Toxoid has a capacity to trigger the immune response and mount immunological response and memory. When the immune system receives a vaccine containing a harmless toxoid, humoral immune system is activated and produces antibodies that lock onto and block the toxin. This is also termed as anatoxin. In aquaculture, few reports of experimental trial of toxoid vaccine with low antibody response are available. Toxoid-enriched inactivated vaccine containing *Photobacterium damselae* subspecies *piscicida* was reported to give 37–41% protection. The toxoid vaccine has also been tried against *A. salmonicida* [107].

## 4.8 Peptide Vaccine

Peptide vaccines are synthetic peptides or small amino acid domains on the surface of a carrier protein, which have the capacity of generating immune responses in the

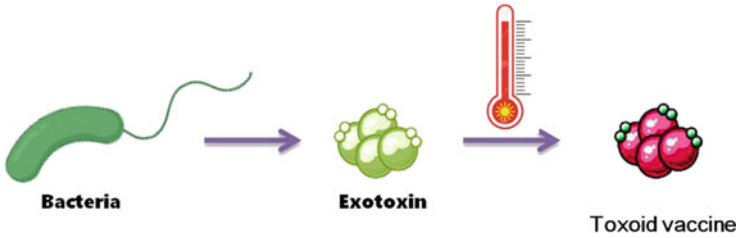


**Fig. 8** Toxoid vaccine

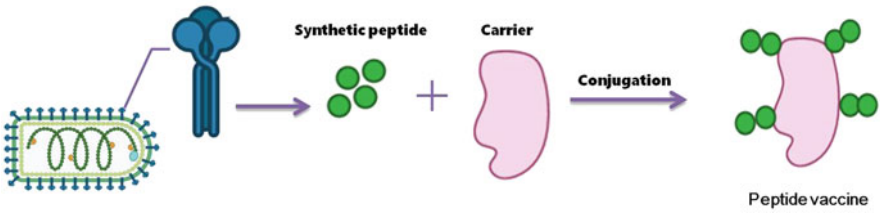


**Fig. 9** Peptide vaccine

recipient host (Fig. 9). The small amino acid domain that has the potential to generate immunogenicity is first identified using bioinformatic tools such as Predict Protein, Prosite, SwissProt and Epitope mapper. The peptide is then synthesized and the synthetic peptide is used as a vaccine to generate the immune response. These are referred to as peptide vaccines as they have the potential to generate immune response and memory. The short peptides are bound to some surface carrier proteins and used as a vaccine. Although, they are very simple and safe, due to low immunogenicity their applications are limited in fish.

## 4.9 Anti-Idiotype Vaccine

This vaccine comprises of antibodies that have three-dimensional immunogenic regions, designated as idiotopes that consist of protein sequences which can bind to cell receptors (Fig. 10). Idiotopes are aggregated into idiotypes, specific to their target antigen. Thus, anti-idiotypes are antigen-mimics that can trigger immune response in the host. These anti-idiotypes can be purified from serum or can be designed using bioinformatics-based molecular docking approach and used as antigen replacement. However, this is yet to be explored in fish vaccination.

## 4.10 Edible Vaccine

Edible vaccines are plant-based vaccines prepared by molecular farming where whole plants or plant cells/tissues are cultured in vitro for the production of

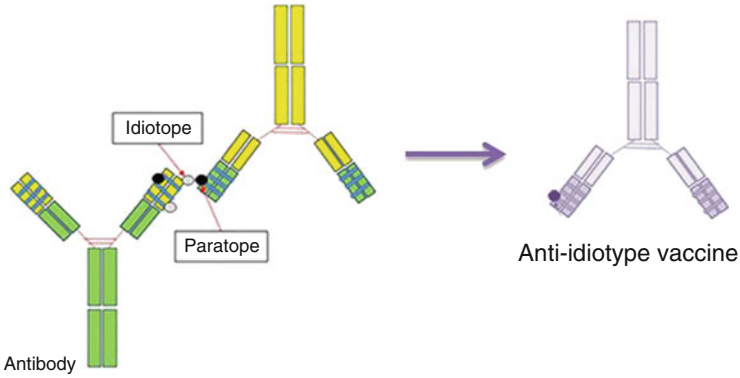


**Fig. 10** Anti-idiotype vaccine

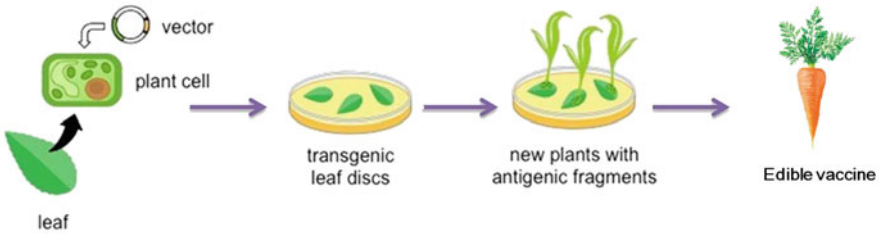


**Fig. 11** Edible vaccine

immunogenic proteins [108] (Fig. 11). These are potentially cheap to produce and are viable alternative to mainstream production systems. Edible vaccines, after consumption, expresses the antigenic proteins, which are then transported via specialized M-cells to the dendritic cells subsequently activating a coordinated immune response involving B-cells and T-helper cells. This vaccine technology is at an early stage for fish vaccines [109] but likely to develop in the near future.

## 5 Conclusion

Vaccination is the best method for disease prevention, and there are several options for vaccine development and application. These methods range from conventional live vaccines to the latest molecular vaccines. Every type of vaccine has its own advantages and disadvantages and the choice of vaccine type depends on the type of target pathogen, immune response, safety of the recipient, and feasibility of the application. The advantages and disadvantages of each type of vaccine are given in Table 1. Vaccination and developing a strategy for successful vaccination in fish have various challenges which can be addressed by modern vaccine methods such as a recombinant protein-based vaccine, VLPs, and synthetic peptides. In the present scenario of emerging diseases which cause serious impact on aquaculture

**Table 1** Advantages and disadvantages of vaccines

| Vaccine type | Advantages | Disadvantages |
|---|---|---|
| Live-attenuated vaccine | Being self-replicating does not need booster immunization<br>Provides long-lasting protective immunity to the host<br>Can be administered easily through oral or immersion method | Possesses the risk of recombination of different strains resulting in the emergence of the new strains<br>Has the risk of reverting to virulent strain<br>Causes serious threat to off-target animals and the aquatic environment<br>Not suitable for immuno-compromised animals as they work on an active immune system |
| DNA vaccine | Induces strong and long-lasting protective immunity to the host<br>Possesses no risk of inadvertent infection<br>DNA vaccines are stable in dried powder or in a solution and do not need a cold chain<br>The vector can encode for multi-valent vaccine against multiple diseases, which could be given in a single administration<br>DNA vaccines are relatively cheap and are easy to produce via identical production processes | Legal restrictions (primarily related to genome integration) for the use of DNA vaccine in food fishes in most of the countries hampers its licensing and commercialization |
| Vector vaccine | Apart from the antigen, the vector has the potential to replicate inside the host cells actively and can activate the immune system like an adjuvant<br>The alphavirus-based replicon has the advantage that it does not spread/re-infect other cells after initial replication<br>The alphavirus replicon has the ability to improve mucosal immunity | Pre-existing antibodies against the vector virus can neutralize or inhibit the viral vector, thereby reducing the targeted immune response against the foreign antigen<br>Vector vaccine technology is still new to fish vaccine development and has been tested to a minimal extent |
| RNA vaccine | RNA vaccines are not made from pathogen particles or inactivated pathogen, so are non-infectious<br>Unlike DNA vaccine, RNA vaccine does not integrate itself into the host genome and gets degraded once the protein is made<br>Limited clinical trial results indicate that these vaccines generate a strong immune response and are well-tolerated by healthy individuals | Very new technology, so tested to a very limited extent in finfish vaccinology |
| Whole-cell inactivated vaccine | Unlike live attenuated vaccines, the inactivated vaccines does not carry the risk of mutating back to their disease-causing state<br>Do not require cold chain for storage and can be easily transported in freeze-dried | Being inactivated, these vaccines induce relatively weaker immune responses, so they need several booster doses for maintaining adequate level of protective immunity over a longer time |

(continued)

**Table 1** (continued)

| Vaccine type | Advantages | Disadvantages |
|---|---|---|
| | form<br>These vaccines are easy to manufacture and are economically feasible | To maximize their effectiveness, they require suitable adjuvant<br>Mostly injection mode of delivery is effective |
| Sub-unit vaccine | Have no live components, thus no risk of inducing disease<br>Safe, stable, and easy to manufacture | Although very effective against human and animal pathogens, in the case of fish vaccine, the administration of the recombinant antigens is found to be inefficient in inducing protective immunity<br>Poor immunogenicity of the antigens, induces a less strong immune response<br>Often a response can be elicited, but there is no guarantee that immunological memory will be formed in the correct manner |
| Toxoid vaccine | Toxoid has the capacity to trigger an immune response and mount immunological response and memory<br>These are extremely safe methods of immunization and are less likely to induce any side effects<br>They can also work in immuno-compromised individuals | May require several doses and usually need an adjuvant<br>Relatively low antibody responses are reported from the limited experimental trial of toxoid vaccine in aquaculture, reducing its applicability |
| Peptide vaccine | They are very simple and safe. | Due to low immunogenicity, their applications are limited in fish vaccinology |
| Anti-idiotype vaccine | Anti-idiotypes can be purified from serum or can be designed using bioinformatics-based molecular docking approach and is used as an antigen replacement | Yet to be explored in fish vaccination |
| Edible vaccine | These are potentially cheap to produce and are a viable alternative to mainstream production systems such as microbes and mammalian cells cultivated in large-scale bioreactors<br>Unlike other recombinant technologies, they are free from undesirable components, e.g., endotoxins in bacteria, and hyperglycosylated proteins produced by yeast<br>There is no limit to their production scale, and the cost of scaling up is low | This vaccine technology is at an early stage for fish vaccines but likely to develop in the near future. |

production, it is important to focus more on developing effective vaccines so that infectious diseases can be prevented and production losses can be minimized.

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# Vaccines Against Bacterial Pathogens of Fish

Gaurav Rathore

**Abstract**

Globally bacterial diseases cause significant economic losses to aquaculture production. Vaccination against bacterial pathogens is one of the most effective measures for preventing bacterial diseases, especially in finfish aquaculture. Both live and inactivated vaccines are predominantly available for use in aquaculture against bacterial diseases in many countries. Most of these vaccines are known to provide high efficacy in eliciting long-term immunity. Besides, several new approaches such as sub-unit vaccines and DNA vaccines have also been successfully demonstrated to provide protection against bacterial pathogens of fish. A summary of bacterial vaccines either in use in aquaculture or under research is compiled in the chapter.

**Keywords**

Fish · Bacterial diseases · Inactivated vaccines · Live vaccines · Sub-unit vaccine · DNA vaccine

## 1 Introduction

Bacterial diseases cause substantial losses to aquaculture production globally impeding both economic and social development in many countries. In USA alone, annual loss due to 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 as well. A total of 13 bacterial genera are known to cause

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disease outbreaks in aquaculture production systems [2]. The majority of bacterial pathogens of fish belong to the families *Enterobacteriaceae, Aeromonadaceae, Psuedomonadaceae, Vibrionaceae, Flavobacteriaceae,* and *Streptococcaceae*. Bacterial diseases can be treated with antibiotics, but the emergence of anti-microbial resistance in bacterial pathogens not only leads to treatment failures but is also a potential threat to public health. On the other hand, vaccines have the advantage of being environmentally friendly, safe, and offer long-term protection.

There are two major types of vaccines available for bacterial diseases: (1) Live vaccines (2) Inactivated vaccines

## 1.1 Live Vaccines

These vaccines include live-attenuated pathogen that replicates inside the host. Some of the common attenuation strategies to develop live vaccines for fish include antigen mimicry, laboratory passage, chemical or physical mutagenesis, and genetic modification using molecular techniques. Major advantages include: (1) live pathogen can stimulate both humoral and cellular immune responses; (2) as live pathogen can multiply inside the host, lesser quantities are needed to induce protection and, therefore, even single administration of vaccine can have high efficacy in eliciting long-term immunity; (3) whole microbes present all the antigens in their natural conformation; hence, they offer better immune response to pathogens; (4) live vaccines can induce mucosal immunity and provide protection at mucosal surfaces that are the main site of entry. Major disadvantages are: (1) live pathogen could sometimes revert to its virulent form and cause disease; (2) live vaccines need to be stored at proper storage conditions and, otherwise, it may affect its viability and efficacy; (3) live vaccines cannot be used in immuno-compromised individuals.

## 1.2 Inactivated Vaccines

These vaccines include an inactivated pathogen that does not replicate inside the host**.** Another category is s*ub-unit vaccines* **–** Sub-unit vaccines are the specific immunogenic proteins of the bacteria purified from a pathogen or expressed in recombinant vectors.

Inactivated or killed vaccines consist of bacteria or other pathogens that have been cultivated in artificial culture conditions and, for the use as vaccine agent, the pathogens are killed using physico-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. Inactivated vaccines have several advantages such as ease of production, ecologically safe, and having low cost of production. Their storage requirements are not as critical as live vaccines. However, they have some disadvantages: (1) as the bacteria are dead and cannot multiply, a large quantity of antigen is needed to confer protection; (2) regular booster doses must be given for continued protection; (3) killed antigens induce only humoral

immunity; (4) depending on the inactivation methods, antigenicity may be altered; (5) incomplete inactivation of the pathogen can lead to vaccine-associated disease.

Sub-unit vaccines use specific epitopes of the antigen that bind to antibodies or T cells. As the sub-unit vaccines contain only specific epitopes of the pathogen, the chances of adverse reactions to these vaccines are less. Sub-unit vaccines are produced in large quantities from organisms either directly or in vitro as recombinant proteins through different eukaryotic and prokaryotic expression. The advantages of sub-unit vaccines are: (1) sub-unit vaccines can be safely given to immunosuppressed animals; (2) side effects are less as compared to whole organism. The disadvantages of the vaccine include: (1) native conformation of the recombinant epitopes may be altered and, therefore, at times, antibodies produced against such epitopes may not recognize the pathogen and fail to induce protection; (2) It has been observed that whole organism stimulates the immune system more efficiently as compared to specific epitopes.

DNA vaccines are genetic constructs containing genes encoding protective antigens of the pathogen. These genetic constructs could contain one or more genes of the pathogen, and when injected into the host lead to the expression of desirable protein from the genes that elicit a protective immune response. In other words, DNA vaccine is a purified bacterial plasmid carrying genes encoding protective antigens of the pathogen. These plasmids have regulatory elements for replication in the bacterial host so that their copy numbers can be increased. The target gene is inserted in the plasmid in correct frame to ensure the expression of the cloned genes in the vaccinated animals. DNA vaccines possess many advantages such as (1) better stability and, hence, storage and transport are easy; (2) the inserted DNA expresses only the proteins of interest, so has less side effects; (3) a DNA sequence can be easily manipulated in the laboratory; (4) as DNA vaccine contains only DNA, the host immune response against the vaccine is minimal. The disadvantages of DNA vaccines include: (1) the plasmid may integrate into host genome leading to insertional mutagenesis; (2) antibodies may be produced against introduced DNA leading to host autoimmune response; (3) continuous expression of the antigen in the host may lead to tolerance.

## 2 Status of Vaccine Development for Major Bacterial Diseases of Fish

### 2.1 Vibriosis (Infections Caused by *Vibrio* Species Mainly *Vibrio anguillarum*, *V. alginolyticus*)

#### 2.1.1 Inactivated Vaccines

A licensed bacterin (GAVA-3) covers O1, O2a, and O2b the highly pathogenic serotypes of Vibrio [3]. Majority of commercial vaccines available appear 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, and the vaccine resulted in efficient protection against all sero-subgroups [5].

### 2.1.2 Live Vaccine

Two live-attenuated vaccines constructed by transposon insertion mutagenesis and one antibiotic-resistant mutant have been demonstrated to provide immunization against homologous as well as a heterologous strain of *V. anguillarum* for at least 2 weeks in rainbow trout [6]. Efficacy of bath vaccination with a live-attenuated *V. harveyi* against vibriosis in fingerling of Asian seabass, *L. calcarifer* has been demonstrated [7].

A live-recombinant vaccine secreting DegQ soluble antigen, against *V. harveyi* in turbot has shown to be effective by intraperitoneal, oral, and immersion [8] routes. A serine endoprotease deletion mutant of *V. harveyi* was successfully evaluated as live vaccine for grouper [9]. Similarly, *V. alginolyticus acfA* gene mutant [10] and ΔclpP mutant [11] have been used as a safe live-attenuated vaccine for control of vibriosis in grouper.

### 2.1.3 DNA Vaccine

A plasmid DNA vaccine construct containing flagellin *flaA* gene has shown 88% relative percent survival (RPS) [12]. Likewise, a bivalent *V. harveyi* DNA vaccine expressing DegQ and Vhp1 immunogens has been tested in Japanese flounder with more than 70% RPS [13]. This vaccine also elicited cross-protection against *V. parahaemolyticus.* DNA vaccine expressing ompW has 92% RPS [14]. 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 [15]. DNA vaccine containing *omp*38 administered intra-muscularly to Asian seabass showed significant rise in serum antibody levels against *V. alginolyticus* with a 55.56% RPS [16].

*omp*38 DNA vaccine when administered orally along with chitosan nanoparticles in Asian seabass gave relatively lower RPS (46%) [17]. Similarly, another *omp*-based DNA vaccine, containing *ompK* was evaluated in black seabream against *V. parahaemolyticus* infection. When vaccine encapsulated in chitosan was fed orally, it evoked immune response within 3 weeks with relative survival rate of 72.03% [18].

### 2.1.4 Sub-Unit Vaccine

Out of four flagellins (rlaA, rflaB, rflaD, and rflaE) of *V. anguillarum*, rflaB vaccine gave higher level RPS in flounder by IP injection [19]. Recombinant protein VirB11 injected through intraperitoneal route offered protection against *V. harveyi* in grouper [20]. Vaccines containing *V. harveyi* recombinant outer membrane protein K (rOmpK), outer membrane protein U (rOmpU), and rOmpK-OmpU fusion proteins elicited protection in orange-spotted grouper by injection [21]. LamB, a member of outer membrane protein (OMPs) family of *Vibrio* species, can serve as an edible plant-based candidate vaccine for vibriosis in zebrafish [22].

## 2.2 Motile Aeromonads Septicaemia Disease (Infection by *Aeromonas hydrophila)*

### 2.2.1 Inactivated Vaccines

Biofilm vaccines appear to have effective responses in many fish species [23–25]. These vaccines elicited high serum antibody and protective response in three carp species up to 60 days [24]. IP administration of biofilm, extracellular product, *omp* and whole-cell vaccines also produced good immune response in goldfish. Immunization with biofilm and *omp* along with immune adjuvant, *Asparagus racemosus* tuber powder showed significantly increased survival after 25–50 days post-vaccination [26]. A formalin-killed whole-cell vaccine showed RPS of 80% [27]. In rainbow trout, IP administration of bacterial lysate significantly lowered mortality [28]. A whole-cell inactivated vaccine obtained via sonication and link to single-walled carbon nanotubes was used as a novel vaccine by immersion in grass carp [29]. PLGA microparticle-encapsulated formalin-killed *A. hydrophila* cells induced longer and more potent immune responses in carp [30]. Vaccination with oil adjuvant-inactivated *A. hydrophila* through intraperitoneal route conferred protection in pacu [31].

### 2.2.2 Live Vaccine

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 [32]. Transposon mini-Tn5 mutagenesis induced growth-deficient mutants used as vaccine resulted in 40% increase in survival [33]. An aroA gene mutated strain conferred significant protection [34]. Effective immunization of *Labeo rohita* with rough lipopolysaccharides containing live-attenuated vaccine having 100,000-fold less virulence has been developed by Swain et al. [35], after simple laboratory passage for 8 years. A novobiocin and rifampicin-resistant live strain demonstrated 86–100% protection in channel catfish and Nile tilapia using intraperitoneal injection [36]. A live *Aeromonas veronii* mutant, Δ*hisJ,* used as attenuated vaccine in the loach was found to be protective [37].

### 2.2.3 Sub-Unit Vaccine

Live-recombinant *Lactococcus lactis* vaccines expressing aerolysin genes provided high RPS [38]. Similarly, recombinant *Lactobacillus casei* (surface-displayed or secretory) expressing the *Omp*AI conferred protection against *A. veronii* in common carp [39]. A dose-dependent protective immunity in rohu was observed when a recombinant *Omp*W was orally administered along with PLGA nanoparticles [40].

Recombinant rOmpR demonstrated significantly reduced mortality 140 days post-immunization [41]. *Omp*TS-based recombinant vaccine immunization of Indian major carp has demonstrated high antibody titres on day 28 post-vaccination [42]. Bath and intramuscular injection immunization by single-walled carbon nanotubes-aerA sub-unit vaccine has shown 80% survival in vaccinated fish [43]. A conserved 46 kD maltoporin (*Omp*) of *A. hydrophila* proved to be a versatile

vaccine candidate in European eel (*Anguilla anguilla*) by injection [44]. Recombinant *Omp*A1, Tdr, and TbpA [45] and ATPase [46] of virulent *A. hydrophila* are known to protect channel catfish against motile Aeromonas septicaemia. Comparative extracellular proteomics of *A. hydrophila* has revealed iron-regulated secreted proteins as potential vaccine candidates [47]. Immunization with extracellular proteins (ECPs) of *A.veronii* can effectively enhance the ability of *Cyprinus carpio* to resist bacterial invasion [48].

## 2.3 Furunculosis (Infection by *Aeromonas Salmonicida* Subsp. *Salmonicida)*

### 2.3.1 Killed Vaccine

Low-frequency sonophoresis (LFS)-adjuvanted bacterin was intraperitoneally given to rainbow trout which resulted in high up-regulation of IgM antibody [49]. Romstad et al. [50], evaluated RPS of Atlantic salmon intraperitoneally immunized with ten commercially available vaccines. They demonstrated that RPS at $LD_{60}$ was maximum in comparison with RPS at $LD_{90}$. Mineral oil-adjuvanted vaccines; AlphaJect 3000® and an experimental autovaccine when tested in rainbow trout resulted in 78% and 56% RPS, respectively [51].

### 2.3.2 Live Vaccine

Rainbow trout vaccinated with A-protein, O-antigen or both deficient strains by immersion resulted in significant protection from challenge with a virulent strain of *A. salmonicida* [52]. A live-attenuated *aro*A-deficient mutant was used to immunize brown trout resulting in significant protection against live infection [53]. Another live-attenuated vaccine developed from *aro*A-deficient mutant of *A. salmonicida* was demonstrated with more than 60% RPS in rainbow trout [54].

### 2.3.3 Recombinant Vaccine

Fourteen proteins of *A. salmonicida* were recombinantly expressed in *Escherichia coli,* and found to be effective as sub-unit vaccines in rainbow trout by injection [55].

## 2.4 Enteric Septicaemia of Catfish (Infections Caused by *Edwardsiella ictaluri*)

### 2.4.1 Live Vaccine

An USDA-approved *E. ictaluri* strain, RE-33-live vaccine, has been shown to provide protection for at least 4 months following a single bath immersion without any risk [56] and, later, the vaccine strain was patented [57]. Modified version of this vaccine, AQUAVAC-ESC™, is being marketed with 87.9% RPS [58]. A novel attenuated *E. ictaluri* vaccine agent (B-50348) developed through selection for novobiocin resistance mutant was successfully tested for immersion and intraperitoneal vaccination [59]. Live vaccine containing a cyclic adenosine

3′,5′-monophosphate receptor protein (CRP) mutant demonstrated high IgM titre after bath immunization in catfish [60]. 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 [61]. A live-attenuated *E. ictaluri* bacterium lacking a viable gene encoding a functional evpB protein protects channel catfish against infection from virulent *E. ictaluri* by immersion [62].

*aroA*-attenuated vaccine against *E. ictaluri* has been demonstrated to be effective in laboratory studies [63]. An O polysaccharide-deficient isolate of *E. ictaluri* was used as a live vaccine, but it did not offer any protection [64]. Potential use of an *E. ictaluri* wzz mutant as a live-attenuated vaccine against enteric septicaemia was successfully tested in *Pangasius hypophthalmus* [65]. Vaccination with haeme/haemoglobin receptor family protein (HemR) mutants of *E. ictaluri* was found to be safe and protective in catfish fingerlings [66].

Oral immunization with rifampicin-resistant live vaccine (S97–773) has offered 82.6–100% RPS [67].

## 2.5 Edwardsiellosis (Infection by *Edwardsiella tarda*)

### 2.5.1 Killed Vaccine

Formalin-killed *E. ictaluri* whole-cells and an *E. ictaluri* rGAPDH combined vaccination adjuvanted with ISA 763A in tilapia showed 71.4% RPS on challenge with virulent *E. tarda* 3 months post-immunization [68]. A killed, but metabolically active (KBMA) *E. tarda* vaccine in olive flounder showed significantly higher survival rates than fish immunized with formalin-killed cells [69]. Starch hydrogel-based oral (SHO) vaccine was found to be better than formalin-killed cells in protection against edwardsiellosis in Japanese eel [70].

### 2.5.2 Live Vaccine

A live-attenuated vaccine, with mutation in aroC gene evaluated in turbot showed long protection [71]. Booster immunization of a temperature-sensitive *E. tarda* mutant induced 100% protection [72]. A recombinant live *E. tarda* mutant, lacking UDP-glucose dehydrogenase showed 76.7% RPS [73].

### 2.5.3 Sub-Unit Vaccine

Recombinant *rFimA* elicited high level of protection in turbot [74]. A recombinant vaccine *rGAPDH* significantly increased transcription levels of immune genes of vaccinated fish [75]. Recombinant *FlgD* protein was used to immunize a zebrafish model and an RPS of about 70% was observed [76]. Recombinant r*Omp*V of *E. tarda* along with cytokines expression plasmids as adjuvants showed significant immune protection against *E. tarda* infection [77]. Similarly, recombinant isocitrate dehydrogenase (rIDH) of *E. tarda*, an immunogenic protein located on the membrane, was found to be a promising vaccine candidate [78].

### 2.5.4 DNA Vaccine

DNA vaccine encoding molecular chaperone GroEL showed an RPS of 60% [79]. A bicistronic DNA vaccine for *L. rohita* against *E. tarda* was constructed using GAPDH gene (37 kDa) of *E. tarda* and IFN-γ as an immune adjuvant. Immunized fish showed significant level of specific and innate immune responses [80]. This DNA vaccine nanoconjugated with chitosan nanoparticles showed an RPS of 81.82% in oral and 72.73% in immersion vaccinated rohu fish [81].

## 2.6 Columnaris Disease (Infection by *Flavobacterium columnare*)

### 2.6.1 Inactivated Vaccines

*Flavobacterium* bacterin has shown to protect carp by bath immunization against experimental challenge. In channel catfish, vaccination by immersion in a bacterin has shown significant decrease in mortality compared to unvaccinated fish [82]. In the US and Canada, a bacterin-based immersion vaccine (Fryvacc 1) is currently being used in salmonids for protection against columnaris disease. A bivalent vaccine named as Fryvacc 2 which contains bacterins of *F. columnare* and *Y. ruckeri* is available in Chile [83]. Coating bacterins in alginate microparticles did not improve the efficacy of vaccine in tilapia [84]. Formalin-killed vaccine complexed with chitosan greatly increases its muco-adhesiveness and protection against columnaris infection [85].

Protection in coho salmon has been demonstrated with heat-killed cells of *F. columnare* incorporated into fish feed [86]. Similarly, feeding for over 3 months of formalin-killed bacteria provided high levels of protection [87]. Significant humoral response was observed in tilapia within 2 weeks after the use of formalin-killed sonicated cells in combination with Freund's complete adjuvant. Rise in the titre of the antibodies was also seen after secondary immunization, which persisted even at 10 weeks post-immunization [88].

### 2.6.2 Live Vaccine

A live vaccine named as Aquavac-Col is currently being used in channel catfish fry in USA for protection against *F. columnare*. In catfish, the RPS ranges between 57 and 94%, while in largemouth bass fry it was between 74 and 94% after challenge with virulent *F.* columnare [89]. A rifampicin-modified *F. columnare* has been developed and patented by USDA-Agricultural Research Service. This vaccine is protective with RPS values ranging from 50 to 76.8% [90]. Vaccinated largemouth bass fry showed 43% lower risk of death by *F. columnare* during the field trial [89].

### 2.6.3 Sub-Unit Vaccine

Recombinant DnaK protein (rDnaK) of *F. columnare* showed significant increase in survival of channel catfish against columnaris disease following bath immunization [91].

## 2.7 Bacterial Coldwater Disease (Infection by *Flavobacterium psychrophilum*)

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

### 2.7.1 Inactivated Vaccines

Positive correlation is seen between specific antibody response and protection against *F. psychrophilum* in rainbow trout [92, 93] and in ayu [94, 95] after intraperitoneal immunization, but not with immersion [92, 96]. Oral vaccinations of ayu [97] and rainbow trout [98] have also been successful. Injectable polyvalent vaccine containing formalin-inactivated *F. psychrophilum* in rainbow trout resulted in 100% protection [99].

### 2.7.2 Sub-Unit Vaccines

Intraperitoneal immunization with a recombinant protein combined with an adjuvant [100] offers a better protection as compared to sub-unit vaccines without the use of conventional adjuvants [94, 101]. In a similar study, high survival rates were seen during IP immunization of rainbow trout with a 70–100 kDa fraction in combination with an adjuvant [102]. However, recombinant heat-shock proteins/high molecular weight proteins or DNA vaccine failed to confer protection [101, 103]. Intraperitoneal immunization of OMPs of the pathogen is reported to be protective [94, 104, 105].

### 2.7.3 Live Vaccines

Several types of live-attenuated strains (grown under iron-limiting conditions [106]) (rifampicin resistant [107]) have been used for vaccination trials with high to moderate success. Live-attenuated *F. psychrophilum* vaccine provided protection against mixed flavobacterium infection [108]. Another live-attenuated immersion vaccine (*F. psychrophilum*; B.17-ILM) has shown to confer significant protection to salmonids [109].

## 2.8 Photobacteriosis (Infection by *Photobacterium damselae* Subsp. *piscicida*)

Photobacteriosis is a bacterial septicaemia and is also known as Pasteurellosis or Pseudotuberculosis. It affects white perch, striped bass, yellowtail, seabream, seabass, and sole.

### 2.8.1 Inactivated Vaccines

Efficacy of inactivated vaccines delivered by immersion can be improved by use of ultrasound treatment [110]. Incorporation of oil adjuvants offers better efficacy for injectable preparations [111]. Use of glucose and/or salt-enriched media to grow the bacterial cells [112] and iron-depleted media has shown to offer better protection [113]. Bacterins prepared from extracellular products (ECP) or LPS [114] and

capsular polysaccharide [115] 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 seabass and yellowtail [116].

### 2.8.2 Live Vaccines

Siderophore-deficient strain has been used as live vaccine [117]. A live vaccine using aro-A-deletion mutant has been patented in the US for use in hybrid striped bass [118].

### 2.8.3 Sub-Unit Vaccine

Recombinant OMP FrpA achieved a 73% RPS against experimental infections with *P. damselae* subsp. *piscicida* in sole by injection [119].

## 2.9 Yersiniosis (Infection by *Yersinia ruckeri*)

Yersiniosis or Enteric redmouth disease (ERM) is caused by *Y. ruckeri*, which mainly affects young salmonids in hatchery. Immersion-based inactivated bacterins are available for protection against Hagerman's O1 biotype 1 and biotype 2 variants of *Y. ruckeri* [120, 121]. Role of antibody-mediated protection of bacterins is not clearly established as *Y. ruckeri* resides within macrophages [122].

### 2.9.1 Inactivated/Sub-Unit Vaccines

The O-antigen of the lipopolysaccharide confers high levels of protection against yersiniosis in trouts [123]. Toxoid of the Yrp1 protease is also known for its protective ability against yersiniosis [124]. Recombinant outer membrane porin F (*Omp*F) of *Y. ruckeri* has been evaluated as a potential antigen for sub-unit vaccine for channel catfish [125].

### 2.9.2 Live Vaccines

A live vaccine using aro-A-deletion mutant has been shown to provide superior protection compared to the bacterin-based vaccines [126].

## 2.10 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 with a body weight of more than 15 g by injection.

### 2.10.1 Inactivated/Sub-Unit Vaccines

Formalin-killed cells along with concentrated extracellular products have been reported to offer significant protection to larger fish, with an RPS of 80% at 30 days post-vaccination [127]. Chen et al. [128] identified ten distinct pulsed-field gel electrophoresis (PFGE) genotypes (A–J) of *S. agalactiae* and used them to develop an inactivated whole-cell bacterial vaccine.

### 2.10.2 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* [129]. Attenuated erythromycin-resistant *S. agalactiae* was found to be a potent vaccine for tilapia [130]. Naturally attenuated *S. agalactiae* strain TFJ0901 has been used as a live vaccine in Nile tilapia [131]. *S. agalactiae* ghost vaccine was developed in culture supplied with penicillin and streptolysin, and then treated with the MIC of sodium hydroxide was able to protect tilapia [132].

## 2.11 Streptococcosis (Infection by *Streptococcus iniae*)

*S. iniae* is a major pathogen of tilapia causing significant production losses.

### 2.11.1 Inactivated/Sub-Unit Vaccines

A number of bacterins have been tested successfully for protection [133–135]. In contrast, some studies have shown that bacterins do not confer protection against different serotypes of *S. iniae* [136, 137]. Preparations containing both formalin-killed cells and ECP have been reported to partially protect Nile tilapia from infection [138–141]. Recombinant PDHA1 and GAPDH were identified as potential vaccine candidates against *S. iniae* infection in flounder [142].

### 2.11.2 Live Vaccine

Use of live-attenuated strains defective in phosphoglucomutase and M-like protein have shown to offer protection [143, 144]. An attenuated novobiocin-resistant strain has been reported to confer protection for a period of 6 months in tilapia [145].

### 2.11.3 DNA Vaccine

DNA vaccine containing putative secretory antigen of *S. iniae* has been known to be protective in turbot [146]. DNA vaccine based on the *sagH* gene, which belongs to the streptolysin S-associated gene cluster, exhibited a high relative percent survival [147].

## 2.12 Lactococcosis (Infection by *Lactococcus garvieae*)

Lactococcosis is a systemic disease of both cultured and wild fish species. The causative agent is *L. garvieae*. Vaccines are available for rainbow trout in Italy, France, and UK; and for yellowtail in Japan [148].

### 2.12.1 Inactivated Vaccines

Injectable formalin-inactivated vaccine showed protection rates of 70–80% for 3 months in trout [149] and yellowtail [148]. Non-mineral oil-adjuvanted vaccine (Aquamun) offers significantly higher protection (92%), as compared to non-adjuvanted vaccine (40%), at 3 months after vaccination [149]. Formalin-inactivated *L. garvieae* bacterin vaccines in combination with Freund's incomplete adjuvant provided longer protection against virulent *L. garvieae* infection in rainbow trout compared to vaccine without the adjuvant [150]. Formalin-inactivated LG vaccine strain (S3) protected grey mullet against infection for a period of 3 months with 100% RPS [151]. An oil-adjuvanted, inactivated whole-cell autovaccine also protected Nile tilapia against infection [152].

### 2.12.2 Live Vaccine

A live vaccine using a strain lacking a virulence-associated capsule has shown to provide long-lasting protection to yellowtail [153].

## 2.13 Piscirickettsiosis (Infection by *Piscirickettsia salmonis*)

Piscirickettsiosis is a septicaemia caused by *P. salmonis,* a facultative Gram-negative bacterium mainly affecting Chilean salmon.

### 2.13.1 Inactivated Vaccines

A number of bacterins with variable protection have been tested including heat or formalin-inactivated bacterins [154, 155]. Birkbeck et al., [156] proved that high antigen concentrations are essential for protection. Commercial bacterins including oral formulation are available in Chile for protection of salmon [157, 158]. Alginate-encapsulated *P. salmonis* antigens incorporated in the feed as an oral vaccine were able to induce the immune response in Atlantic salmon (*Salmo salar*) [159].

### 2.13.2 Sub-Unit Vaccines

Recombinant OspA gave high protection in intraperitoneal challenge [160]. Heat-shock proteins and flagellin have also been tested as vaccine candidates [161, 162].

### 2.13.3 Live Vaccine

Live vaccine "Renogen" developed to control BKD shows significant reduction of mortality due to piscirickettsiosis under farm conditions [163].

## 2.14 Bacterial Kidney Disease (Infection by *Renibacterium salmoninarum*)

### 2.14.1 Inactivated/Sub-Unit Vaccines

Studies have shown that whole-cell bacterins offer variable protection to BKD [164, 165]. A highly abundant, 57 kDa extracellular major soluble antigen (MSA or p57 protein) of *R. salmoninarum* plays an important role in pathogenesis and eliciting immune response. However, inclusion of MSA in the bacterin confers very less protection through either oral or intraperitoneal route [166–169]. Studies showed that lowering the levels of the MSA in vaccine preparation by either heat treatment [168, 170] or using low MSA-producing strains [171] is effective.

### 2.14.2 Live Vaccines

Live-attenuated strains with reduced or normal cell-associated MSA have been tested as vaccines [171–173] with little or no protection. Significantly, *Arthrobacter davidanieli*, a virulent bacterium whose surface carbohydrate resembles *R. salmoninarum* has been tested as vaccine candidate. It provides significant protection in Atlantic salmon [172–174] and is marketed as "Renogen" in several countries.

## 3 Polyvalent Vaccines

In recent years, a few studies have been carried out on developing polyvalent vaccines for use in aquaculture. A pentavalent vaccine for rainbow trout has been developed for control of *A. salmonicida subsp. salmonicida, V. anguillarum,* and *Y. ruckeri* in Danish aquaculture [175]. Polyvalent vaccines against *V. alginolyticus* and *E. tarda* were developed by shuffling six genes of *omp*A family. These vaccines had strong ability in eliciting the innate immunity [176]. Recombinant Hsp33 and *Omp*C protein were shown to serve as promising divalent vaccine with protection against *V. anguillarum* and *E. tarda* in flounder [177].

In summary, lot of research work on vaccine development against bacterial pathogens of finfish is underway in many different countries. The vaccines which are being used in aquaculture or in experimental stages are summarized as follows: Inactivated vaccines (Table 1); live vaccine (Table 2); sub-unit vaccines (Table 3) and DNA vaccines (Table 4).

## 4 Conclusions

To meet the ever-increasing demand for fish production, new fish species are being brought into culture. Intensification of aquaculture leads to stress and, as a result, new strains or variants of bacterial pathogens are emerging. Use of antibiotics to control bacterial infection remains a health and environmental concern. Therefore, preventing bacterial disease outbreaks through vaccination is the best strategy. In

**Table 1** List of inactivated bacterial vaccines used in aquaculture including vaccines under experimental trials

| S. No | Pathogen | Fish species | Name of vaccine/ type | Mode of delivery | Reference |
|---|---|---|---|---|---|
| 1 | *Vibrio anguillarum* | Salmon | GAVA-3 | Bath | [3] |
| 2 | *Vibrio* species | Seabass | Alpha-Marine, alpha dip, and aqua-Vac *vibrio* | Bath | [4] |
| | *V. anguillarum* | Atlantic cod | Formalin inactivated | Bath | [5] |
| 3 | *Aeromonas hydrophila* | Magur | Biofilms vaccines | Oral | [23] |
| | | Carps | Biofilms vaccines | Oral | [24] |
| | | Murrel | Biofilms vaccines | Oral | [25] |
| | | Goldfish | Biofilms vaccines | Oral | [26] |
| | | Carps, trout | Formalin killed | IP | [27, 28] |
| | | Carp | Whole-cell linked to single-walled carbon nanotubes | Bath | [29] |
| | | Carp | PLGA microparticle-encapsulated formalin-killed | Oral | [30] |
| | | Pacu | Oil adjuvant inactivated | IP | [31] |
| 4 | *Aeromonas salmonicida* | Rainbow trout | Low-frequency sonophoresis-adjuvanted bacterin | IP | [49] |
| | | | Oil adjuvanted vaccine | IP | [50] |
| | | | AlphaJect 3000® | IP | [51] |
| 5 | *Edwardsiella tarda* | Tilapia | Formalin-killed and an *E. ictaluri* rGAPDH | IP | [68] |
| | | Flounder | KBMA *E. tarda* | IP | [69] |
| 6 | *Flavobacterium columnare* | Channel catfish | Formalin-killed cells | Bath | [82] |
| | | Salmonids, tilapia | *Flavobacterium* bacterin (Fryvacc 1) bacterins of *F. columnare* and *Y. ruckeri* (Fryvacc 2) | Bath | [83] |
| | | Tilapia | Formalin killed vaccine complexed with chitosan | Bath | [85] |
| | | Coho salmon | Heat-killed cells/ formalin-killed | Oral | [86, 87] |
| | | Tilapia | Formalin-killed sonicated cells+ Freund's complete adjuvant | IP | [88] |
| 7 | *Flavobacterium psychrophilum* | Rainbow trout | Formalin-killed cells/heat killed with FCA | IP | [92, 93] |
| | | Ayu | Formalin-killed cells with adjuvant | | [94, 95] |

(continued)

**Table 1** (continued)

| S. No | Pathogen | Fish species | Name of vaccine/ type | Mode of delivery | Reference |
|---|---|---|---|---|---|
| | | Ayu | Formalin-killed cells | Oral | [97] |
| | | Rainbow trout | Formalin-killed cells | Oral | [98, 99] |
| 8 | *Photobacterium damselae* | Seabream | Whole-cell bacterin with ultrasound | Bath | [110] |
| | | | Oil adjuvant | IP | [111] |
| | | | Use of glucose/ iron depleting conditions | IP | [112, 113] |
| | | | LPS/ capsular antigen | IP | [114, 115] |
| | | Sea bass, yellowtail rainbow trout | Formalin-killed bacterin + over-expressing protective protein (Photogen) | Bath/IP | [85–87] |
| 9 | *Yersinia ruckeri* | Trout | AquaVac® ERM formalin inactivated | Bath | [120, 121] |
| 10 | *Streptococcus agalactiae* | Tilapia | Formalin-killed cells and concentrated extracellular products | IP | [127] |
| | | | Inactivated whole-cell bacterial vaccine (VA–VJ- PFGE type) | IP/bath | [128] |
| 11 | *Streptococcus iniae* | Nile tilapia | Bacterins | IP | [133–135] |
| | | | Oralject | Oral | [141] |
| 12 | *Lactococcus garvieae* | Trout, yellow tails | Formalin-killed cells | IP | [148, 149] |
| | | Trout | Non-mineral oil adjuvanted vaccine (Aquamun) | IP | [149] |
| | | Trout | Formalin-killed cells with FCA | IP | [150] |
| | | Grey mullet | Formalin-killed cells | IP | [151] |
| | | Tilapia | Inactivated whole-cell autovaccine | IP | [152] |
| 13 | *Piscirickettsia salmonis* | Salmon | Heat or formalin-inactivated bacterins | IP | [154–156] |
| | | | Heat or formalin-inactivated bacterins | Oral | [157, 158] |
| | | | Alginate-encapsulated | Oral | [159] |
| 14 | *Renibacterium salmoninarum* | Salmon | Heat or formalin-inactivated bacterins | IP/Bath | [164, 165] |
| | | | Reduced p57 cells | IP | [170, 171] |

**Table 2** List of live bacterial vaccines used in aquaculture including vaccines under experimental trials

| S. No | Pathogen | Fish species | Name of vaccine/ expressed part | Mode of delivery | Reference |
|---|---|---|---|---|---|
| 1 | *Vibrio anguillarum* | Rainbow trout | Avirulent mutants | Bath | [6] |
| 2 | *V. harveyi* | Asian seabass, turbot | Live attenuated | Bath | [7, 8] |
| | | Grouper | Serine endoprotease deletion mutant | IP | [9] |
| | *V. alginolyticus* | Grouper | *acfA* gene mutant and ΔclpP mutant | IP | [10, 11] |
| 3 | *Aeromonas hydrophila* | Channel catfish | Rifampicin attenuated | Immersion | [32] |
| | | Blue gourami | Tn5 induced protease-deficient mutants | IP | [33] |
| | | Rainbow trout | aroA mutant | IP | [34] |
| | | Rohu | Rough attenuated variants | IP | [35] |
| | | Tilapia & channel catfish | Rifampicin & Novobiocin resistant strains | IP | [36] |
| | | Loach | Mutant Δ*hisJ* | IP | [37] |
| 4 | *A. salmonicida* | Rainbow trout | Mutants lacking A-layer & O-antigen | Bath | [52] |
| | | Rainbow trout | Live attenuated *aro*A | IP | [53, 54] |
| 5 | *Edwardsiella ictaluri* | Channel catfish | RE-33, AQUAVAC-ESC™ | Bath | [56–58] |
| | | Channel catfish | Novobiocin resistance mutant | Bath/IP | [59] |
| | | Channel catfish | Crp mutant | Bath | [60] |
| | | Channel catfish | TCA and one-carbon (C-1) metabolism mutants | Bath | [61] |
| | | Channel catfish | evpB mutant protein | Bath | [62] |
| | | Channel catfish | aroA mutant | Oral | [63] |
| | | *P. hypophthalmus* | Wzz mutant | IP | [65] |
| | | Channel catfish | HemR | IP | [66] |
| | | Channel catfish | Rifampicin resistant | Oral | [67] |
| 6 | *E. tarda* | Turbot | AroC mutant | IP | [71] |
| | | Turbot | Temperature sensitive mutants | IP | [72] |
| | | Turbot | Recombinant live *E. tarda* + lacking UDP-glucose dehydrogenase | IP | [73] |

| | | | | | |
|---|---|---|---|---|---|
| 7 | *Flavobacterium columnare* | Channel catfish, Large mouth bass | Aquavac-col | Bath | [89] |
| | | Channel catfish | A rifampicin modified | Bath | [90] |
| 8 | *F. Psychrophilum* | Rainbow trout | *exbD* loci mutant, rifampicin resistant | IP | [106, 107] |
| | | | Live attenuated | Bath | [108, 109] |
| 9 | *Photobacterium damselae* | Yellow tail | Siderophore-deficient strain /aroA mutant | IP | [117] |
| | | Hybrid striped bass | Aro-A-deletion mutant | IP | [118] |
| 10 | *Yersinia ruckeri* | Rainbow trout | Aro-A-deletion mutant | IP | [126] |
| 11 | *Streptococcus agalactiae* | Nile tilapia | Sparfloxacin-resistant *S. agalactiae* | IP | [129] |
| | | | Erythromycin-resistant | IP | [130] |
| | | | Naturally attenuated | IP | [131] |
| | | | Ghost cell vaccine | IP | [132] |
| 12 | *S. Iniae* | Nile tilapia | Defective phosphoglucomutase and M-like protein/ attenuated novobiocin-resistant | IP | [143–145] |
| 13 | *Lactococcus garvieae* | Yellowtail | Strain lacking a virulence-associated capsule | IP | [153] |
| 14 | *Piscirickettsia salmonis and Renibacterium salmoninarum* | Atlantic salmon | *Arthrobacter davidanieli*, a virulent bacterium whose surface carbohydrate (Renogen) | IP | [163] |
| 15 | *Renibacterium salmoninarum* | Chinook salmon | Live & Killed strains of strain MT239; live *Arthrobacter davidanieli* (Renogen) | IP | [163] |
| | | | Live mutant strains | IP | [172–174] |

**Table 3** List of bacterial sub-unit vaccines used in aquaculture including vaccines under experimental trials

| S. No | Pathogen | Fish species | Name of vaccine/ expressed part | Mode of delivery | References |
|---|---|---|---|---|---|
| 1 | *Vibrio anguillarum* | Flounder | rflaB | IP | [19] |
| 2 | *V. harveyi* | Grouper | VirB11 | IP | [20] |
| | | | rOmpK) + (rOmpU) + rOmpK-OmpU fusion proteins | IP | [21] |
| | | Zebrafish | Edible plant-based | Oral | [22] |
| 3 | *Aeromonas hydrophila* | Tilapia | *Lactococcus lactis* expressing aerolysin genes | Oral | [38] |
| | | Common carp | *Lactobacillus casei* expressing the OmpAI | Oral | [39] |
| | | Rohu | OmpW with PLGA | Oral | [40] |
| | | Rohu | Recombinant rOmpR, ompTS | IP | [41, 42] |
| | | Grass carp | Single-walled carbon nanotubes-aerA | IP & bath | [43] |
| | | Eel | 46 kD maltoporin | IP | [44] |
| | | Channel catfish | Recombinant OmpA1, Tdr, and TbpA and ATPase | IP | [45, 46] |
| 4 | *A. salmonicida* | Trout | Fourteen recombinant proteins | IP | [55] |
| 5 | *Edwardsiella tarda* | Turbot | Recombinant *rFimA,* | IP | [74] |
| | | | *rGAPDH,* | IP | [75] |
| | | Zebrafish | *FlgD* protein | IP | [76] |
| | | Flounder | rOmpV along with cytokines expression plasmids/rIDH | IP | [77, 78] |
| 6 | *Flavobacterium columnare* | Channel catfish | Recombinant DnaK protein | IP | [91] |
| 7 | *F. psychrophilum* | Trout | Elongation factor-Tu, SufB Fe-S assembly protein and ATP synthaseβ | IP | [100] |
| | | | L10-like protein with adjuvant | IP | [101] |
| | | | 70–100 kDa fraction | IP | [102] |
| | | | Recombinant heat shock proteins | IP | [103] |
| | | | OmpH, OmpA | IP | [104, 105] |

| | | | | | |
|---|---|---|---|---|---|
| 8 | *Photobacterium damselae* | Sole | Outer membrane protein FrpA | IP | [119] |
| 9 | *Yersinia ruckeri* | Trout | O-antigen/inactivated Yrp1 protease | IP | [123, 124] |
| | | | Recombinant outer membrane porin F (OmpF) | IP | [125] |
| 10 | *Streptococcus iniae* | Tilapia | Extracellular polysaccharide | IP | [137] |
| | | Flounder | Recombinant PDHA1 and GAPDH | IP | [142] |
| 11 | *Piscirickettsia salmonis* | Salmon | OspA | IP | [160] |
| | | | Heat-shock proteins and flagellin | IP | [161, 162] |

**Table 4** List of DNA vaccines for bacterial diseases used in aquaculture including vaccines under experimental trials

| S. No | Pathogen | Fish species | Name of vaccine/ expressed part | Mode of delivery | Reference |
|---|---|---|---|---|---|
| 1 | *Vibrio anguillarum* | Red snapper | Flagellin *flaA* | IP | [12] |
| | | Asian seabass | *omp*38 | IP | [16] |
| | | Asian seabass | *omp*38 + chitosan nanoparticles | Oral | [17] |
| 2 | *V. harveyi* | Japanese flounder | DegQ and Vhp1 | IP | [13] |
| 3 | *Vibrio parahaemolyticus* | Crimson snapper | ompW | IP | [14] |
| | | Black seabream | *ompK* encapsulated in chitosan | Oral | [18] |
| 4 | *V. alginolyticus* & *E. tarda* | Zebra fish | OmpA | IP | [15] |
| 5 | *Edwardsiella tarda* | Flounder | Chaperone GroEL | IP | [79] |
| | | Rohu | GAPDH and IFN-γ | IP | [80, 81] |
| 6 | *Flavobacterium psychrophilum* | Trout | Bacterial ribosomal protein L10 | IP | [101] |
| 7 | *Streptococcus iniae* | Turbot | Secretory antigen of *S. iniae* | IP | [146] |
| | | Flounder | *sagH* gene | IP | [147] |

India, bacterial disease outbreaks would continue to remain a challenge due to lack of vaccines and vaccination procedures. Sustained efforts are needed to develop effective vaccines for bacterial diseases of fish, especially with the use of local bacterial strains and effective modes of delivery.

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# Viral Vaccines for Farmed Finfish

Makesh M. and Rajendran K. V.

**Abstract**

Fish viral diseases cause more damage to aquaculture due to its rapid spread causing acute mortalities and are not amenable to any treatment measures. Prophylaxis through vaccination is a reliable method for controlling viral diseases of fish. Several vaccines have been developed against many viral diseases of fish such as infectious pancreatic necrosis, Koi herpesvirus disease, infectious salmon anaemia, viral nervous necrosis, infectious haematopoietic necrosis, etc. Several of these vaccines are commercially available either as monovalent or multivalent vaccines along with other bacterial vaccines. Most of the vaccines available are inactivated injectable vaccines although recombinant and DNA vaccines are available for a few viral diseases. The commercially available fish viral vaccines are listed.

**Keywords**

Fish viral diseases · Vaccines · Inactivated · Recombinant · DNA vaccines · Immune response

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## 1 Introduction

World fish production, including both inland and marine sector, reached 179 million tonnes in the year 2018 out of which aquaculture has contributed 82 million tonnes, valued at USD 250 billion [1]. Of the 82 million tonnes of aquaculture produce, 54.279 million tonnes was contributed by finfish [1]. The new height scaled in aquaculture production in recent years is due to intensification of culture practices and diversification of cultured species. Aquaculture production has recently surpassed capture fisheries production. However, the sustainability of the growth of aquaculture is constrained by various diseases affecting farmed fish species. A trend has been observed in disease occurrence where a new disease emerges every 3–5 years causing major production losses. Such diseases are most often caused by viruses and to a lesser extent by a bacterium or a parasite [1]. The direct impact of such diseases is the loss of production due to mortality and reduced growth rate. It is estimated that the loss due to diseases exceeds 25% worldwide [2]. Among the diseases, viral diseases cause more damage as it spreads rapidly causing acute mortalities and are not amenable to any treatment measures. Viral disease management involves adoption of biosecurity protocols, good management practices, and early diagnosis to contain the spread of the disease. However, specific antiviral drugs are not readily available for most viral pathogens affecting fish. Nevertheless, as fish have a well-established adaptive immune system capable of mounting a long-term specific immune response, prophylaxis through vaccination is a reliable method for controlling viral infections.

## 2 History of Fish Vaccination

The first report of vaccination in fish is probably made by Snieszko et al. in 1938 (as cited by Van Muiswinkel [3]) where the authors reported protective immunity in carp against infectious dropsy caused by *Aeromonas punctata* when immunized with killed bacterin. Following this, Wilhelm Schaperclaus showed that intraperitoneal injection of fish with killed or attenuated *Pseudomonas punctata* (*Aeromonas hydrophila*) evoked protective immunity upon challenge. The first report of oral vaccination in fish is by Duff [4] in 1942 where the author demonstrated that administering fish with a chloroform-killed *Aeromonas salmonicida* through feed induced protection against furunculosis when challenged by injection or by cohabitation. Not much work was reported on fish vaccines during the next 2–3 decades, probably, because of the availability of antibiotics. Most of the earlier work on fish vaccines were for bacterial diseases of salmonids. The first vaccine for fish to be licenced was *Yersinia ruckeri* bacterins in 1976 to control enteric redmouth disease [5]. Infectious pancreatic necrosis virus is the first fish virus to be isolated in vitro using tissue culture [6]. However, the first commercially available viral vaccine was for spring viraemia of carp, an attenuated oil-adjuvant vaccine produced by a Czechoslovakian company (Bioveta) in 1982 [7]. Since then, many vaccines against bacterial and viral diseases have been reported to be successful under experimental

conditions. Many vaccines, mostly inactivated vaccines, have been licensed for use in Europe and North America as monovalent and polyvalent vaccines. The first commercially available DNA vaccine for finfish is for infectious haematopoietic necrosis virus (IHNV) marketed by Aqua Health Ltd., Novartis, licensed for use in Canada and USA [2].

## 3 Viral Diseases and Vaccines for Farmed Finfish

The common viral diseases of farmed finfish along with the hosts affected, causative agents, and their characteristics are given in Table 1. As vaccination is an effective method to control diseases of farmed finfish, a number of vaccines have been developed and are commercially available for the control of viral diseases of fish. The commercially available vaccines for some of the common viral diseases of fish are given in Table 2. Most of the vaccines available commercially are inactivated vaccines containing formalin- or heat-killed viruses, as they are safe to administer. Although live-attenuated vaccines have advantages such as induction of high protective immunity and low dose requirement, they are not commonly used due to the risk of reversion of virulence of the attenuated virus [25]. The level of protection offered by recombinant vaccines is often low and not reproducible [26]. Although many successful DNA vaccines against viral diseases are reported, only one DNA vaccine against infectious haematopoietic necrosis virus is available commercially because of the possible consideration of the DNA vaccinated fish as genetically modified organisms [25].

Vaccines are usually administered by intraperitoneal injections and, hence, they are used only for large-sized fishes as it is practically not possible to inject small fishes which are less than 10 g. As many viral diseases affect early stages of fish, alternate vaccination methods such as immersion and oral routes are increasingly being explored. Immersion vaccines are easy to administer especially for smaller fish in large batches at the time of stocking without causing much stress to the fish. However, administration of booster dose is a problem. Oral vaccines are easy to administer to fishes of all sizes and repeated booster doses can also 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 a certain extent by micro- or nano-encapsulation of the antigen in particles such as chitosan, alginate, etc. [27]. However, more efforts are required to increase the efficacy of oral vaccines which is a promising method of vaccinating farmed finfish with least stress. Specialized adjuvants that help in the sustained release of antigen and improved presentation of the antigen to the antigen-presenting cells are also available for vaccine preparation. Multivalent vaccines targeting more than one pathogen are also available commercially to reduce the vaccination stress.

The efficacy of a vaccine can be assessed using various parameters. Assessment of the relative percent survival (RPS) using the formula [1 − (mortality in vaccinated fish/mortality in non-vaccinated fish)] × 100 is a direct method to assess the protection offered by the vaccine. To evaluate a vaccine based on RPS, it is

**Table 1** Major viral disease of farmed finfish and the causative agents

| Disease | Host species | Pathogen/ classification | Viral morphology | Viral genome | References |
|---|---|---|---|---|---|
| Diseases caused by DNA viruses | | | | | |
| Epizootic haematopoietic necrosis | Redfin perch and Rainbow trout | Epizootic haematopoietic necrosis virus<br>Genus: *Ranavirus*<br>Family: *Iridoviridae* | Large (130–170 nm) enveloped icosahedral virus | Linear dsDNA, 125–127 kbp | [8, 9] |
| Koi herpesvirus disease | Carps and carp varieties such as Koi carp and ghost carp | Cyprinid herpesvirus 3<br>Genus: *Cyprinivirus*<br>Family: *Alloherpesviridae* | Icosahedral enveloped virus 100–110 nm | dsDNA, 295 kbp | [10] |
| Red seabream iridoviral disease | Red seabream and more than 30 other marine cultured species | Red seabream iridovirus<br>Genus: *Megalocytivirus*<br>Family: *Iridoviridae* | Non-enveloped, icosahedral, 120–200 nm in diameter | dsDNA, 112 kbp | [11] |
| Diseases caused by RNA viruses | | | | | |
| Viral nervous necrosis (Viral encephalopathy and retinopathy) | Asian seabass, European seabass, turbot, halibut, Japanese parrotfish, red-spotted grouper, and striped jack | Nervous Necrosis Virus<br>Genus: *Betanodavirus*<br>Family: *Nodaviridae* | Icosahedral, non-enveloped, 25 nm in diameter | Two positive-sense ssRNA molecules—RNA1 (3.1 kb) and RNA2 (1.4 kb) | [12] |

| | | | | | |
|---|---|---|---|---|---|
| Infectious haematopoietic necrosis | Salmons and trout | Infectious haematopoietic necrosis virus<br>Genus: *Novirhabdovirus*<br>Family: *Rhabdoviridae* | Enveloped, bullet-shaped 150–190 × 65–75 nm | Non-segmented, negative-sense, ssRNA, 11 kb | [13, 14] |
| Viral haemorrhagic septicaemia | Rainbow trout, turbot, Japanese flounder as well as a broad range of wild freshwater and marine species | Viral haemorrhagic septicaemia virus<br>Genus: *Novirhabdovirus*<br>Family: *Rhabdoviridae* | Bullet-shaped measuring about 180 nm long and 70 nm in diameter | 11 kb negative ssRNA | [15] |
| Spring viraemia of carp | Common carp, grass carp, silver carp, bighead carp, crucian carp, goldfish, etc. | Spring viremia of carp virus<br>Genus: *Vesiculovirus*<br>Family: *Rhabdoviridae* | Bullet-shaped, 80–180 nm in length and 60–90 nm in diameter | Negative-sense, linear ssRNA of ~11 kb | [16] |
| Infectious pancreatic necrosis | Rainbow trout, brook trout, brown trout, Atlantic salmon | Infectious pancreatic necrosis virus<br>Genus: *Aquabirnavirus*<br>Family: *Birnaviridae* | Non-enveloped, icosahedral measuring about 70 nm in diameter | Bi-segmented, dsRNA virus, Segment A: ~3.0 kbp; segment B: 2.4 kbp | [17–19] |

(continued)

**Table 1** (continued)

| Disease | Host species | Pathogen/ classification | Viral morphology | Viral genome | References |
|---|---|---|---|---|---|
| Infectious salmon anaemia | Atlantic salmon | Infectious salmon anaemia virus<br>Genus: *Isavirus*<br>Family: *Orthomyxoviridae* | Enveloped virus, 100–130 nm in diameter | Eight single-stranded, negative-sense RNA segments | [20] |
| Pancreas disease (PD) or sleeping disease (SD) | Atlantic salmon, common dab, rainbow trout, and Arctic charr | Salmonid alphavirus<br>Genus: *Alphavirus*<br>Family: *Togaviridae* | Enveloped, spherical, 60–70 nm in diameter | Single-stranded, positive-sense RNA virus, with a genome of ~12 kb | [21, 22] |
| Tilapia lake virus disease | Wild tilapia, farmed tilapia, commercial tilapia hybrids, and giant gourami | Tilapia lake virus (TiLV)<br>Genus: *Tilapinevirus*<br>Family: *Amnoonviridae* | Enveloped, 55–75 nm in size | Negative-sense, single-stranded RNA virus with ten segments, 10.323 kb in total length | [23, 24] |

**Table 2** Commercially available viral vaccines for finfish

| Name of the vaccine | Type of vaccine | Delivery method | Disease/Pathogen targeted | Target Species | Produced by |
|---|---|---|---|---|---|
| AQUAVAC® IPN Oral | Recombinant | Oral | Infectious pancreatic necrosis (IPN) virus | Atlantic salmon | MSD Animal Health<br>www.msd-animal-health-me.com/ |
| NORVAX® Compact PD | Inactivated vaccine | Intraperitoneal injection | Salmonid Alphavirus (SAV)/Pancreas disease | Atlantic salmon | MSD Animal Health<br>www.msd-animal-health-me.com/ |
| ALPHA JECT micro® 1 PD | Inactivated | Intraperitoneal injection | Salmonid alpha virus (SAV) | Atlantic salmon | PHARMAQ AS, Norway<br>www.pharmaq.no/ |
| Clynav | DNA vaccine | Intramuscular injection | Salmonid Alphavirus 3 (SAV)/Pancreas disease | Atlantic salmon | Elanco GmbH<br>www.ema.europa.eu/ |
| NORVAX® Minova 6 | Inactivated, multivalent vaccine | Intraperitoneal injection | Furunculosis, classical vibriosis, coldwater vibriosis, wound disease, and infectious pancreatic necrosis (IPN) | Atlantic salmon | MSD Animal Health<br>www.msd-animal-health-me.com/ |
| KV3 Vaccine | Attenuated virus vaccine | Immersion and Injection | KHV disease | Common carp and Koi carp | Phibro Animal Health Corpn. |

(continued)

**Table 2** (continued)

| Name of the vaccine | Type of vaccine | Delivery method | Disease/Pathogen targeted | Target Species | Produced by |
|---|---|---|---|---|---|
| ALPHA JECT micro® 6 | Inactivated, multivalent | Intraperitoneal injection | *A. salmonicida*, *Vibrio salmonicida*, *Listonella anguillarum*, *Moritella viscosa* and IPN | Atlantic salmon | PHARMAQ AS, Norway<br>www.pharmaq.no/ |
| ALPHA JECT® 6-2 | Oil adjuvant vaccine | Injection | Furunculosis, Vibriosis, Coldwater vibriosis, Winter sore, IPN | – | PHARMAQ AS, Norway<br>www.pharmaq.no/ |
| ALPHA JECT micro® 7 ILA | Oil adjuvant vaccine | Injection | Furunculosis, Vibriosis, Coldwater vibriosis, Winter sore, IPN, ISA | – | PHARMAQ AS, Norway<br>www.pharmaq.no/ |
| ALPHA JECT® 2-2 | Inactivated | Intraperitoneal injection | Furunculosis, IPN | Atlantic salmon | PHARMAQ AS, Norway<br>www.pharmaq.no/ |
| ALPHA JECT micro® 1 Noda | Inactivated | Intraperitoneal injection | Viral nervous necrosis | European seabass | PHARMAQ AS, Norway<br>www.pharmaq.no/ |
| ALPHA JECT® micro 2 | Oil adjuvant vaccine | Injection | IPN, Salmon Rickettsial Septicaemia (SRS) | – | PHARMAQ AS, Norway<br>www.pharmaq.no/ |
| ALPHA JECT® micro 3 | Oil adjuvant vaccine | Injection | Vibriosis, IPN, SRS | – | PHARMAQ AS, Norway<br>www.pharmaq.no/ |

| | | | | | |
|---|---|---|---|---|---|
| Icthiovac® VNN | Inactivated | Intraperitoneal injection | Viral nervous necrosis | European Sea bass | Hipra, Spain www.hipra.com/ |
| ALPHA JECT® IPNV-Flavo 0,025 | Oil adjuvant vaccine | Intraperitoneal | IPN, Flavobacteriosis | – | PHARMAQ AS, Norway www.pharmaq.no/ |
| ALPHA JECT® micro 1 ISA | Inactivated | Intraperitoneal injection | Infectious salmon anaemia | Atlantic salmon | PHARMAQ AS, Norway www.pharmaq.no/ |
| ALPHA JECT® 4-1 | Oil adjuvant vaccine | Injection | Furunculosis, Vibriosis, IPN, SRS | – | PHARMAQ AS, Norway www.pharmaq.no/ |
| ALPHA JECT® 5-1 | Oil adjuvant vaccine | Injection | Furunculosis, Vibriosis, IPN, ISA, SRS | – | PHARMAQ AS, Norway www.pharmaq.no/ |
| ALPHA JECT® 1000 | Oil adjuvant vaccine | Injection | IPN | – | PHARMAQ AS, Norway www.pharmaq.no/ |
| BLUEGUARD® IPN + SRS + As + Vo + ISA INJECTABLE | Multivalent | Injectable | *Piscirickettsia salmonis*, *Vibrio ordalii*, *A. salmonicida*, *IPNV*, *ISAV* | Atlantic salmon | Centrovet www.centrovet.virbac.com/ |
| Apex-IHN® | DNA vaccine | Intramuscular injection | *Infectious haematopoietic necrosis* | Salmonids | Aqua health Ltd (Novartis) [2] |
| AQUAVAC® IridoV | Oil-adjuvant vaccine | Intraperitoneal injection | *Iridovirus* | Tilapia and Asian sea bass | MSD Animal Health (Intervet) www.aquavac-vaccines.com/ |

important to select a challenge model that best reflects the natural route of infection [28]. In case of non-lethal viruses, the ability of the vaccine to protect fish from histological changes following challenge with virulent virus can also be used to assess the efficacy of a vaccine. Measurement of specific antibodies in serum/mucous by ELISA is another direct method of assessing the efficacy of a vaccine, provided, anti-fish antibody is available. Serum neutralization test or competitive ELISA can also be used to quantify the antibodies if anti-fish antibodies are not available.

### 3.1 Epizootic Haematopoietic Necrosis

Epizootic haematopoietic necrosis (EHN), an OIE-notifiable disease, is a systemic disease characterized by extensive visceral tissue damage leading to mortality [29]. The disease is caused by epizootic haematopoietic necrosis virus (EHNV) belonging to the genus *Ranavirus* within the sub-family *Alphairidovirinae* and family *Iridoviridae* [30]. ENHV is an enveloped icosahedral virus assembled in the cytoplasm and released by budding, measuring 130–170 nm, and has up to 36 major polypeptides [8]. Its genome comprises of dsDNA of size 125–127 kbp [9]. Fish species susceptible to this virus are redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*). Experimentally, the virus can infect many Australian native species including Murray cod (*Maccullochella peelii*), Macquarie perch (*Macquaria australasica*), and Murray River rainbowfish (*Melanotaenia fluviatilis*) [31]. The virus is shed from infected tissue and disintegrating carcass [15]. The disease affects all age groups, although the clinical signs are more severe in juveniles and fingerlings. The target organs of the virus are kidney, spleen, and liver. Isolation of the virus in cell culture and antibody-capture ELISA are used for targeted surveillance [15]. EHN is endemic to Australia but similar viruses like European catfish virus (ECV), European sheatfish virus (ESV) and Santee-Cooper ranavirus have been reported in other countries associated with diseases of fish and frogs [32]. The clinical signs of the disease include distended abdomen, petechial haemorrhage at the base of fin and gills, and multifocal necrosis of haematopoietic tissue of kidney and spleen with enlargement of kidney and spleen. No report of vaccine against EHN is available and no commercial vaccines are available for this disease.

### 3.2 Koi Herpesvirus Disease

Koi herpesvirus disease (KHVD) is a highly contagious disease affecting common carp and its varieties like koi carp and ghost carp causing significant mortality [33]. It is an OIE-notifiable disease. Koi herpes virus disease is caused by koi herpesvirus (KHV) also called as cyprinid herpes virus-3 (CyHV-3) which belongs to the family *Alloherpesviridae* and the genus *Cyprinivirus*. CyHV-3 is the type species of the genus *Cyprinivirus* which also contains the species CyHV-1 and CyHV-2. It is an

enveloped virus measuring 100–110 nm with a dsDNA genome of size 295 kbp [10]. Surviving fish become persistent carriers for long time [34] and the virus is shed through faeces, urine, gills, and gill mucus [15]. The virus is transmitted usually horizontally through water and vectors, although vertical transmission cannot be ruled out. Gills and skin are the major portals of entry of the virus [35, 36]. The disease has a 100% morbidity rate and mortality ranges from 70% to 80% [33, 37].

Control of the disease is by following strict biosecurity measures and introduction of disease-free fish into the culture system. Many researchers have reported the protective effect of recombinant and DNA vaccines expressing different genes encoding the antigenic proteins of the pathogen. Boutier et al. [38], described the development of a recombinant attenuated vaccine against KHV for mass vaccination of carps through immersion route which induced protective mucosal immune response at portals of pathogen entry. A DNA vaccine expressing the glycoprotein gene offered better protection to common carp (*Cyprinus carpio*) [39, 40]. A DNA construct expressing ORF149 of KHV coupled to single-walled carbon nanotubes (SWCNTs) when injected intramuscularly resulted in better immune response, higher RPS, and longer protection [41]. However, oral administration or single intramuscular injection of DNA vaccine expressing ORF25 did not confer protection against KHV when challenged by immersion or cohabitation, although multiple doses induced strong protection [28]. Constructs expressing multiple antigenic proteins such as ORF25, ORF148, ORF149, ORF81, ORF72, and ORF92 could trigger a protective immune response [28]. A siRNA targeting DNA polymerase gene of KHV could inhibit the in vitro viral replication in common carp brain cells. However, its potential to inhibit viral replication in vivo needs to be established [42]. A live-attenuated vaccine has been licenced for emergency use in Israel [15].

### 3.3 Red Seabream Iridoviral Disease

Red seabream iridoviral disease (RSIVD) is an OIE notifiable disease of farmed red seabream, *Pagrus major*, and several other farmed marine fishes [43] causing up to 100% mortality. The disease was first reported in Japan in 1990 [44] and since then, the disease has spread to many East and South-East Asian countries viz. China, Hong Kong, Korea, Malaysia, Philippines, Singapore, Thailand [15], and India [45]. The disease causes mortality ranging between 0% and 100% depending on the age, size, species of the affected fish, water temperature, and other culture conditions. The disease outbreaks are mostly observed in summer when the water temperature is above 25 °C [15]. The disease is caused by red seabream iridovirus (RSIV) belonging to the genus *Megalocytivirus* under the family *Iridoviridae*. The virus is icosahedral measuring 120–200 nm in diameter. The genome is made up of dsDNA of about 112 kbp and is highly methylated [11]. Infectious spleen and kidney necrosis virus (ISKNV), turbot reddish body iridovirus (TRBIV), and rockbream iridovirus (RBIV) are closely related viruses having high sequence similarity with RSIV. Further investigation needs to be carried out to decide whether these viruses should be included under RSIV. The clinical signs include lethargy, anaemia,

petechiae on the gills, and enlargement of the spleen [44, 46]. The target organs of the virus are gills, intestine, kidney, spleen, and heart. The disease spreads horizontally through water.

A formalin-inactivated vaccine is commercially available in Japan for RSIVD for red seabream (*P. major*), striped jack (*Pseudocaranx dentex*), Malabar grouper (*Epinephelus malabaricus*), and orange-spotted grouper (*Epinephelus coioides*) [47, 48]. This is the first viral vaccine for marine fish [49]. The vaccine when administered intraperitoneally to juvenile red seabream, resulted in higher survival upon challenge [47] and the antigen could not be detected in the spleen of the vaccinated group [48]. This vaccine did not offer protection to the genus *Oplegnathus*, as it is highly susceptible to RSIV [49]. However, another formalin-inactivated vaccine could offer significant protection to yellowtail (*Seriola quinqueradiata*), amberjack (*Seriola dumerili*), kelp grouper (*Epinephlelus moara*), striped jack (*P. dentex*), and spotted parrotfish (*Oplegnathus punctatus*) [50]. A recombinant vaccine consisting of formalin-killed *Escherichia coli* expressing the 351R capsid protein of RSIV expressed in fusion with GAPDH of *Edwardsiella tarda*, when injected intraperitoneally resulted in significantly higher survival on challenge [51]. A DNA vaccine consisting of plasmids encoding the major capsid protein (MCP) and a transmembrane domain against RSIV could provide better protection to red seabream than the unvaccinated control fish [52]. Small interfering RNAs (siRNAs) targeting the MCP gene of RSIV could inhibit the replication of RSIV in vitro, in a cell culture system, indicating the potential of siRNA in protecting cultured fish against RSIVD [53]. A commercial oil-adjuvanted vaccine is available for tilapia and Asian seabass for intraperitoneal injection.

## 3.4 Viral Nervous Necrosis

Viral nervous necrosis (VNN), also called as viral encephalopathy and retinopathy (VER), is an acute viral disease affecting more than 120 species of marine, brackish water, and freshwater fishes belonging to 30 families [54]. The disease causes up to 100% mortality in larval and early juvenile stages. Adult fish when infected are asymptomatic, but become carrier of the virus. Affected fish exhibit dark or pale colouration with abnormal swimming such as circling, darting, belly up, and whirling [54]. The disease is caused by nervous necrosis virus (NNV) belonging to the genus *Betanodavirus* under the family *Nodaviridae*. NNV is a non-enveloped virus of size 25 nm with a bi-segmented single-stranded positive-sense genome [12]. The RNA-1 segment is about 3.1 kb long coding for the RNA-dependant RNA polymerase of size 110 kDa while the RNA-2 segment is about 1.4 kb long coding for the single capsid protein of the virus of size 42 kDa [12, 55]. The betanodaviruses are classified into four genotypes based on the phylogenetic analysis of T4 variable region of RNA-2: tiger puffer nervous necrosis virus (TPNNV), striped jack nervous necrosis virus (SJNNV), barfin flounder nervous necrosis virus (BFNNV), and red-spotted grouper nervous necrosis virus (RGNNV) [55]. The virus

is classified into three serotypes based on serum neutralization test and optimal growth temperature [56]. The virus is transmitted horizontally through infected fish and water and vertically through egg and milt [57, 58]. The practical way to control the disease is to use disease-free brooders and vaccinate them with a potent vaccine against NNV [59–61].

The first report of vaccine against NNV was that of Húsgag et al. [62], where a recombinant vaccine against SJNNV produced specific immune response and protection in turbot, *Scophthalmus maximus* and Atlantic halibut, *Hippoglossus hippoglossus*. Since then, several reports of experimental trials with different forms of vaccine have been reported. Inactivated vaccine administered through intraperitoneal injection gives the best immune response in terms of specific serum antibody titre [59, 63]. Among the inactivating agents, formalin inactivation results in higher antibody production compared to β-propiolactone and heat treatment [63], while UV inactivation could only provide partial protection [64]. Vaccination of broodstock with inactivated vaccine results in the transfer of maternal antibodies to the eggs and larvae and prevents vertical transmission of the virus [60]. Pakingking Jr. [61] demonstrated that annual vaccination of Asian seabass with an inactivated NNV vaccine is the practical way to prevent the vertical transmission of NNV to offspring. The immune response elicited by the inactivated vaccine is directly proportional to the antigen dose administered. However, antigen dose greater than 200 μg had a negative effect on serum antibody titre [65].

Recombinant capsid protein expressed in prokaryotes could produce neutralizing antibodies and offer protection to fish challenged with NNV [65–69]. To overcome the drawbacks of injection vaccine, recombinant protein can be administered orally [70]. The recombinant protein can also be expressed in plants for immunizing fish [71]. Live NNV can be used as vaccine when administered intramuscularly to fish and maintained at natural seawater temperature (17 °C) [72, 73]. However, use of live virus has the risk of infection when the water temperature rises to optimal level for virus replication.

A DNA vaccine expressing the viral capsid protein when injected intramuscularly to Asian seabass juveniles was reported to produce 77.33% RPS, after intramuscular challenge with betanodavirus [74]. No cross-protection between SJNNV and RGNNV has been observed [75], as both the genotypes belong to different serotypes. Two inactivated vaccines against VNN are commercially available: ALPHA JECT micro® 1 Noda marketed by PHARMAQ AS, Norway and Icthiovac® VNN marketed by Hipra, Spain. Both are recommended for European seabass for intraperitoneal administration.

## 3.5 Infectious Haematopoietic Necrosis

Infectious haematopoietic necrosis (IHN) is a viral disease of salmonids, such as salmon and trout, caused by infectious haematopoietic necrosis virus (IHNV), a negative-sense single-stranded, RNA virus. The disease is an OIE-notifiable disease. IHNV belongs to the genus *Novirhabdovirus* under the family *Rhabdoviridae*. It is a

bullet-shaped virus measuring 150–190 nm in length and 65–75 nm in width [13]. The genome consists of a non-segmented, negative-sense, single-stranded RNA genome of size ~11 kb encoding six proteins: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G), a non-virion protein (NV), and a polymerase (L) [14]. Kidney, spleen, and other internal organs are the target organs of the virus and the virus is excreted through urine, ovarian fluids, and mucus. The virus enters through the gills and the bases of fins [76]. The disease is more prevalent in fry stages and, adult fish are relatively resistant. The disease spread horizontally through infected fish and water, while vertical transmission through eggs has also been reported [77]. The disease is prevalent in the northern hemisphere in North America, Europe, and Asia [15]. Cumulative mortality may reach up to 95% in acute cases and varies with species, temperature, culture conditions, and virus strain [76]. The control measures include adoption of strict biosecurity measures and disinfection of fertilized eggs.

An inactivated autogenous vaccine and a DNA vaccine have been licensed for commercial use in North America [15]. The DNA vaccine against IHNV is commercially available in Canada and USA and no outbreak of IHNV has been reported in the vaccinated fish [78]. Many other researchers have patented their inventions on DNA vaccines for IHNV. Vaccination through injection results in stress to the fish and, hence, other routes of administration have been explored. Ballesteros et al. [79], demonstrated the dose-dependent increase in immune response and protection offered by the orally administered DNA vaccine expressing the glycoprotein (G) of IHNV, encapsulated into alginate microspheres. Fish can also be vaccinated through nasal route where the nasal mucosal immune system is stimulated. The use of the nasal route for vaccination in rainbow trout, where a live-attenuated IHNV vaccine was administered through nares, has been demonstrated to be safe [80–82].

## 3.6 Viral Haemorrhagic Septicaemia

Viral haemorrhagic septicaemia (VHS) is an OIE-notifiable viral disease of farmed rainbow trout in Europe [83]. In addition to rainbow trout, a large number of marine and freshwater fish are susceptible to the disease. The disease has been reported in about 80 species of fish in the Northern hemisphere, including North America, Europe, and Asia. VHS is caused by VHS virus (VHSV), an enveloped bullet-shaped virus measuring about 70 × 180 nm belonging to the genus *Novirhabdovirus*, within the family *Rhabdoviridae*. The genome is a negative-sense, single-stranded RNA of size approximately 11 kb [15]. The genome (3′ N-P-M-G-NV-L-5′) encodes six proteins: a nucleoprotein, N; a phosphoprotein, P; a matrix protein, M; a glycoprotein, G; a non-virion protein, NV, and a polymerase, L [84]. The neutralizing antibodies are targeted against the G protein and, hence, G protein is often the target for developing sub-unit or DNA vaccines [25]. The VHSV isolates have been grouped into four genotypes based on the genomic sequences. VHSV causes disease and mortality in all life stages. However, the disease is more common in young fish not previously infected [15]. The target organs of the virus are

kidney, heart, and spleen. In chronic cases, high virus titre is observed in brain [85]. Fish surviving the infection becomes the carrier of the virus. Virus is shed through urine and reproductive fluids, although there is no evidence of vertical transmission. Transmission is horizontal through contact with contaminated fish or water. Mortality is low at lower temperatures but the cumulative mortality is high. Although disease outbreaks occur during all seasons, it is more common during spring and when the water temperature is fluctuating [15]. Young rainbow trout fry is more susceptible with mortality reaching close to 100%. Control of the disease is by periodic surveillance and stamping out carrier fishes.

Currently, no commercial vaccine is available for VHS. A thermoresistant live-attenuated VHSV strain-induced high levels of protection [86]. However, the vaccine is not commercially available, probably, because the vaccine did not perform well under field conditions [25]. Another live-attenuated viral vaccine administered orally along with polyethylene glycol resulted in significant protection against VHSV [87]. Attempts were made to produce recombinant/sub-unit vaccine by expressing the G protein in *E. coli* [88], or *A. salmonicida* [89]. However, these recombinant/sub-unit vaccines did not offer protection due to the lack of post-translational modifications in the recombinant proteins produced in the prokaryotes. There are many reports of development of successful DNA vaccines against VHSV [90–94]. However, the efficacy of these vaccines was tested against homologous virus. Prevalence of many genotypes and the limited protection offered against heterologous strains are the disadvantages for commercial use of these vaccines. Further, DNA vaccines are not being approved in Europe, as the DNA-vaccinated fish may be considered as genetically modified organism [25]. An inactivated VHSV vaccine conjugated with poly lactic-co-glycolic acid (PLGA) nanoparticles, when administered through immersion route followed by a booster dose by oral route gave 73.3% RPS [95].

## 3.7 Spring Viraemia of Carp

Spring viraemia of carp (SVC) is an acute viral disease of carps and some other cyprinid and ictalurid species causing haemorrhagic and contagious viraemia [15]. It causes significant mortality during spring [16]. This disease is OIE-notifiable and is caused by SVC virus (SVCV), a member of the genus *Vesiculovirus* under the family *Rhabdoviridae* in the order *Mononegavirales*. The virus is enveloped and bullet-shaped, measuring approximately 80–180 nm in length and 60–90 nm in diameter. The genome of the virus is non-segmented, single-stranded negative-sense RNA consisting of 11,019 nucleotides encoding five proteins: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G), and an RNA-dependent RNA polymerase (L). The non-virion (NV) gene found in between the G and L genes in *Novirhabdovirus* genus of *Rhabdoviridae* family is missing in this virus [16]. Based on the partial G gene sequence, SVCV isolates are classified into four genotypes, 1a, 1b, 1c, and 1d. Isolates from North America and Asia belong to genotype 1a, while isolates from Europe belong to genotypes 1b, 1c, and 1d [96].

Carps less than 1 year are more susceptible to the virus, although all age groups can be affected with mortality ranging from 1% to 40% [15]. Liver and kidney are the target organs of the virus, while lower virus titre is observed in spleen, gills, and brain [97]. Virus is transmitted horizontally, although vertical transmission through eggs has not been ruled out. Disease outbreaks usually happen in spring in temperate countries when the temperature ranges between 11 and 17 °C, and mortalities are less at temperatures below 10 °C and above 22 °C [98]. Control measures to prevent disease outbreaks include adopting strict biosecurity measures and reducing stocking density during winter and early spring. In farms with environmental control, raising water temperature above 19 °C will prevent disease outbreaks [15].

Very few published reports are available on vaccination against SVCV. An inactivated oil-adjuvanted vaccine comprising two strains of SVCV was commercialized by a Czechoslovakian company (Bioveta) in 1982 [7]. This has been the first viral vaccine for fish; however, the vaccine is currently unavailable. The DNA vaccines for SVCV are not as efficacious as that of *Novirabdovirus* DNA vaccines [25]. A DNA vaccine expressing the full-length glycoprotein (G) gene of SVCV could produce 48% RPS on challenge with heterologous strain of SVCV in common carp [99], while 50–88% RPS was recorded in koi carp [100]. Embregts et al. [101], reported up to 100% protection in European common carp (*C. carpio carpio*) vaccinated with experimental DNA vaccine against SVCV by intramuscular route. However, the same vaccine when administered orally by alginate encapsulation method did not offer any protection [102]. Recombinant sub-unit vaccines conjugated to single-walled carbon nanotubes could induce specific and non-specific immune parameters and protect common carp vaccinated through immersion route suggesting the potential of single-walled carbon nanotubes for use in immersion vaccines [103]. No commercial vaccines are currently available for SVCV.

## 3.8 Infectious Pancreatic Necrosis

Infectious pancreatic necrosis (IPN) is a viral disease of salmonids affecting young fry of rainbow trout (*Salmo gairdnery*) and post-smolt of Atlantic salmon (*Salmo salar*) resulting in close to 100% mortality [104]. The disease is characterized by anorexia and abnormal corkscrew and erratic swimming [104]. The disease is caused by IPN virus (IPNV), a non-enveloped, icosahedral bi-segmented dsRNA virus [17] measuring around 70 nm [18]. The virus is the type species of the genus *Aquabirnavirus* under the family *Birnaviridae*. IPNV is the first fish virus to be isolated in vitro using tissue culture [6]. This is one of the most widely prevalent viruses infecting most of the farmed fishes causing high mortality in juvenile salmon when they are transferred from freshwater to seawater [25]. The genome consists of two segments—A and B. Segment A is of size 2962–3097 bp and segment B is around 2400 bp [19]. RNA-A codes for a polyprotein and a non-structural protein, VP5. The polyprotein is cleaved to generate VP2 (capsid protein), VP3, and VP4. RNA-B codes for VP1 which is the RNA-dependent RNA polymerase [105]. Based

on the sequence of VP2, IPNV has been classified into six genogroups comprising of ten serotypes [106]. Fish which recover from the infection become life-long asymptomatic carriers [107]. The virus is transmitted horizontally through water and vertically through eggs. The virus enters through gills, intestinal epithelium, and skin [19].

Several attempts have been made to develop vaccines against IPNV. However, attempts to protect fish at early stages against IPNV were not successful because the fish were not immunocompetent when they were the most susceptible to IPNV [25]. Inactivated whole-virus vaccine could protect Atlantic salmon better than sub-unit or DNA vaccine [108]. Recombinant vaccines containing capsid protein (VP2) of IPNV either alone or as a fusion protein with VP3 when injected to rainbow trout elicited increased levels of specific IgM and reduced viral load in challenged fish [109, 110]. Immature viral particles (provirus) of IPNV triggered neutralizing antibodies when injected to rainbow trout fry [111]. Virus-like particles of IPNV have also been demonstrated to be an excellent candidate for protecting fish against IPNV when administered intraperitoneally [112]. However, as administration of vaccine by injection is stressful and is not feasible for small fry, many oral vaccines have been developed which could protect salmonids from IPNV. A live-vector vaccine containing the coding region of VP2 of IPNV when administered by immersion route resulted in 88.24% RPS [113]. Live-recombinant *Lactobacillus casei* expressing the VP2 when administered orally to rainbow trout resulted in high neutralizing antibodies and low viral load after challenge [114, 115]. Many researchers have developed DNA vaccines containing plasmid constructs expressing VP2 of IPNV for administration through injection or orally through feed after conjugating with alginate microspheres or chitosan and tripolyphosphate nanoparticles [116–122]. The efficacy of the DNA vaccines was dose-dependent [121] eliciting specific IgM response, low viral load on challenge, and upregulation of various immune-related genes. Recombinant live virus has also been reported to protect rainbow trout from IPNV. A recombinant vaccine with IHNV as the backbone vector expressing VP2 of IPNV provided better protection and survival when challenged with IPNV and IHNV demonstrating the potential of recombinant viruses to protect fish against two or more viruses [123]. Many commercial vaccines, either as monovalent vaccine or as multivalent vaccine along with other inactivated bacterial and viral pathogens, are available against IPN for salmonids (Table 2).

## 3.9 Infectious Salmon Anaemia

Infectious salmon anaemia (ISA) is a systemic viral disease of salmonids, affecting mainly Atlantic salmon (*S. salar*), characterized by severe anaemia. It is an OIE-listed disease. The disease is caused by ISA virus (ISAV), the type species of the genus *Isavirus* under the family *Orthomyxoviridae*. Two variants of the virus exist, the highly polymorphic region (HPR)-deleted pathogenic ISAV, and the non-pathogenic HPR0 (non-deleted HPR) ISAV. HPR deleted ISAV is associated with clinical disease, while the HPR0 ISAV is not associated with clinical disease in

Atlantic Salmon [124]. ISAV is an enveloped virus measuring 100–130 nm in diameter and the genome consists of eight single-stranded, negative-sense RNA segments [20] coding for at least ten proteins [125] out of which four are major structural proteins. The haemaglutinin-esterase (HE) protein is one of the most variable proteins and is responsible for the receptor-binding activity and generates neutralizing antibodies. Hence, HE protein is commonly used for developing sub-unit or DNA vaccines [25, 126, 127]. The major route of infection is through gills and to a lesser extent through skin and intestine. The primary target for the virus is endothelial cells lining the blood vessels of gills, heart, liver, kidney, and spleen [128]. Diseased fish exhibit severe anaemia, haemorrhage, and necrosis in several organs [15]. The virus is excreted though urine, faeces, and mucus. The disease affects all life-stages and outbreaks are common in seawater cages. The disease has been reported in Norway, Canada, the United Kingdom, the Faroe Islands, the USA, and Chile [125]. Morbidity and mortality are typically low and the daily mortality ranges from 0.5% to 1%. The cumulative mortality also remains low, although in severe outbreaks mortality may exceed 90% over several months [15]. Control of ISA is through early diagnosis and culling all fish in the affected cages. Disinfection of eggs is an important control measure.

Many vaccines against ISAV have been licensed for use in Norway, Chile, Ireland, Finland, and Canada [25]. Many of them are inactivated, injectable vaccines either monovalent or multivalent with the addition of inactivated pathogens, usually pathogenic bacteria. An oral vaccine against ISA containing a recombinant hemagglutinin-esterase and fusion protein as antigens induced high level of specific IgM antibodies and protection in Atlantic salmon [129]. Inactivated vaccines, when injected along with adjuvants, elicited higher immune response and protection in a dose-dependent manner [130]. Subsequent oral administration of one or more booster doses of vaccines is beneficial and results in high level of specific serum IgM [131]. Several oral immunizations are required in the field to maintain high antibody levels and protection. A salmonid alphavirus-based replicon vaccine expressing HE of ISAV protected Atlantic salmon against the viral challenge [132, 133].

## 3.10 Pancreas Disease or Sleeping Disease

Pancreas disease (PD) or sleeping disease (SD) is an OIE-listed disease caused by infection with salmonid alphavirus (SAV) in Atlantic salmon, rainbow trout, common dab [21], and Arctic charr [22]. The disease is a systemic viral disease characterized by necrosis of exocrine pancreatic tissue, cardiomyocytic necrosis, and white skeletal muscle degeneration [15]. SAV is an enveloped spherical virus measuring 60–70 nm in diameter belonging to the genus *Alphavirus* and family *Togaviridae*. The genome consists of a positive-sense, single-stranded RNA of approximately 12 kb. The genome codes for eight proteins, four of which are non-structural proteins (nsP1–nsP4), and the remaining four are capsid glycoproteins (E1, E2, E3 and 6K). Glycoprotein E2 induces neutralizing antibodies [21] and,

hence, is an ideal candidate for developing sub-unit or DNA vaccines. Based on the sequences of protein E2 and nsP3, SAV isolates have been divided into six genotypes, SAV1–SAV6 [134]. Gills and intestinal track are the portals of entry. Although the predilection sites of the virus are not known, in acute cases, high titre of the virus is found in kidney, heart, blood, and several other organs. Virus is shed through mucus and faeces. Disease outbreaks are influenced by environmental factors, and increase in water temperature favours the virus [135]. The disease affects all life-stages in both freshwater and seawater [136]. The virus persists in the infected fish for several months and is transmitted horizontally through water [137].

A BEI inactivated SAV-1 vaccine administered to Atlantic salmon provided protection against cohabitant challenge with the wild-type virus [138]. Xu et al. [139], compared the efficacy of inactivated whole-virus vaccine with sub-unit vaccine and DNA vaccine expressing E1 and E2 spike proteins of salmonid alphavirus sub-type 3 (SAV-3) and concluded that the immunogenicity of inactivated whole virus was superior than sub-unit and DNA vaccines. Karlsen et al. [140], also demonstrated the near-total protection of Atlantic salmon immunized with inactivated SAV3 genotype. Two inactivated vaccines are commercially available for Atlantic salmon.

### 3.11 Tilapia Lake Virus Disease

Tilapia lake virus (TiLV) disease is a new and emerging disease, first observed in Israel in 2009 in tilapia [141]. Since then, the disease has spread to many countries in Asia, Africa, and America [142]. The disease is caused by TiLV, classified under the genus *Tilapinevirus*, family *Amnoonviridae*, and order *Articulavirales* [143]. TiLV is enveloped with icosahedral symmetry measuring 55–75 nm [141]. The genome consists of ten segments of linear, negative-sense, single-strand RNA of about 10.323 kb total length [23, 141]. The disease is characterized by high mortality during hot summer months with more than 80% mortality. The clinical signs include black discoloration, skin abrasions, and ocular degeneration. Histological changes observed include congestion of the internal organs such as kidney and brain with foci of gliosis and perivascular cuffing of lymphocytes in the brain cortex and ocular inflammation [141]. The disease is restricted to tilapines including wild, farmed, and hybrid tilapia and giant gourami [24, 141]. Other fishes co-habitated with infected tilapia or experimentally injected with TiLV did not exhibit clinical signs [24, 144]. No reports on vaccine development or commercial vaccines for TiLV are available.

## 4 Immune Response to Vaccines

The innate immune molecules such as interferon are induced rapidly in response to vaccination. Subsequently, the adaptive immune system of fish comes into play when the fish encounters a pathogen or after immunization. The B lymphocytes,

upon antigen presentation, differentiate into plasma cells and secrete IgM which are found in the serum and mucus of gills, skin, and intestine. IgM is the major immunoglobulin of fish. The specific serum IgM level is maximum following intraperitoneal vaccination compared to other routes of vaccination. IgM is also secreted into the mucus when immunized by immersion or by oral routes. In addition to mucosal IgM, systemic IgM is also produced, although at a lesser magnitude, upon immersion and oral vaccination. IgT, an intestinal immunoglobulin, equivalent to IgA of mammals but, phylogenetically distant from IgA, is secreted in the intestine upon exposure to antigens [145]. IgD is also a mucosal immunoglobulin, the transcripts of which are upregulated many folds in the gills upon immersion-vaccination in fish [146] suggesting that this immunoglobulin might play an important role in the mucosal immunity. Several factors are known to affect the modulation of fish immune response. In general, the adaptive immune response increases with age [147] and temperature [148] and decreases with chronic stress [149].

## 5 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, besides providing long-term protection. Hence, vaccination of fish is an important tool in fish 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 and, this shows the 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, establishment of protective antibody titre required to withstand a natural infection, and improved vaccine delivery methods for mass vaccination are the areas which require increased attention in the field of fish vaccinology.

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# Vaccines for Parasitic Diseases of Fish

B. Kar, A. Mohapatra, S. Parida, and P. K. Sahoo

**Abstract**

Globally, incidences of fish diseases caused by parasites have been on a rise, especially in intensive aquaculture practise, leading to considerable economic losses. The traditional control measures and therapeutics used to manage parasitic infections are associated with numerous limitations as well as risks. Vaccines have emerged as an effective means for control of pathogens; the use of vaccines for bacterial fish diseases has successfully cut down the use of antibiotics in aquaculture. However, development of vaccines for parasitic diseases of fish has seen limited success with availability of only commercial vaccine against sea lice. Nevertheless, significant strides have been made in understanding host-parasite interactions, which provides researchers with the arsenal of information required for identification of vaccine candidates and their development. In this review, we discuss protective responses reported in fish against major group of parasites and various efforts made in the field of vaccine development for important parasite groups of both marine and freshwater fish.

**Keywords**

Parasite · Diseases · Fish · Aquaculture · Vaccine

## 1 Introduction

The horizontal and vertical rise in aquaculture production in the last few years due to intensification and diversification of aquaculture operations is associated with a corresponding increase in fish diseases, especially transmissible fish parasites

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[1]. In all types of intensive aquaculture practised, the risk of parasitoses runs high owing to the ease of disease transmission in high-density monoculture as well as polyculture of fish species. Owing to increase in the incidences of fish parasitoses, there is a greater emphasis on studies related to biology, pathogenicity, transmission, and treatment of fish parasites. Additionally, the fish immune system is well-researched and in recent years there has been a considerable progress in our understanding of defence responses of fish against parasitic diseases.

A wide range of parasites occurs on farmed as well as wild fish populations. Fish act as favourable hosts for parasitic lifestyle because of a number of reasons. Water provides a physiologically stable, buffered environment, and its viscosity smoothens the dispersal and survival of eggs and fragile free-living stages [2] of various parasite groups. Fish offer a large surface area for encounter and colonisation of parasites. Additionally, fishes are mobile, creating the potential for dispersal of parasites. Therefore, fish are rarely found to be free of parasitic infections both in natural as well as in artificial habitats. Fish serve as hosts to many parasites from numerous invertebrate taxa—protozoan flagellates and coccidian species, metazoan cestodes, nematodes, crustacean copepods and branchiurans. In fact, it is estimated that the number of species of fish parasites of marine fish alone might be an astounding 100,000 species [3]. These parasitic associations differ considerably across different parasite taxa. Some of these parasites may exhibit host-specificity, and some may not. Some parasites utilize multiple host species to complete their life cycle, and many parasites have complex life cycles with several stages. More broadly, these parasites can be divided into ecto- and endoparasites of fish. Many of these parasites are reported to cause economic losses in fish: arthropod ectoparasites called sea lice (*Lepeophtheirus* and *Caligus* spp.) leading to serious concerns in both wild and farmed salmonid and gadidae species [4], *Trichodina* spp. affecting both freshwater and marine hatcheries [5], *Tetracapsula* spp. causing proliferative kidney disease (PKD) of salmonids [6], the ciliate *Ichthyophthirius multifiliis* causing Ichthyophthiriasis [7], *Argulus* spp. causing argulosis [8] and *Loma*, an intracellular fungal parasite infecting the gills of salmonids and other species [9]. Traditionally, the answer to parasitic infections has been the use of anti-parasitic chemotherapeutics, which have been widely used in spite of their devastating effect on the environment and non-target effect on other organisms. A concern that is associated with the use of these drugs and chemicals is the development of resistant parasite populations due to repeated exposure. Today, vaccination is an integral part of large-scale aquaculture for control of bacterial diseases, and this has cut down the use of antibiotics significantly. However, development of vaccination regimes against parasitic diseases has not been realized fully, except for a parasite vaccine launched in Chile against sea lice: first ever sea lice vaccine launched in Chile.

Parasitism has been recognized as one of the most successful ways of existence in nature [10]. Complex immunological processes are a part of the host-parasite interactions that take place in case of a parasitic infection. The immunity to parasitic diseases is described as a game of chess where the parasite evolves ways to evade the immune response and the immune system evolves to thwart the evasion [11]. Perhaps, the success of parasitism as a way of existence is based on this ability of the

parasites to evade immune recognition and subsequent responses. Most of these parasites undergo transformations with several phases or stages, even on or in the body of the host during the infection process, in which their habitat, mode of nutrition, reproduction and antigenic profile may change. In such infection models, owing to the immune-evasion strategies, designing effective anti-parasitic vaccines becomes a great challenge. Additionally, cultivation of parasites in laboratories, which often requires the natural hosts obligatorily, is a limiting factor. Nevertheless, a considerable development has been achieved in the characterization of fish immune responses and pathways involved in fish parasitoses. Recent studies on fish immunity to parasite infection have aided in the better understanding of responses involved in parasitic infections; these studies have been reviewed and compiled extensively with respect to fish parasites in general or a specific group of fish parasites [1, 12–17]. However, vaccine development against fish parasites continues to be elusive. In this chapter, major studies on the development of vaccines against various groups of fish parasites are discussed.

## 2 Anti-Parasitic Drugs and Disease Resistance

Drug resistance has been reported for most of the anti-parasitic drugs used in animals. Similar is the case with fish; for example, resistance of salmon louse and freshwater fish louse to common pesticides like organophosphates, synthetic pyrethroids, and avermectins have been reported. It is difficult to ascertain the exact mechanism of drug resistance, as this might be due to the induction by drug applications or due to the existence of resistant strains in the initial population [18]. The intensive use of parasitic drugs leads to accumulation of drug residues in products, which is a public health concern. Hence, the current practice of parasite control is not sustainable. Prevention or management of parasitic diseases using vaccination would facilitate environmental and economic sustainability in aquaculture. Further, targeted drug development rather than screening of existing molecules would be a very complex and time-consuming process.

## 3 Immune Memory in Fish: The Foundation of Vaccination

The immune system has been compartmentalised into two—the innate, non-adaptive immune system that learns to recognise pathogens through natural selection in the process of evolution working at the population level and, the acquired, adaptive immune system that works at the individual level and mounts better responses to threats based on its memory [19] (as described in detail in earlier chapters). However, the molecules of adaptive system responsible for its specificity and memory can neither be found in all organisms nor can they be traced back from their present form to precursor molecules in the process of evolution. The adaptive system seems to have appeared in the jawed vertebrates—first in the jawed fish, then passed on to higher vertebrates, and is not found in the jawless fish and other primitive lineages.

Both the innate and adaptive immunity work in tandem to provide protection against infections; however, adaptive immunity is more effective for the elimination of recurring infections because of memory cells and pathogen-specific membrane-bound as well as soluble receptors. Acquired/adaptive immunity in fish includes both humoral and cell-mediated responses. Similar to higher vertebrates, adaptive responses viz., immunoglobulins, T-cell receptors, cytokines, and major histocompatibility complex molecules (MHC) are existing in fish. While the B-cells are involved in the humoral response, the T-cells are responsible for the cell-mediated response. The B-cells proliferate and differentiate into long-lasting memory cells and plasma cells secreting specific antibody (Ab) following activation by a specific antigen of either soluble form or in association with the MHC marker on antigen-presenting cells (APC). In teleosts, four types of immunoglobulins (Igs) have so far been recorded, namely, IgM, IgD, IgZ or IgT [20], and IgM–IgZ chimera [21] that mediate the humoral response. Production of memory T-cells is initiated only when T-cells recognize a pathogen associated with MHC markers on the APCs. The correct presentation of antigen leads to a cascade of events that includes cytokine production, which regulates or enhances the cellular response. Many studies suggested that at the time of parasitic infections, levels of antibody-secreting cells (ASC) increase at the site of infection. *Ichthyophthirius multifiliis* infection induces production of many adaptive immune molecules such as T cell receptor β (TCRβ), cluster of differentiation 4 (CD4), CD8, IgM, and MHC II along with ASC. Channel catfish develop protective immunity to *I. multifiliis* re-infection, which confirms the presence of humoral immunity facilitated by $IgM^+$ memory B cells. Similarly, salmonids develop resistance to myxozoan parasite, *Ceratomyxa shasta* by increasing $IgT^+$ B-cells. Generally, host develop specific-antibody response against almost all parasites of different taxa. Certain limitations do exist in adaptive system of fish in comparison with mammals like the restricted repertoire of immunoglobulins caused by the limited organization of clonal selection and absence of lymph nodes, and restricted secondary response and memory owing to the absence of Ig class switch [22]. Very few reports suggest Th1/Th2 switching response in fish with respect to parasitic infections. Nevertheless, all the basic features of adaptive immunity that are involved in immunological memory are present in fish and, though relatively delayed, it does provide long-lasting immunity in fish, which is crucial for anti-parasite vaccination.

The specific Ab responses against parasites have been reported for several parasitic infections of fish, which in some cases have been related to protection; thus, development of commercial vaccines against fish parasites is definitely a possibility. This article discusses different protective responses reported in fish against major group of parasites and various efforts made in the field of vaccine development for important parasite groups of both marine and freshwater fish and the details of which are enlisted in Table 1.

**Table 1** List of parasitic diseases and host-parasite interaction influencing modulation of immune molecules

| S. No. | Taxonomic Group | Pathogen | Disease | Major fish affected | Immune response | Available Vaccines | Reference (s) |
|---|---|---|---|---|---|---|---|
| 1 | Ciliophora | *Ichthyophthirius multifiliis* | Ichthyophthiriosis | *Oncorhynchus mykiss* | ↑ Pro-inflammatory molecules: IL1β, TNFα, CXCa, CXCR1, CXCR2 | Not available | [7] |
| | | | | | ↑ Complement factor: C3 | | |
| | | | | | ↑ Anti-microbial peptides: Histone-like proteins, Piscidine 2 | | |
| | | | | | ↑ Acute-phase proteins: SAA | | |
| | | | | | ↑ Adaptive immune response: IL-22, CD4, CD8, MHCII$^{+}$, TCRβ, IgT | | |
| | | | | *Cyprinus carpio* | ↑ Pro-inflammatory molecules: IL1β, TNFα, CXCa, CXCR1, CXCR2 | | |
| | | | | | ↑ Complement factor: C7 | | |
| | | | | | ↑ Acute-phase proteins: SAA, factor B/C2-A | | |
| | | | | | ↓ β2m, (PGDS) Prostaglandin D2 synthase | | |
| | | | | | ↑ Adaptive immune response: CD8α+, MHCII$^{+}$ | | |
| | | | | | ↑ Adaptive immune response: Abs | | |

(continued)

**Table 1** (continued)

| S. No. | Taxonomic Group | Pathogen | Disease | Major fish affected | Immune response | Available Vaccines | Reference (s) |
|---|---|---|---|---|---|---|---|
| | | | | *Ictalurus punctatus* | ↑ Pro-inflammatory molecules: IL1β, TNFα | | |
| | | | | | ↑ Adaptive immune response: Abs | | |
| | | | | *Oreochromis niloticus* | ↑ Pro-inflammatory molecules: IL1β, TNFα | | |
| | | | | | ↑ Adaptive immune response: Abs | | |
| | | *Cryptocaryon irritans* | Cryptocaryosis | *Chelon labrosus* | ↑ Pro-inflammatory molecules: IL1β, TNFα | Not available | [23, 24] |
| | | | | | ↑ Adaptive immune response: Abs | | |
| | | | | *Epinephelus coioides* | ↑ Pro-inflammatory molecules: IL1β, TNFα | | |
| | | | | | ↑ Adaptive immune response: Abs | | |
| | | | | *Lates calcarifer* and other fish species | ↑ Pro-inflammatory molecules: IL1β, TNFα | | |
| | | | | | ↑ Adaptive immune response: Abs | | |
| | | Scuticocillatida | Scuticocilliatosis | *Psetta maxima* | ↑ Adaptive immune response: Abs | Not available | [25] |
| | | | | *Paralichthys olivaceus* | ↑ Adaptive immune response: Abs | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2 | Kinetoplastida | *Cryptobia salmositica* | Cryptobiosis | *O. mykiss*, *Oncorhynchus* spp. | ↑ Complement factor: all classical and alternative pathways factors | Not available | [26] |
| | | | | | ↑ PAMP: Cysteine protease | | |
| | | | | | ↑ Acute-phase proteins: α2 microglobulin | | |
| | | | | | ↑ Adaptive immune response: Mϕs, Abs | | |
| | | *Trypanoplasma borreli* | | *C. carpio* | ↑ Pro-inflammatory molecules: IL1β, TNFα, CXCR2 | | [27] |
| | | | | | ↑ Complement factor: C3 | | |
| | | | | | ↑ Acute-phase proteins: SAA, α2 microglobulin | | |
| | | | | | ↑ Adaptive immune response: Abs | | |
| | | *Trypanosoma carassii* | | Cyprinids | ↑ Pro-inflammatory molecules: IL1β, TNFα, CXCL-8, CCL-1 | | [28, 29] |
| | | | | | ↑ Adaptive immune response: IgM | | |
| 3 | Amoebozoa | *Neoparamoeba perurans* | Amoebic gill disease (AGD) | *O. mykiss* | ↑ Pro-inflammatory molecules: IL1β, TNFα, IL-8 | Not available | [17] |
| | | | | *Salmon salar* | ↑ Pro-inflammatory molecules: IL1β | | [30, 31] |
| | | | | | ↑ Adaptive immune response: Mϕs, Abs | | |
| | | | | | ↓ IFN-ϒ, MHC genes | | |

(continued)

**Table 1** (continued)

| S. No. | Taxonomic Group | Pathogen | Disease | Major fish affected | Immune response | Available Vaccines | Reference (s) |
|---|---|---|---|---|---|---|---|
| 4 | Dinoflagell-ata | *Amyloodinium ocellatum* | Amyloodiniosis | *O. mykiss*, Hybrid srtiped bass, *Oreochromis aureus*, *Amphiprion frenatus* | ↑ Pro-inflammatory molecules: IL1β, TNFα | Not available | [17, 32] |
| | | | | | ↑ Anti-microbial peptides: Histone-like proteins | | |
| | | | | | ↑ Adaptive immune response: Abs | | |
| 5 | Microsporidia | *Loma salmonae* | Microsporidiosis | *S. salar*, *O. mykiss* | ↑ Lectin complement pathway factors | Not available | [17, 33] |
| | | | | | ↑ Adaptive immune response: Abs | | |
| | | *Tetramicra brevifilum* | | *P. maxima* | ↑ Adaptive immune response: Abs | | |
| | | *Glugea* spp. | | *Platichthys flessus*, *Plecoglossus altivelis*, *Epinephelus akaara* | ↑ Lectin, complement pathway factors | | |
| | | | | | ↑ Adaptive immune response: Abs | | |
| 6 | Myxozoa | *Myxobolus cerebralis* | Whirling disease | *O. mykiss* | ↑ Pro-inflammatory molecules: IL1β, COX-2, TGFβ | Not available | [17, 34] |
| | | | | | ↓ Mϕ proteins (Nramp α and β) | | |
| | | | | | ↑ Adaptive immune response: Abs | | |
| | | *M. artus* | | *C. carpio* | ↑ Adaptive immune response: Abs | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Enteromyxum leei* | Enteromyxosis | *P. maxima*, *Sparus aurata*, *Diplodus puntazzo* | ↑ Pro-inflammatory molecules: IL-β1 | | |
| | | *E. scophthalmi* | | | ↑ Adaptive immune response: MHCI, MHCII, $IgM^+$ | | |
| | | | | | ↓ TNF α | | |
| | | *Tetracapsuloides bryosalmonae* | Proliferative kidney disease (PKD) | *O. mykiss* | | | |
| | | *Ceratomyxa shasta* | Ceratomyxosis | *O. mykiss* | ↑ Pro-inflammatory molecules | | |
| | | | | | ↑ Adaptive immune response: $IgT^+$ | | |
| | | *Sphaerospora dicentrarchi* | Sphaerosporosis | *Dicentrarchus labrax* | ↑ Adaptive immune response: Abs | | |
| | | *Thelohanellus kitauei* | | *C. carpio* | ↑ Cytokines: IL-6, IL-1β and TNF-α | | [35] |
| | | | | | ↑ Adaptive immune response: IgM | | |
| | | *Sphaerospora molnari* | | *C. carpio* | ↑ Cytokine profile | | [36] |
| | | | | | ↑ Adaptive immune response: IgM, $IgM^+$ B lymphocytes | | |
| 7. | Crustaceans | *Lepeophtheirus salmonis* | Sea lice infestation | *S. salar* | ↑ Stress response—IL-1β, TNF-α, TGF-β, COX-2, MHII Biphasic immune response: Inflammatory response (↑ initial response: IL-1β, IL-1R, IL-12, CD4/8, | Not available | [37–54] |

(continued)

**Table 1** (continued)

| S. No. | Taxonomic Group | Pathogen | Disease | Major fish affected | Immune response | Available Vaccines | Reference (s) |
|---|---|---|---|---|---|---|---|
| | | | | | TNF α, prostaglandin E2, etc.) followed by a major down-regulation of genes and then a second innate inflammatory spike (IL-1, prostaglandin E2, MMPs, IL-10, etc.) | | |
| | | | | | ↓ Splenic proteases and APCs | | |
| | | | | | ↑ Arginase1, IL-10, and (TGF)-β, known alternative macrophage signals and TH2 drivers | | |
| | | | | | ↓ Antiviral responses: (MH class I-b, Mx, galectin 9, TRIM 16 and TRIM 25, etc.) | | |
| | | | | | ↑ Adaptive immune response: Abs | | |
| | | *Argulus japonicus* | Argulosis | *C. carpio* | ↑ Pro-inflammatory molecules: CXCa, CXC1, TNFα | | [55, 56] |
| | | *Argulus siamensis* | | *L. rohita* | ↑ Innate immune molecules in skin: TNFα, TLR22, NKEF-B, MnSOD | | [57–59] |
| | | | | | ↓ Innate immune molecules in kidney: C3, | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | lysozymes, TLR22, CXCa | | |
| | | | | | ↑ Adaptive immune response: IgM, IgZ, and IgD | | |
| | | *Sinergasilus major* | | *Ctenopharyngodon idella* | ↑ Immune molecules: C3, MHC I, α-2-macroglobulin, source of immunodominant MHC-associated peptides (SIMP) and TNF receptor-associated factor 2 binding protein (T2BP) | | [60] |
| 8. | Monogenea | *Gyrodactylus derjavini* | Gyrodactylosis | *O. mykiss* | ↑ Pro-inaflammatory molecules: IL-1β, TNF-α, iNOS, COX2 | Not available | [61] |
| | | *Gyrodactylus salaris* | Gyrodactylosis | *S. salar* | ↑ Mc1-1 related to IL-1β | | [61] |
| | | | | Baltic *S. salar* | ↑ MHC I, Mx, IFN-g and CD8a genes | | [62] |
| | | *Neoheterobothr-ium hirame* | | *Paralichthys olivaceus* | ↑ Matrix metalloproteinases-9 and 13, leukotrine-B4 receptor, CD20 receptor, MHC I and II b chain, Ig H and L chains | | [63] |
| | | *Diplectanum aequans* | Diplectanosis | *Dicentrarchus labrax* | ↑ Pro-inflammatory cytokines: IL-1β and TGF-β | | [64] |
| | | | | *D. labrax* | ↑ Adaptive immune response: Abs | | [65] |

(continued)

**Table 1** (continued)

| S. No. | Taxonomic Group | Pathogen | Disease | Major fish affected | Immune response | Available Vaccines | Reference (s) |
|---|---|---|---|---|---|---|---|
| | | *Dactylogyrus catlaius* | Gill-fluke infection | *L. rohita* | ↑ Inflammatory response: IL-1β | | [66] |
| | | | | | ↓ MHCI, MHCII, β2-microglobulin, TLR22, TNFα | | |
| | | *Pseudodactylogyrus* spp. | | Eels | ↑ Adaptive immune response: Abs | | [67, 68] |
| | | *Discocotyle sagittata* | | *O. mykiss* | ↑ Adaptive immune response: Abs | | [69] |
| | | *Heterobothirum okamatoi* | | *Takifugu rubripes* | ↑ Adaptive immune response: Abs | | [70, 71] |
| | | *Neobenedenia melleni* | | *Oreochromis mossambicus* | ↑ Adaptive immune response: Abs | | [72] |
| 9. | Digenea | *Diplostomum spathaceum* | Diplostomosis | *O. mykiss* | ↑ Adaptive immune response: Abs | Not available | [73, 74] |
| | | *Rhipidocytle fennica* | | *Rutilus rutilus* | ↑ Adaptive immune response: Abs | | [37] |
| | | *Sanguinicola inermis* | Sanguinicolosis | *C. carpio* | ↑ Adaptive immune response: Abs | | [75] |
| | | *Cardicola forsteri* | | *Thunnus maccoyii* | ↑ Adaptive immune response: Abs | | [76] |
| 10. | Cestoda | *Diphyllobothrium* spp. | | *O. mykiss* | ↑ Adaptive immune response: Abs | Not available | [38] |
| 11. | Nematoda | *Anguillicola crassus* | | *Anguilla japonica* and *A. anguilla* | ↑ Adaptive immune response: Abs | Not available | [77] |

## 3.1 Ciliophora

Ciliates are the most commonly encountered protozoan parasites of marine and freshwater fish. The quantum of infection decides the course of the disease; mortality generally occurs only when the parasite is excessively accumulated in the host system. Skin and gill irritation are the common pathological symptoms.

***I. multifiliis*** is a highly pathogenic ciliated protozoan parasite that causes white spot disease 'ich' and infects all species of freshwater fish. Significant losses are estimated due to infection of this parasite in channel catfish and trout. The biology and economic importance of this parasite have been reviewed extensively. This parasite possesses a simple life cycle including a reproductive tomont, an infective theront, and a parasitic trophont. Once the fish is exposed to the parasite, its highly motile theronts get attached to the epithelial surface of skin forming an extracellular amoeboid trophont in few minutes. These trophonts remain on fish and grow for approximately 7 days. After maturation, these are released from the fish to the free-living state producing a translucent proteinaceous cyst and this will attach to any substratum available in water bodies. This encapsulated tomont stage is resistant to medication because of the thick gelatinous covering. After a series of palintomic divisions, many tomites or daughter cells are produced and these, in turn, transform into the infective stage called theronts, which infect other susceptible fish.

As a result of host-parasite interaction, the motile theronts extensively damage the skin and gill epithelia by using perforatorium, which initiates inflammatory responses at the site of infection. It has been demonstrated that neutrophils migrate to the area of invasion within 24 h of infection [78]. Extravasation and influx of neutrophils are facilitated by increased expression of chemokines such as CXCa, CXCR1, CXCR2 in the skin and blood. In the early phase of infection, many proinflammatory cytokines like interleukin-1β (IL-1β) and tumour necrosis factor α (TNF-α) and C3 transcript levels were upregulated [79, 80] providing evidence that complement has some role to play during infection. Many monocytes and macrophages produce acute-phase proteins and serum amyloid protein A (SAA) at the site of the target tissue—the skin, which acts as a chemo-attractant for phagocytes and T-lymphocytes. *I. multifiliis*-specific antibodies (Abs) have been reported in skin and gills of infected hosts. Many adaptive immune molecules such as CD4, CD8, TCR β, and genes-encoding Abs IgM and IgT have been detected at the infection site. It has been suggested that Abs bind to parasite cilia to alter its behaviour and induce an escape reaction whereby specific IgT takes a central role in protection against *I. multifiliis* [81]. Infected fish mainly produce a type of Ab against immobilization antigens or i-antigens that immobilize the parasite in vitro. A simple infection model showing cellular and humoral mechanism of mucosal and systemic immunity was described, that clearly explains how recruitment of inflammatory cells, mainly neutrophils and eosinophils, and other pro-inflammatory molecules attract B and T lymphocytes to the mucosal epithelium [7]. These lymphocytes

proliferate and produce more and more antibody-secreting plasma cells and memory cells against specific i-antigen.

Over the years, many approaches were developed and tested for conforming protection against *I. multifiliis*. The most common method was to immunize the host with killed or inactivated parasites—theronts and trophonts [82, 83]. More rapid and strong immune responses in hosts were observed when live-attenuated parasites were used instead of killed ones. Three different isolates of formalin-killed parasites from different origins were mixed to form a trivalent formulation and immunization was carried out with an adjuvant, Montanide ISA 763A [1]. Challenge with live parasites resulted in up-regulation in lysozyme and complement levels both in the control and immunized hosts. So different formulations of killed parasites were tried in medium-scale vaccination trials, where trivalent antigen without adjuvants provided better results than monovalent and pentavalent antigens. As *I. multifiliis* is an obligate parasite, collection of it could be possible only from live infected fish because in vitro culture system is yet to be developed. This acts as a major constraint in studies related to host-parasite interactions. In channel catfish, ciliated membrane protein and purified protein antigens were used as subunit vaccines [84]. However, the most efficient candidate is i-antigen-derived vaccines, which include either purified i-antigens or passive Ab transfer raised against i-antigens. A major limitation of using purified i-antigen was that it provides protection against a specific serotype while many serotypes are present. DNA vaccine constructs with two i-antigen genes—52A and 52B variants—alone or together and in combination with plasmids expressing the glycoprotein G of Atlantic salmon haemorrhagic septicaemia virus were injected but failed to produce any protective effect against *I. multifiliis*. Similarly, i-antigens were also targeted for recombinant vaccine production of a closely related ciliate, *Tetrahymena termofila*, using delivery system of i-antigen genes. It causes serious health issues in the host, as cadmium originated from gene promoter accumulates in the fish epidermis and produces toxic effects. To increase the depth of study for vaccine candidature, transcriptional profiling of two major stages, theronts and host-associated trophonts, was done [85]. *I. multifiliis* genome sequence revealed the presence of 17 different i-antigen genes of which five serotypes, A–E, were characterized based on immobilization of i-antigen-specific antisera. This provided very important genomic information, which will further help to identify new drug targets, efficient delivery systems, and promising vaccine candidates against *I. multifiliis* infection. Additionally, the immunogenic parasite protein i.e., neurohypophysial domain protein #10 is now known to give significant protection against *I. multifiliis* and it might be a probable vaccine candidate supplementing the protective i-antigen in future vaccine trials [86].

***Cryptocaryon irritans*** is a ciliated ectoparasite, which causes white spot disease, commonly called marine ich in marine fishes. It has a similar life cycle as that of *I. multifiliis* possessing both trophont and infective theront stages. Its length of life cycle depends on temperature, salinity, and host. When the parasites adhere to the host epithelium, it produces inflammatory responses involving both mucosal and systemic immunity. It has been reported that *Chelon labrosus* showed acquired

protection against *C. irritans* after being immunized with controlled infection, and humoral Abs were detected in immunized *Lates calcarifer* serum [23, 87]. Protective immunity also developed in grouper (*Epinephelus coioides*) against *C. irritans* after immunization with live and formalin-killed theronts [24]. A high Ab titre in the skin mucus and serum was observed after eighth week of immunization providing good protection against *C. irritans*. The immobilization antigen (iAg) has been demonstrated as a protective immunogen against *C. irritans* infection. From the transcriptome profile of *C. irritans*, nine putative I-antigen transcripts were found [88]. The parasitic Hsp70C when co-administered with iAg to fish, provides the evidence of humoral and cellular immune responses in immunized fish against *C. irritans* [89].

Scuticociliates are one of the dominant ciliate taxa in many estuaries and coastal waters. Scuticociliates can infect fish and behave as histophagous parasites, thus causing severe mortalities in farmed fish. Scuticociliates pathogenic to fish include several species of the genera *Miamiensis*, *Pseudocohnilembus*, and *Uronema*. Scuticociliate-infected fish show ulcers and dark patches on the body surface, exophthalmia, and distension of the peritoneal cavity along with internal signs such as liquefaction of the brain, haemorrhages, and muscle ulceration.

Unlike most other fish parasites, scuticociliates can be cultured in vitro; this has facilitated studies on vaccine development. Vaccines against scuticociliates have been reported to elicit good Ab response and protection. For example, turbot vaccinated with whole (formalin-killed), lysed or even ciliate components of *Philasterides dicentrarchi* or other scuticociliate species as antigen and olive flounder immunized with lysed *P. dicentrarchi* or *Miamiensis avidus* showed humoral immunity and protection [25, 90, 91]. Similarly, sonicated *Uronema marinum* ciliate antigen induced a good immunological response in the kelp grouper [92]. Several adjuvants, including oil-based adjuvants (Freund's complete adjuvant or Montanide), microparticles (made of poly [D,L-lactide-co-glycolide] or chitosan-Gantrez), and GERBU (based on a glycopeptide derived from *L. bulgaricus* cell walls), have been standardized to increase the immunogenicity of the antigens tested [25]. Few protective antigens have also been identified and characterized as potential vaccine candidates. Immobilization antigens expressed by *P. dicentrarchi* have been identified to be important in inducing protection against this pathogen. Surface antigens expressed constitutively during infection by *P. dicentrarchi* have been shown to be a stronger inducer of immune responses in comparison to immobilization proteins [91]. In terms of internal antigens, b-tubulin from *Pseudocohnilembus persalinus* has been explored as a vaccine candidate against this ciliate [93].

Despite the above-mentioned advances, a commercial vaccine does not exist for these parasites. An important hindrance is the presence of many serotypes of ciliates, which calls for the characterization of all serotypes and subsequent development of multi-valent vaccines addressing the differences in antigens of the serotypes. Besides, in vitro culture of infective stages of the parasite species is an area that needs to be developed.

## 3.2 Kinetoplastida

***Cryptobia salmositica*** is a flagellated parasite known to cause cryptobiosis in both marine and freshwater fishes. This parasite mainly affects all types of *Oncorhynchus* spp. To study the protective response of *Oncorhynchus mykiss*, it was immunized with the live-attenuated parasite. Cell-mediated responses were detected by an increased level of macrophages stimulating respiratory burst activity and release of superoxide anions. Complement cascade was activated by complement molecules fixing Abs forming immune complexes on parasite membrane, which resulted in the lysis of the parasite. A high Ab titre was detected by ELISA after *C. salmositica* challenge [94]. *C. salmositica* contains cysteine protease and metalloprotease, which function as pathogen-associated pattern recognition molecules along with surface carbohydrate molecules. In an experiment, it was demonstrated that parasitaemia in brook charr declined when neutralization of metalloprotease by α2 macroglobulin occurred both in vivo and in vitro [95]. A less infective and non-pathogenic avirulent strain was produced by extended in vitro culture, which produced no disease and gave protection to rainbow trout by complement-fixing Abs production [26]. A monoclonal antibody (mAb-001), produced against carbohydrate moieties of metalloprotease and cysteine protease, reduced infectivity, multiplication, and survival of parasite [14]. DNA vaccine constructs, which were produced with metalloprotease and cysteine protease genes, were tested on *O. mykiss* and *Salmo salar*. After infection, lower parasitaemia, delayed peak parasitaemia, and faster recovery with detection of agglutinating Abs against *C. salmositica* were observed in metalloprotease-vaccinated fish. But cysteine-protease-vaccinated fish and plasmid alone could not render protection to the disease. In a separate experiment, isometamidium chloride, a prophylactic drug that gives 30% protection to infected fish was conjugated with mAb-001, and polyclonal Abs were isolated from recovered fish [96]. When polyclonal antibodies-conjugated drugs (PAIC) were injected into the infected host, it caused lysis of the parasite spreading throughout the kinetoplast. The above studies highlight the developments of a fish vaccine against *C. salmositica*.

***Trypanoplasma borreli*** is an extracellular blood flagellate that infects populations of common carp (*Cyprinus carpio*) and tench (*Tinca tinca*). Carp infected with *T. borreli* stimulate host immune response by producing nitric oxide (NO), reactive oxygen species (ROS) from neutrophils [97] along with up-regulation in the expression of TNFα and IL-1β. Transcript levels of acute-phase proteins such as C3, SAA, and α-2 macroglobulin were high in flagellate injected carps [98]. When carp were injected with live *T. borreli*, specific Abs for the parasite were detected, which help in lysis of the parasite and decrease parasitic load in the host by activating the classical complement pathway.

***Trypanosoma carassii*** is an extracellular parasite, which is resistant to complement lysis in the host because of its potent evolutionary evasion mechanism. Calreticulin of *T. carassii* binds to C1q complement factor inhibiting the complement cascade

[99]. Goldfish infected with this parasite developed an adaptive immune response. Recombinant hsp70 isolated from *T. carassii* upregulated different pro-inflammatory cytokines (TNF-α, IL-1β, and IL-12) and chemokines (CCL-1 and CXCL-8) in goldfish macrophages [28]. Fish recovered from the infection developed IgM, which can be used for passive immunization against *T. carassii*.

## 3.3 Amoebozoa

***Neoparamoeba*** is an economically important group and causes a potentially fatal disease in some marine fish called amoebic gill disease (AGD). In a molecular-based approach, *Neoparamoeba perurans* was identified as the predominant species of amoeba responsible for the induction of AGD. Immune response of host during this infection was widely studied suggesting up-regulation of pro-inflammatory molecules along with inflammatory cellular reactions in gills and internal organs of salmon and trout. Increased level of MHC II molecules, receptors of the immunoglobulin superfamily, helps to imitate immune responses, and this was detected within AGD lesions of *S. salar* [100]. MHC polymorphism has been reported to be associated with salmon resistance to AGD [101]. Specific Abs have been detected in *S. salar* sera against the disease but this confers no protection to AGD [102]. However, multiple exposures to the disease help in developing resistance to AGD, which is associated with the presence of anti-*Neoparamoeba* sp. Abs in the serum of Atlantic salmon [103]. The transcriptome analysis of *N. perurans* revealed new sequence information of a mannose-binding protein-like factor, r22CO3, which helps in the attachment of the parasite and induced production of systemic and mucosal Abs that bind to the surface of *N. perurans*. High level of serum Ab production was induced in gill and skin of *S. salar* after immunization with recombinant protein r22CO3. However, after *N. perurans* infection, neither difference was observed in mortality curve in control and immunized groups nor did it have any effect on the size of the lesions of AGD. The infected fish developed *Yersinia ruckeri* co-infection, which may have interfered with the survival of the challenged fish. The serum Abs detected after immunization with r22CO3 confirmed activation of adaptive immunity but no protection to *N. perurans* infection [30, 104].

## 3.4 Dinoflagellata

Dinoflagellate ectoparasite, *Amyloodinium ocellatum* causes amyloodiniosis and possesses a tri-phasic life cycle that includes: the infective dinospore that attaches to host; trophont, which is the attached feeding stage and tomont, the reproductive stage. Immune response of host in case of amyloodioniosis is not widely studied. But some fish develop resistance to amyloodioniosis upon repeated exposure to non-lethal doses of the trophont stage of the parasite [105]. The protective response in *Oncorhynchus mykiss* and hybrid striped bass (*Morone saxatilis* male × *M. chrysops* female) was mainly developed by administration of

anti-microbial peptides named as HLP-1 (histone-like proteins), which caused severe developmental abnormalities in the parasite [32]. Specific Abs were also detected by ELISA from serum samples of *Oreochromis aureus* immunized with *A. ocellatum* [106], cultured striped bass recovered from an amyloodioniosis outbreak [107], and in *Amphiprion frenatus* [105, 108]. Naturally infected Mediterranean seabass was also found to produce specific Abs after the infection of amyloodiniosis; this confirmed the association of acquired immunity with Ab response [109].

## 3.5 Microsporidia

***Microsporidia*** are eukaryotic, unicellular organisms belonging to the phylum Microspora. All microsporidia are obligate, spore-forming, intracellular parasites that invade vertebrates and invertebrates. These parasites affect major farmed fishes such as salmonids, flatfish, eels, and ornamental fishes like zebrafish and killifish. Fishes are host to 156 recorded species of microsporidia assigned to 14 genera. The infected cells usually enlarge to accommodate the parasite, and these enlarged cells are termed as xenomas that help to protect the parasite from the host immune response and localize the parasite from further spreading in the host. Microsporidia are classified into two groups based on the ability to form xenomas. *Glugea*, *Tetramicra*, *Loma*, *Ichthyosporidium*, *Jirovecia*, *Microfilum*, *Microgemma*, and *Nosemoides* form xenomas; *Nucleospora*, *Pleistophora*, *Heterosporis*, and *Thelohania* do not form xenomas [110].

***Loma salmonae*** is one of the most important emerging microsporidians affecting Pacific salmon, *Oncorhynchus* spp. of Pacific Northwest. This parasite mainly causes Microsporidial Gill Disease of Salmon (MGDS)—an infection resulting in pale gills with petechial haemorrhages, inflammation, hyperplasia, and formation of white cysts termed xenomas. Protective humoral response has been recorded in microsporidian infections. When naive rainbow trout were passively immunized using the serum of the fish infected by *L. salmonae*, a limited protection against the parasite in the gut was observed [111]. Studies related to specific cellular immunity have been carried out as well. Immunization with spores of *L. salmonae* had a proliferative effect on the head kidney mononuclear cells (MNC), which also protected from xenomas formation [33]. Vaccination against *L. salmonae* infection is associated with the cellular immune response. An attempt to develop a vaccine was made by using a less virulent variant of *L. salmonae* (SV strain); this showed a substantial lowering of the infection [112]. An inactivated spore-based vaccine has been demonstrated to provide protection 3 weeks after exposure and gradually a complete resistance by a reduction in the xenoma intensity [113]. This vaccine has a simple production protocol and is highly recommended due to its very low possibility of transmission because of the use of dead spores.

***Heterosporis saurida***, a microsporidian parasite of lizardfish, *Saurida undosquamis*, causes severe economic losses in marine aquaculture. Novel treatment strategies like small interfering RNA molecules causing knockdown expression of ATP/ADP antiporter 1 and methionine aminopeptidase II genes reduced *H. saurida* infection levels in eel kidney cells by 40% and 60%, respectively, as measured by qRT-PCR and spore counts [114].

## 3.6 Myxozoa

Myxozoans are intracellular and intercellular parasites of fish and include some economically important parasite species. The genera *Myxidium*, *Sphaerospora*, *Chloromyxum*, and *Myxobolus* parasitize fish in freshwater habitats. Other genera, such as *Ceratomyxa*, *Parvicapsula*, *Ortholinea*, and *Kudoa*, occur mostly as marine parasites. *Myxobolus cerebralis*, the causative agent of whirling disease of salmonids, is an economically important parasite owing to the high losses it can cause. The three species of the genus *Enteromyxum* (*E. leei*, *E. fugu*, and *E. scophthalmi*) infect various economically important fish species like turbot, fugu, and puffer fish resulting in high mortality rates along with other pathological complications.

Some aspects of humoral and cellular immune responses against the myxozoan parasites have been characterized with the primary motive of developing immunological interventions for control. As early as 1979, Ferguson and Ball [115] reported that fish recovering from previous infection of *Tetracapsuloides bryosalmonae* are relatively more resistant to challenge infections. In case of several myxozoan infections such as *Ceratonova shasta* [116], *M. cerebralis* [117], *Myxobolus artus* [118], and *E. scophthalmi* [119], specific humoral response has been reported. The immune response of European seabass after intra-coelomic immunization with spores of *Sphaerospora dicentrarchi* and adjuvant was evaluated, and it was concluded that the immunization predominantly activates the innate immunity; specific Abs were not detected [120]. Both turbot and gilthead sea bream can mount a specific immune response against *Enteromyxum* spp. [119, 121], but the pace of Ab production is relatively slow. However, specific anti-*E. scophthalmi* Abs were detected in the turbot that survived enteromyxosis infection [122] and antibody was detected as soon as 48 days post-infection, if fish had previous exposure [122], whereas naïve animals died at 40–49 days post-infection [123, 124]. In experimental infection of turbot with *E. scophthalmi*, an increase in $Ig^+$ cells was detected in the intestine [125]. Some information is available on the parasite structures stained with fish Abs [121] but the specific antigen is unknown. Identification of potential antigens as vaccine candidates is yet to be done in the case of myxozoans.

Salmonids have been reported to develop some resistance against *Ceratomyxa shasta*. In a recent study of *C. shasta* infections in rainbow trout, a large accumulation of $IgT^+$ cells was demonstrated mainly in the lamina propria of the intestine of survivor fish 3 months post-infection [34, 126]. This increase in $IgT^+$ B cells also coincided with the IgT level against the parasite, mostly in the intestinal mucus

[126]. Recently it was reported that IgM and cytokines such as IL-6, IL-1β, and TNF-α levels were highly up-regulated during *Thelohanellus kitauei* infection in carp. High level of IgM secretion indicates its anti-parasitic immune response in carp [35]. Similarly, *Sphaerospora molnari* causes infection in *C. carpio* and modulates both innate and adaptive immune responses. It mainly activated proinflammatory response during early infection followed by B-cell-mediated response during 35 days post-infection producing high levels of IgM transcripts [36]. A serine protease MyxSP-1 gene was identified from *M. cerebralis* [127]. Genomic and transcriptomic assemblies of two distinct myxozoan species, *Kudoa iwatai* and *M. cerebralis*, were also done [128]. Such identification of proteins important for physiological pathways of the parasite might pave the way for vaccine development.

The inability to culture myxozoans in vitro and poor knowledge on host-myxozoan interaction, particularly on the parasite antigens that trigger the host immune response seems to be a major bottlenecks in designing a vaccine.

## 3.7 Monogenea

Monogenean trematodes, also called as flukes, are host and site-specific parasites of fish which have a direct life cycle with no intermediate hosts. Though studies related to protective immunity and efforts on vaccine development are meagre on this group of parasites, some data on the humoral Ab responses and presence of acquired protection against monogeneans are available [16]. Infection with *Pseudodactylogyrus bini* and *Pseudodactylogyrus anguillae* in eels produced specific Abs; however, the production of these Abs could not be related to protection in the host against the infection [39]. It was reported that infection with the monogenean *Dicocotyle sagittata* in farmed rainbow trout elicits specific immunoglobulin production. Further studies to relate the Ab production to protection revealed that immunization of *O. mykiss* with *D. sagittata* extracts conferred partial protection and elicited significant titres of Abs, which were believed to mediate protection. However, the high titres of Abs could not be correlated to parasite burdens on the immunized fish, which were re-challenged [69, 129, 130]. *Seriola dumerili* and *Paralichthys olivaceus* recovering from *Neobenedenia girellae* infection acquired partial protection against re-infection; however, the underlying mechanism was poorly explored [131]. Another monogenean parasite, *Neobenedenia melleni* was exposed to *Oreochromis mossambicus* for 4 months, and it was found that systemic Ab level was very high in mucus and plasma at 120 days, significantly decreasing the parasite load [72]. In case of infection with *Heterobothrium okamatoi*, specific Abs were detected in tiger puffer [70], though there was no clear understanding between the acquired protection induced by persistent infection and the serum Ab levels [71]. Other important genus of pathogenic monogeneans *Gyrodactylus* and *Dactylogyrus* have received attention only with respect to immune gene responses in a few studies, which investigate the expression level of immune-relevant genes during infection [61, 62, 66, 132, 133]. Anything in the direction of potential protective responses is yet to be established. All the above studies suggest the role

of specific immunity in protection against monogeneans, which reflects that in near future there is a possibility of vaccine development if antigen determination and screening are taken up systematically.

## 3.8 Digenea

In case of digenean parasites, fish usually serve as the intermediate host becoming infected with the cercaria (shed from a primary host like mollusc), which transforms into metacercaria in the fish host tissue. The most notable group of digenean parasites that remain encysted as larval stages in the fish are the eye flukes (*Diplostomum* sp.). Other encysted parasites also exist. Though vaccine development in case of these parasites has not been addressed yet, there do exist some reports of protective responses wherein protective Ab responses have been recorded. Abs are produced in several fish hosts against *Cryptocotyle lingua* and *Diplostomum spathaceum*. The participation of an antibody-dependent cell-mediated cytotoxic mechanism has been reported for the protection against *D. spathaceum* [73]. Fish are known to mount an immune response against *D. spathaceum*, which reduces the establishment in subsequent exposures. More specifically, *D. spathaceum* elicits responses of both non-specific (e.g. activation of macrophages) and specific (e.g. T-cell activation) immunity against the parasite in exposed fish. In general, specific responses develop in a few weeks after the first exposure because of parasite antigens, which have been demonstrated experimentally using both live and attenuated parasite infective stages [74]. Eye flukes are immunologically privileged as they reside in the eye where they are protected from host responses. Therefore, host responses must prevent the parasites within 24 h from exposure before they migrate towards the eye and this hinders the development of any adaptive response.

A specific Ab response against *Rhipidocytle fennica* in roach immunized with homogenized cercariae was generated and a strong response was elicited by infecting fish with living cercariae emerging from infected clams [37]. The subsequent challenge experiment with *R. fennica* indicated that previous infection of fish gave some protection against *R. fennica*. Specific Abs were also found in *C. carpio* only when the host was injected with cercariae of *Sanguinicola inermis* and not when it was challenged with the parasite [75]. However, in *Thunnus maccoyii*, an Ab response and the development of acquired resistance were found in the case of natural infection with sanguinicolid, *Cardicola fosteri* [76].

## 3.9 Cestoda

Cestodes are the tapeworm parasites that have economic and medical importance. Generally, these parasites penetrate the intermediate host tissue and then develop into a metacestode stage and ingestion of the infected tissue by the definitive host completes the life cycle. Although not much has been investigated about this

parasitic group, there is a report on the production of specific Abs from naturally infected rainbow trout in response to *Diphyllobothrium* spp. [38].

## 3.10 Nematoda

Nematodes or roundworms are common in wild fish that are exposed to the intermediate hosts. The life cycle of the parasite involves only one intermediate host, a planktonic crustacean such as cyclopoid or diaptomid copepod or an ostracod and fish act as definitive hosts. However, sometimes fish may act as transport or intermediate hosts for larval nematode forms that infect higher vertebrate predators, including humans. Important parasites of fish from this group belong to the genera such as *Capillaria* and *Anguillicola*.

It has been reported that the host immune response after parasitic invasion does not solely depend on the chemical composition of parasite but also on its life cycle. Owing to their complex life cycles, these parasites can induce different immune responses, which help in Th1 to Th2 switching. Up-regulation of SAA and IgM genes was observed in *S. salar* against intracellular parasitic infection whereas intraperitoneal infections with the larva of *Anisakis simplex* in brown trout induced an encapsulation response. TLRs 2 and 4 have been described to bind to helminth antigens thus focusing on their roles in anti-parasitic responses. Some models describe that fish like salmonids, which possess MHC II and CD4+ molecules, may demonstrate a switch from Th1 to Th2 response against nematodes but fish like Atlantic cod that lack MHC II and CD4+ may induce some alternative mechanism during the infection.

The efficacy of irradiated third stage larvae of the nematode *Anguillicola crassus* was tested in its original host, the Japanese eel (*Anguilla japonica*), and the recently colonized European eel (*Anguilla anguilla*) by immunization and challenge [77]. The immunization induced a significant reduction in the number of adult worms in challenged *A. japonica*, but not in *A. anguilla*. Both eel species also developed an Ab response against *A. crassus*. It was opined that Ab response was not a factor that provided protection because the Ab levels were not positively correlated with protection from infection. *Anguilla* spp. also produces specific Abs against *A. crassus* surface antigens and against the detoxifying enzyme glutathione-*S*-transferase that might serve as a candidate for vaccine development [16, 39].

It has been noticed that specific Abs against the anisakids are being produced naturally in some Antarctic fish; for example, anti-*Contracaecum osculatum* Abs were detected in plasma and anti-*Pseudoterranova decipiens* Abs in bile and plasma of infected fish [40, 41]. Many secretory and excretory products of above-mentioned anisakids recently got attention because of their role in diagnosis and therapeutic potential. These antigens elicit immune responses in lower vertebrates including production of Ab [42]. Recently, transcriptome analysis of the liver of *Gadus morhua* infected with the anisakid nematode *C. osculatum* was carried out and the genes related to metabolism, immune responses, and growth in the host were found

to be associated with the infection. This may pave a new way towards vaccine production against anisakid nematodes in future [43].

## 3.11 Crustaceans

Most common crustacean parasites affecting the fish species belong to the orders Copepoda (typified by *Ergasilus*, *Lernaea*, and *Caligus*), Branchiura (*Argulus*), and Isopoda (*Gnathia* and typical isopod forms). Some of these crustacean parasites like the anchor worm (*Lernaea*) are obligatory parasites of finfish, and some are intermittent parasites like fish lice (*Argulus*).

The model parasite in the crustacean group is sea lice (*Lepeophtheirus salmonis* and *Caligus rogercresseyi*) owing to the sheer amount of research focus it has attracted, being one of the most economically devastating parasites of salmon culture. Sea lice infections are endemic in most countries where salmonids are cultured. In the search for immunoprophylaxis against this parasite, the immune responses of the host species have been studied extensively in sea lice infestations [134, 135]. Other crustacean parasites like *Argulus* and *Lernea* have also received attention with respect to characterization of the host immune responses. However, little data exists on the Ab responses, which is a prerequisite for vaccine development. In silico prediction of protective antigens like cysteine and serine proteases, galectins, paramyosin, GSTs, and aquaporins of *Lernaea* was done using VaxiJen software for vaccine candidates [136]. Paramyosin inhibits the classical pathway by binding to IgG, and GSTs interfere with detoxification of free radicals, which can be targeted for future trials. The characterization of the immunoglobulin responses against *L. salmonis* showed a ten-fold increase in IgT and IgM in infected Atlantic salmon, but these increases have not been correlated to protective immunity [135]. Recently, a rapid upregulation in IgM, IgD, and IgZ transcripts has been recorded in our laboratory in response to *Argulus siamensis* infection in the skin, skin mucous, and head kidney of infected rohu. Nevertheless, such Ig responses suggest the possibility of immunostimulation of host responses by the vaccine to enhance host protective responses against this parasite. Acquired immunity in case of parasitic copepod infections was demonstrated first as early as 1978 in goldfish resulting in erratic re-infection [137]. The egg production and fitness of *L. cyprinacea* offspring were shown to be negatively impacted in previously infected kissing gouramis (*Helostommma temminicki*) [138].

Vaccine development, particularly in case of sea lice, has come a long way. Many researchers have reported humoral responses to *L. salmonis* antigens in both naturally infected and *L. salmonis* homogenate-immunized Atlantic salmon. Atlantic salmon was shown to be carrying fewer ovigerous female *L. salmonis* with lower fecundity following immunization with *L. salmonis* extracts compared to non-immunized fish [44]. The protective antigens in *L. salmonis* were screened using monoclonal and polyclonal Abs developed against recombinant louse proteins expressed as β-galactosidase and maltose-binding fusion proteins. These antigens, when used for immunization, resulted in reduced fecundity of *L. salmonis* [45]. The

first recombinant anti-sea lice vaccine prototype was developed targeting *L. salmonis* trypsin, which provided only 20% protection in Atlantic salmon smolts [46]. Another recombinant anti-sea lice vaccine targeting vitellogenin of the parasite was used against *L. salmonis*, which was effective in reducing development of lesion induced by the parasite and the number of adult female parasites; however, it did not decrease numbers of male lice [47]. A major breakthrough has been the fully-sequenced *L. salmonis* genome, which in conjunction with RNAi or gene knock-down technology now provides opportunities for identifying more vaccine candidates by testing antigenic targets within the parasite. These techniques helped in identifying a novel akirin 2-like protein in *L. salmonis* and *C. rogercresseyi*, and screening of recombinant my32 protein as a vaccine candidate in Atlantic salmon [48]. This recombinant vaccine again has been tested against *C. rogercresseyi* and *L. salmonis* infections by two different laboratories providing mixed results in terms of efficacy [48, 49]. Very recently, the salmon louse midgut function and blood digestion were targeted for the screening of candidate antigens to control infestations. New candidate protective antigens, putative Toll-like receptor 6 (P30), potassium chloride, and amino acid transporter (P33) were evaluated in Atlantic salmon; vaccination with these antigens provided protection in Atlantic salmon by reducing adult female sea lice. These antigens bind to specific IgM Abs on intestinal cell membranes and thus affecting protein function resulting in the reduction of viability of sea louse [139]. Vitellogenin-like proteins, SEP proteins, and trypsins are some of the other proteins of the parasite being evaluated as candidate antigens for vaccine development [46].

In the case of freshwater cousin of sea lice, the branchiuran fish parasite *Argulus* that has a devastating effect on carp culture, little data exist related to protective immunity. The presence of humoral Ab response in rainbow trout was demonstrated on immunization with antigen extract of *A. foliaceus* [140]. To screen candidate antigens for vaccine development, the immunodominant polypeptides of the *A. siamensis* were characterized recently [57]. Several polypeptide bands ranging from 130.55 to 16.22 kDa were detected in whole lice homogenates by SDS-PAGE and two polypeptide bands of 75.78 and 79.6 kDa were found to be immunodominant polypeptides. A crucial step in the studies on this parasite has been the transcriptomic analysis of *A. siamensis* [141], which would facilitate the availability of the genomic information of the parasite and a better understanding of the proteins and metabolic pathways critical to the development of vaccine candidates. Several genes associated with mucosal immunity (IL-6, IL-15, and IL-1β, TLR-22, β2 microglobulin, lysozyme G, and NKEF-B) were found to be up-regulated in *L. rohita* infected with *A. siamensis* which expands our understanding of mucosal-immune responses during this infection [142]. The ribosomal protein P0 of *A. siamensis* was evaluated as a candidate antigen in a vaccination and challenge trial using *L. rohita* and it demonstrated low efficacy [143]. The whole-genome sequence using single-molecule long-read sequencing from PacBio was performed to generate information about *A. siamensis*. It revealed around 130 male-specific genes and ten female-specific genes, which are currently in process of screening as possible vaccine candidates (unpublished data).

Despite the existence of nearly 50 years of evidence of acquired immunity against crustacean parasites in host fish, development of prototype vaccines has been staggeringly slow.

## 4 Challenges in Parasite Vaccine Developments

One of the major hindrances in vaccine development for parasitic diseases is the complex structure and life cycle of the parasites. It is further impossible to in vitro cultivate any parasite of homologous make-up in large quantity for vaccine trials. Development of a challenge model for vaccine screening remains to be an obstacle during candidate screening. Vaccine efficacies further vary based on the fish species, the age of fish, culture conditions, and environmental temperature during the infection process. Some hosts do not mount ample Ab production at lower temperature when a parasite takes upper hand to infect the fish. Finally, several existing species within a genus express antigenically distinct variants of stage-specific molecules [18] that may serve as potential factors for disease pathogenesis and could act as strong candidate antigens. In addition, the infection site influences the development of protective immune response. For example, unlike mammals, investigating immune molecules for ectoparasites that do not suck enough blood and targeting a novel molecule of quite effective nature is a big challenge. In this regard, the detailed characterization of Ab molecules in fish species at the mucosal sites is necessary. Same is the case with intestinal parasites that only remain as an attachment to the gut wall. Targeting novel antigens with secretory Ab at the mucosal site would play a crucial role. Recent attempts to produce parasite antigens through genetically modified microorganisms have improved the possibility of some parasite vaccines. However, production of recombinant protective candidate proteins has been quite laborious because of incorrect folding or improper post-translational modifications of recombinant proteins. Hence, basic research on fish parasite immunology should be strengthened.

## 5 Conclusion

In this chapter, the protective immune responses and studies related to vaccine development have been reviewed for different groups of fish parasites. Although Abs are produced in response to several parasites, these Abs do not necessarily ensure mounting of a protective response. Therefore, characterization of the Abs with an integrated study of characterization of mechanisms involved in protective immunity is necessary.

Development of vaccines for control of parasitic diseases should follow a routine starting from the study of host-parasite molecular interactions, which include knowledge of the parasite contributing factors (parasite proteins, secretions, molecules mediating pathological changes, etc.) as well as the host contributing factors (molecules of innate and adaptive immunity and other responses). Such complete

understanding of host-parasite interactions is yet to be achieved even in some of the major human pathogens, so in comparison, fish parasitoses are still at infancy. However, technological advancements like transcriptome and whole-genome studies aid the procurement of huge amount of valuable data in a short time. With the advent of reverse vaccinology, it is now possible to access all the proteins encoded by an organism using transcriptome data and computational analysis. In silico prediction tools can screen proteins on the basis of characteristics like cellular localization and protective antigenicity, and these candidates can be developed into recombinant proteins and validated by vaccination trials. These advancements will certainly facilitate developments in parasite vaccinomics. Many vaccines may find their greatest and most immediate application in integrated control strategies considering use with chemotherapeutics. The synergies offered by a combination of vaccines and parasiticides should be thoroughly explored, as this approach may lead to a substantial reduction in the use of parasiticides.

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# Bacterial Biofilm for Oral Vaccination in Aquaculture

Shankar K. M.

**Abstract**

Conventional bacterial free cell oral vaccines provide poor, inconsistent immune response and protection due to antigen destruction in stomach. A bacterial biofilm with high density of cells enmeshed in glycocalyx matrix could be better gut destruction-resistant oral vaccine. Against this background, biofilm of *Aeromonas hydrophila*, evaluated as oral vaccine in carps, provided high antibody titre and protection upon challenge compared to that with free cell vaccine. Biofilm antigens retained for a longer duration in immune responsive sites—kidney, lower gut and spleen—also boosted gut immunity with higher mucosal antibody titre and expression of immune beneficial proteins. Besides carps (herbivores), biofilm of *A. hydrophila* was also demonstrated with significant higher antibody titres and protection upon challenge in omnivore catfishes, carnivore murrel and seabass. A biofilm of *Vibrio alginolyticus* was also demonstrated to be an effective immunostimulant in *Penaeus monodon* against *Vibrio* and WSSV. Biofilm could be demonstrated to successfully deliver subunit antigens.

**Keywords**

Bacterial biofilm · Oral vaccine · Aquaculture

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## 1 Introduction

Aquaculture, the fastest growing food production sector, contributes more than 50% of the total fish produced in India, and the sector will witness further growth as fish production from natural waters is reaching a plateau. Intensive aquaculture with high stocking density, artificial feeding and fertilizers is profitable which, however, often alters the aquatic environment favouring pathogens resulting in disease outbreaks and economic loss [1]. Further, the disease management through chemotherapy deteriorates water quality and the overall aquatic environment. Accumulation of residues, development of resistance to antibiotics and chemicals by microbes and parasites also adversely affect the health of fish and human. Aquatic animals suffer from a number of infectious diseases caused by parasites, viruses, bacteria and fungi. Aquaculture health management often depends on chemotherapy similar to that practised in higher animals. However, mechanism of action and kinetics of the chemicals/drugs in cold-blooded vertebrates and invertebrates are quite different from that of warm-blooded animals. Chemotherapy in aquaculture is considered as a last option and, further, there are several limitations which deter the use of drugs in aquaculture. Therefore, avoidance of pathogens through biosecurity and prophylactic measures such as use of vaccines and immunostimulants are preferred. Several conventional and biotechnology-based effective vaccines have been developed for aquatic animals world over.

## 2 Oral Vaccines

Oral vaccine is most ideal for mass immunization in aquaculture, as this would facilitate the administration of vaccines to a large number of fish of any size (feeding stages) with least stress. However, as in higher animals, oral vaccines in fish tried world over have given poor and inconsistent immune response and protection. This poor performance is believed to be due to destruction of vaccine in the hostile stomach/foregut with low pH and high enzyme activity, leading to poor availability of vaccine antigen to immune responsive sites. On the other hand, administration of vaccine through anus has given better response and protection in fish. Furthermore, posterior gut in carps has high uptake of macromolecules [2] and, hence, antigen/vaccine adsorbed in the hindgut are carried to intestinal macrophage for the activation of the immune system. Thus, the major limitation to the successful oral fish vaccine is the destruction of antigens by gastric and pancreatic secretions. Strategies for protection of oral antigens including microencapsulation that can prevent deteriorative actions of digestive secretions on the antigens have been tried to deliver vaccines to the immune sensitive hindgut with minimum alteration [3]. However, these strategies are complex and costly. Oral administration of vaccines has obvious advantages in aquaculture. However, it is apparent that greater quantities of antigens may be required to elicit a desired response than that is required in injection or immersion treatments [4].

## 3 Biofilm of a Bacterial Pathogen as a Novel Oral Vaccine

In aquatic system, bacterial population occurs predominantly in biofilm mode as surface associated population [5]. Solid surfaces in water bodies attract and adsorb inorganic and organic nutrients, which microbes in sessile biofilm make use of. Biofilm development on industrial and pathogenic system [6, 7], leading to biofouling to plugging, corrosion and reduced efficiency of thermal exchangers [8] has been a problem. Biofilm cells produce a polymer glycocalyx, which cover the growing colonies serving as a capsule. The glycocalyx matrix provides protection to biofilm cells from surfactants [9], antibiotics [10], antibodies [11] and even phagocytosis [12, 13].

Making use of the glycocalyx-based resistance of the biofilm cells to antibiotics, antibodies, enzymes and disinfectants, a novel biofilm oral vaccine model for fish has been developed. It was hypothesized that biofilm vaccine can withstand hostile foregut/stomach conditions and reach the immune responsive hindgut for eliciting appropriate immune response and protection. Furthermore, bacteria infect and thrive in a host in biofilm mode. Therefore, biofilm antigens of a bacterial pathogen are mirror image of the antigens in infection mode and, hence, serve as appropriate antigens for vaccination. Biofilm of *Aeromonas hydrophila,* a common fish pathogen, was developed on chitin substrate and evaluated for immune response and protection in the commonly cultivated fishes of India such as carps (herbivores) [14–19], murrel [20], seabass (carnivores) [21] and *Clarias* (omnivore) [22] having varying gut enzymes and pH profile. Recently, biofilm of *A. hydrophila* has been successfully evaluated in striped catfish *Pangasianodon hypophthalmus* [23].

In a study on herbivores comprising of catla, rohu and common carp fed with biofilm (BF) of *A. hydrophila* developed on chitin and administered through feed resulted in higher agglutinating antibody titre and relative percent survival (RPS) compared to free cell (FC)-fed fish [16]. Monoclonal antibody-based immunofluorescence for antigen localization [16] showed higher retention of BF vaccine antigen for a longer duration in immune responsive sites such as hindgut, kidney and spleen [16]. Glycocalyx matrix/capsule is believed to protect biofilm cells in the foregut from destruction, as the BF was retained for 48 h compared to only 6 h with free cells. Additionally, biofilm might have presented an appropriate immunogenic material which is reflected in the antibody titre and protection upon challenge [16]. A dose of $10^{10}$ CFU $g^{-1}$ fish administered through feed for 15 days was found to be optimum, resulting in better antibody titre and RPS, although the onset of immune response and peak antibody titre post-vaccination differed widely between species [16].

Biofilm of *A. hydrophila* when administered orally to walking catfish, *Clarias batrachus*, and *Channa striatus* resulted in significantly higher serum agglutination antibody titre and RPS compared to control fish fed with free cell [20, 22]. Similarly, Asian seabass, *Lates calcarifer*, administered orally with heat-killed biofilm of *Vibrio anguillarum* produced higher antibody response and RPS compared to fish administered with heat-killed free cells of *V. anguillarum* [21].

## 4 Antigen Expression in Biofilm

Altered growth conditions of bacteria reflect changes in cell surface structure, cell wall composition, virulence and exo- and capsular polysaccharides. It has also been established that protein and lipopolysaccharide profile undergo changes when bacteria switches from free planktonic to sessile biofilm form. In a study on the antigen expression in biofilm of *A. hydrophila, V. anguillarum* and *Vibrio alginolyticus,* biofilm of *A. hydrophila* showed three additional proteins, with repression of nine proteins [24] while biofilm of *V. alginolyticus* [25] and *V. anguillarum*, each had three additional proteins and repression of four and ten proteins, respectively. Further, it was observed that S layer protein was lost, while lipopolysaccharide possessed additional high molecular weight band, which is known to improve protection. Earlier studies have shown a difference of 30–40% in proteins between biofilm and free planktonic cells. Normally a pathogen thrives in a host in biofilm mode. Therefore, antigen expression in biofilm mode is the mirror image of antigen expression in vivo and, hence, biofilm mode is more appropriate as a vaccine antigen. Biofilm oral vaccine besides providing better antigen delivery, also provides additional appropriate antigens to boost immune response and protection.

## 5 Gut Mucosal Immunity with Biofilm Oral Vaccine

Developing an effective oral vaccine that resists gastric digestion and facilitate enhanced uptake for presentation of antigen to gut associated lymphoid tissue at the mucosal layer is a challenge. Enteropathogenic agents invade mucosal surface of gut and, therefore, mucosal immunity is very important. Leucocytes are extensively present in fish intestine where lymphocytes, plasma cells, macrophages and granulocytes are present under the epithelium [21]. Lectin/adhesin of pathogen elicited mucosal antibody response and inhibited the adhesion of the gut pathogen in mammals [22]. Oral and anal delivery of soluble and particulate antigens elicit systemic and mucosal immune response in fish—both humoral and cellular [4]—indicating the existence of similar mucosal immune system in fish and mammals. Common carp fed with *A. hydrophila* biofilm-incorporated feed at $10^{10}$ CFU $g^{-1}$ fish $day^{-1}$ for 10 days had higher antibody response in both gut mucosa and blood and the immune related genes were upregulated compared to control fish. Thus, it is clear that BF oral vaccine boosts gut immunity in fish.

## 6 Biofilm as Immunostimulant in Shrimp

Infectious microbial diseases cause huge loss in shrimp culture [26]. There is a growing interest in immunostimulants as there are limitations in using antibiotics or vaccines for the management of diseases in crustaceans. Several preparations such as live and killed bacteria, glucans, peptidoglycans and lipopolysaccharide (LPS) have been used as immunostimulants in shrimp [27]. Oral route is reported to be the most

beneficial for the administration of immunostimulants to shrimp [28]. However, gut destruction of immunostimulants administered orally is a common hindrance in shrimp leading to poor response. Therefore, a gut-resistant form of immunostimulant would be ideal for oral administration in shrimp. Immune response in *Penaeus monodon* fed with biofilm (BF) and free cells (FC) of *V. alginolyticus* [29] had higher haemocyte count, phenoloxidase activity and antibacterial activity compared to FC-fed or control shrimp. Among the different doses of BF of *V. alginolyticus* tested, 109 CFU $g^{-1}$ shrimp $day^{-1}$ for 2 weeks could elicit higher immune response. BF-fed shrimp were more resistant to injection challenge with *V. alginolyticus* and white spot syndrome virus (WSSV) with significantly higher RPS compared to FC-fed and control shrimp. Better resistance was also reflected by rapid clearance of *V. alginolyticus* and WSSV from the haemolymph of biofilm-fed shrimp.

## 7 Bacterial Biofilm as a Carrier of Subunit Vaccine

Similar to whole-cell vaccine, subunit vaccine is susceptible to gut destruction during oral vaccination. Therefore, use of biofilm for delivery of subunit vaccine in fish is preferred for protecting the vaccine molecule from gut destruction. Genes encoding for immunogenic proteins of pathogens can be cloned and expressed in prokaryotes such as *Escherichia coli*. The recombinant bacteria cultured as a biofilm on chitin microspheres can be administered orally for protecting fish against the pathogen.

## 8 Conclusion

Oral vaccination is the most ideal route of vaccination for fish as this method of vaccine administration can be used for any size of fish (feeding stages) with least stress. However, this method has the disadvantage that the antigen is degraded in the hostile stomach/foregut with low pH and high enzyme activity, leading to poor availability of antigen to the immune responsive sites. This disadvantage can be overcome by using biofilm of bacterial pathogens. Bacterial population normally occurs as biofilm in aquatic ecosystems as surface-associated population and infect and thrive in a host in biofilm mode. Biofilm cells produce a polymer, glycocalyx, which cover the growing colonies and provide protection from surfactants, antibiotics, and even phagocytosis. Furthermore, biofilm cells also express additional proteins and polysaccharides which are essential in a vaccine to offer protection against pathogen. Biofilms grown on substrates like chitin when administered orally can withstand hostile foregut/stomach conditions and reach the immune responsive hindgut for eliciting appropriate immune response and protection. Biofilm vaccines have the added advantage that it can also elicit mucosal immune response in the gut. Biofilm can also be used as immunostimulant in shrimp and for oral delivery of subunit vaccine for fish.

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# RNA Interference Vaccines for Disease Control in Aquaculture

P. Gireesh-Babu and Aparna Chaudhari

**Abstract**

Prevention and control of diseases is a significant factor contributing to sustainable aquaculture. Discovery of RNA interference (RNAi) mechanism has opened new vistas in developing effective vaccines against viral diseases in aquaculture species, especially shrimps. Since the mechanism of action does not involve the host immune system, these are not vaccines in the true sense, but the RNAi molecules being developed globally to control diseases have both prophylactic and therapeutic effects. In general, a crucial viral gene essential for replication/pathogenesis is targeted for degradation by RNAi through post-transcriptional gene silencing. Several double stranded RNA (dsRNA) substrates like siRNA, shRNA and lhRNA can be utilized for this purpose. Single stranded antisense RNA (asRNA) molecules are also shown to exert gene silencing by RNAi mechanism. Several software are available for designing RNAi molecules and care should be taken to avoid off-target effects. Although the dsRNA / asRNA molecules can be delivered transiently for short-term silencing, more effective gene silencing can be achieved by employing plasmid vector-based dsRNA / asRNA expression constructs. Further, DNA vector-based RNAi molecules are more stable and can be delivered to aquaculture species through the immersion route that is least stressful, effective and economical. However, plasmid-based RNAi vaccines need to be designed in accordance with the regulatory guidelines.

**Keywords**

RNA interference · RNAi construct · Tissue distribution · Off-target effect

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## 1 Introduction

The discovery of RNA interference (RNAi) phenomenon [1] in 1998 amazed the scientific community as it was difficult to believe that a biological phenomenon of such great significance could have escaped discovery for so long. Although it was first described in the model organism *Caenorhabditis elegans*, it has since been reported from a large number of eukaryotes including fish and shellfish [2–7]. RNAi is a post-transcriptional process by which dsRNA induces degradation of homologous mRNA transcripts resulting in target gene silencing. This technique is now popular as a gene knockdown tool for a number of applications [8–10]. Several RNAi-based therapeutics are currently under clinical trials with majority in Phase III trials. The first RNAi therapeutic to be clinically approved is not far off considering the advancements in design and delivery of RNAi molecules [9]. With its ability to target single or multiple genes involved in metabolic pathways, RNAi can be helpful in treating neuro-degenerative diseases and cancer. In the latter case, it has been used to check the expression of multiple drug resistance (MDR) protein that makes cancer cells resistant to chemotherapy [11].

Apart from these applications, RNAi is emerging as an alternative method for management of many viral diseases and has been shown to check viral infection effectively in both plants and animals. Promising results have been reported against HIV, hepatitis A and B in humans and FMD virus in cattle where mostly the viral structural proteins or polymerases are targeted for silencing [9, 12]. Several studies have been reported from fish and shellfish [13–18]. Although conventional vaccines have been effective against bacterial pathogens, RNAi-based vaccines have greater potential against viral diseases. In addition, they are particularly attractive for use in farmed invertebrate species as they lack a well-defined adaptive immune system. Inhibition of viral multiplication by siRNA molecules has been seen in fish cell lines and also studies conducted in vivo [17]. Two groups [19, 20] reported delay in mortality of vaccinated rainbow trout juveniles and rock bream fingerlings challenged by viral haemorrhagic septicemia virus (VHSV) and rock bream iridovirus (RBIV), respectively. However, this outcome was seen to be mediated by non-specific IFN response instead of gene-specific silencing. Crustaceans, on the other hand, do not have an interferon response system, and more effective gene-specific knockdown by RNAi molecules has been reported in shrimps [6, 14, 21]. About 20 viruses have been reported to infect shrimp globally, of which six including white spot syndrome virus (WSSV), monodon baculovirus (MBV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), Taura syndrome virus (TSV) and yellow head virus (YHV) are especially important due to their epizootic spread and economic impact [22].

## 2 Principle of RNA Interference

RNA interference is believed to have evolved as a defence mechanism against RNA viruses [23] and also post-transcriptional gene regulation [24]. The process is initially triggered when dsRNAs are expressed in vivo or introduced exogenously into a cell (Fig. 1). In the cytoplasm, the double-stranded viral RNA is cleaved by the multi-domain ribonuclease III enzyme, Dicer [25], into 21–23 nucleotide (nt) fragments with characteristic 2-nt 3′ overhangs. These dsRNA fragments termed small interfering RNAs (siRNAs) are recognized by the RNA-induced silencing complex (RISC) [26] and lead to degradation of targeted viral mRNA. RISC is a multi-enzyme unit that separates the two strands of the siRNAs. The sense-strand is released and is reported to trigger further dsRNA synthesis by RNA-dependent RNA polymerase in some organisms [27]. RISC uses the bound antisense-siRNA to guide it to the target mRNA containing the complementary sequence. Argonaute, another RNase III family enzyme that is also part of RISC, cleaves the target mRNA and silences its expression [28]. The efficiency of siRNA depends on perfect complementarity of the seed sequence (positions 2–6) with the target mRNA. In case of imperfect base pairing between the guide RNA and the target mRNA, if a bulge is created in miRNA/siRNA then the transcript is marked for translational repression. If the bulge is created in the mRNA, the complex continues with cleavage [29]. If the pathway is directed towards translational inhibition, the mRNA is reportedly decapped and ultimately degraded in processing bodies (P-bodies) [27, 30, 31].

Another class of naturally occurring molecules that result in the formation of siRNAs is the microRNA (miRNA). These are short hairpin-shaped RNA duplexes of 21- to 25-nt, bearing a 2-nt overhang at each 3′ terminus and a phosphate group at each recessed 5′ terminus with ~10-nt distal loops [32]. The mature miRNAs are derived from longer precursors called primary miRNAs (pri-miRNA) that are transcribed in the nucleus and have nuclear as well as cytoplasmic maturation stages. A multi-protein complex comprising mainly Drosha (RNase III family enzyme) and DiGeorge syndrome critical region gene 8 (DGCR8) processes the pri-miRNA in nucleus resulting in ~65 to 70-nt precursor miRNA (pre-miRNA) which is then transported to cytoplasm with the help of Exportin-5 and RanGTP. In cytoplasm, the processing pathways converge for both miRNA and exogenous siRNA molecules resulting in the production of dsRNA duplex of the appropriate size for loading onto an Argonaute protein and the subsequent target mRNA degradation through RISC complex. The mature miRNA molecules are believed to have a major role in the regulation of gene expression [33, 34].

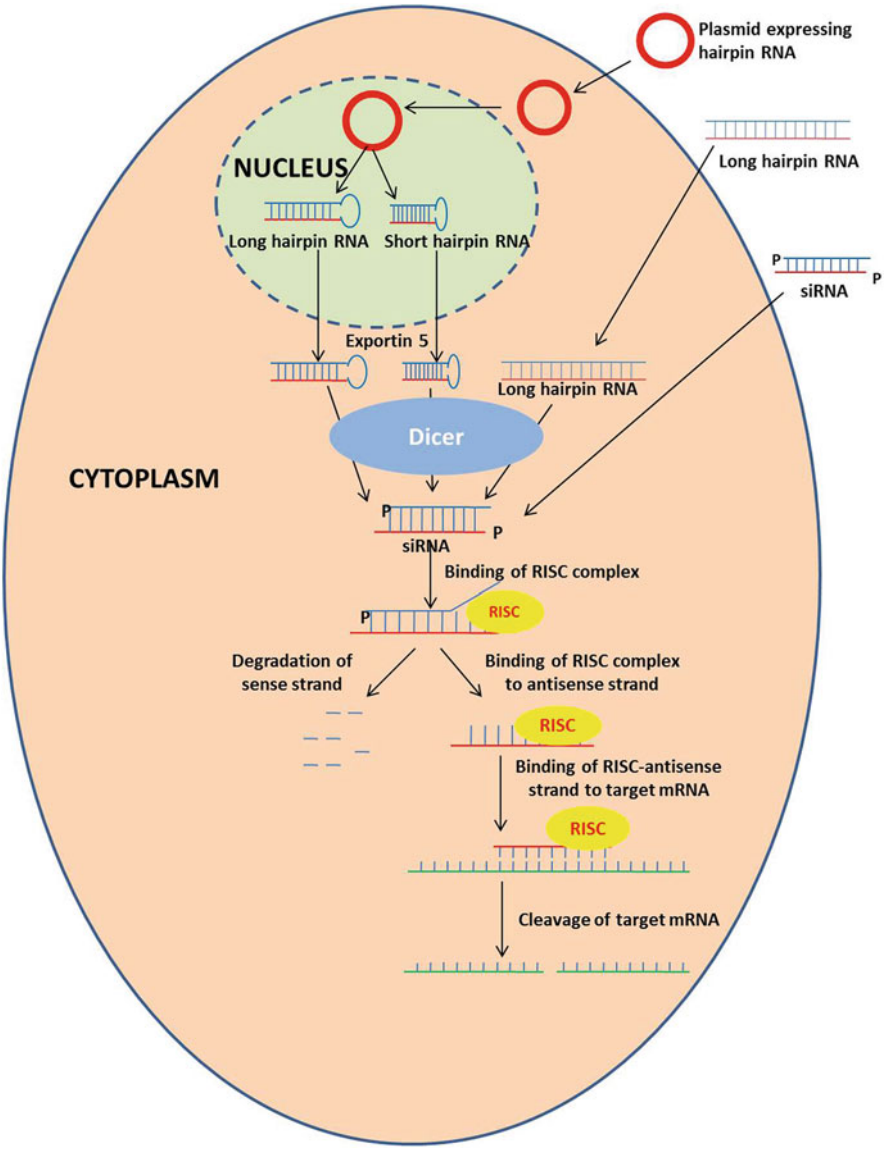


**Fig. 1** Mechanism of RNA interference (RNAi) along with various sources of dsRNA. (dsRNA- double stranded RNA, siRNA- short interfering RNA, RISC- RNA induced silencing complex)

# 3 Critical Features of RNAi-Based Vaccines

## 3.1 Target Gene

The pathogenic gene to be targeted for silencing is the most critical choice to be made for developing RNAi vaccine/therapy. The RNAi molecule used is expected to check the spread of the pathogen without causing any adverse effects to the host. Among fish pathogens, the viral genes that have been used as targets include major capsid protein of RBIV [20] and nucleoprotein-N and glycoprotein of VHSV [19, 35]. RNAi-based therapies hold more promise for shrimps and have shown to be effective against YHV, WSSV and TSV. A number of WSSV structural genes have been used as RNAi targets. These include *vp28* [14, 36–38], *vp19* [14, 39, 40], *vp15* [36], *vp281* [37] and ICP11 [41]. The YHV protease gene was targeted by some researchers [42–44], while Yodmuang et al. [44] targeted RNA polymerase and Tirasophon et al. [42] targeted helicase and RdRP. Significant reduction in target gene expression was recorded by all researchers, but in some cases even non-specific genes were able to produce similar levels of knockdown [37, 40]. It has been suggested that RNAi-induced systemic immunity is caused by both sequence-specific gene silencing and triggering of the non-specific immune response pathway [44].

As an alternative strategy, it is also possible to target host factors contributing to viral propagation, provided the host cell viability is not affected. This approach was adopted by Zhou et al. [45] for control of HIV-1, and it avoids the chances of 'viral escape' by mutation of the target gene. Use of siRNA cocktail that targets more than one viral gene can also achieve the twin objectives of higher protection efficacy and lower likelihood of viral resistance because the number of mutations required for escape could lead to loss of virulence [46, 47]. If, however, shRNA cocktail is used, it could lead to saturation of cellular RNAi pathway or induction of off-target effects and it becomes important to control the dosage.

## 3.2 Target Sequence Selection

The specific sequence in the target gene against which the RNAi molecule is designed should be unique (confirmed by BLAST search), conserved across reported strains of the virus, and has a suitable thermodynamic profile that allows inclusion of the guide strand into RISC [48]. Even after careful selection, it is possible to observe in vivo toxicity, and it is best to screen 4–5 options in pilot tests in order to select the most effective molecule for in vivo trials. Even a single mutation in the target sequence can render the RNAi molecule ineffective [49], and RNA viruses can escape sooner than DNA ones as they accumulate point mutations 107 times more rapidly than DNA viruses [50].

### 3.3 The RNAi Molecules

Various RNAi molecules like siRNA, micro-RNA (miRNA), shRNA and lhRNA are incorporated into the RNAi pathway at different stages [51], and synthetic molecules that mimic the natural ones can be designed as follows:

1. *siRNA*: These molecules are 21 nt long and have 2-nt 3′ overhangs [52] and can be designed by identifying the target sequence with the help of various online design software. The selected sense and antisense sequences are synthesized chemically and siRNAs are produced by mixing and annealing them at room temperature. As siRNAs directly enter RISC, they do not overload the cellular machinery and fewer off-target side effects are observed. However, they are not protected against nuclease-degradation and being relatively unstable in vivo and, therefore, require frequent administration [53]. Xu et al. [38] reported complete eradication of WSSV when three successive injections of vp28-siRNA were administered daily to juvenile *Penaeus japonicus*. Making the molecule resistant to nuclease-degradation by chemical modification or devising slow release mechanisms can help overcome the requirement of repeated administration.
2. *shRNA*: These molecules are designed to mimic pre-miRNAs and have a small apical loop and a UU overhang at the 3′ end [54]. The sequence is inserted in a plasmid vector in sense as well as antisense orientation and expressed under the control of a Pol III promoter to form hairpin RNA. After shRNAs are synthesized in the nucleus, exportin-5 translocates them to the cytoplasm where it is converted into functional siRNAs by Dicer. Unlike siRNAs, these molecules can provide long-term silencing. In addition, it is possible to obtain tissue-specific delivery and inducible expression.
3. *Long dsRNA/lhRNA*: These molecules are longer (100–200 bp) than shRNAs, but very similar. They are also designed to be expressed from plasmid constructs and offer the advantage of producing several siRNAs on being processed by Dicer, and this prevents the virus from escaping by a point mutation. However, it has been reported that lengths higher than 30 bp result in the induction of interferons in vertebrate organisms, which in turn cause phosphorylation of eIF2α (eukaryotic initiation factor) to inhibit the host translational machinery. Longer molecules can be of use in invertebrates where the interferon response pathway is absent. As these molecules need to be processed into the effector molecules, saturation of endogenous cellular machinery and adverse effects resulting from it should be avoided by controlling the dosage. Robalino et al. [40] used dsRNA synthesized in vitro as vaccine against WSSV in *Penaeus vannamei*, while similar molecules were used in *Penaeus monodon* [55] and *Penaeus chinensis* [37]. Ongvarrasopone et al. [56] used dsRNA to protect *P. vannamei* against TSV; and Yodmuang et al. [44] used it against YHV in *P. monodon*. Sarathi et al. [57] observed 100% silencing of WSSV vp28 gene when bacterially expressed long dsRNA was orally administered to *P. monodon*. In most of the above cases, protection did not last more than a few days. Tirasophon et al. [43] used this molecule in a therapeutic mode and YHV-specific dsRNA could completely

check viral progression in shrimp when it was administered within 12 h of the onset of viral infection. In another study, protection was provided by a DNA construct expressing long hairpin RNA targeting WSSV vp28 transcript in *P. monodon* [14]. The construct could be PCR amplified from various organs after 5 days of intramuscular injection and persisted for a month at least.

4. *Antisense-RNA*: A fragment of RNA designed to be complementary to a targeted transcript is called antisense-RNA and can bring about mRNA knockdown by stoichiometric inhibition of ribosome assembly. It is also possible for antisense RNA to trigger the RNAi pathway as has been seen in some transgenic plants that have been modified to express antisense-RNA [58, 59]. TSV-resistant transgenic *P. vannamei* designed to express antisense RNA targeting a TSV coat protein had partial resistance to TSV infection [60].

## 4 Plasmid Vectors for RNAi-Based Vaccines

Wherever RNAi molecules are designed to be expressed in vivo from plasmid constructs, the choice of promoters and selectable markers needs special consideration.

The most frequently used promoters in shRNA stable expression constructs are U6 and H1. These are compact and efficient Pol III promoters known to have a well-defined transcription start site. The transcription terminators associated with them have a stretch of four or more thymidines and, hence, the transcribed RNA has one to four uridines at the 3′ end. While U6 can commence transcription only at a 'G' nucleotide, H1 promoter can initiate at any nucleotide and is also shorter compared to U6. Both promoters can also be used for bidirectional expression. Cytomegalovirus immediate early promoter (CMVp) is a Pol II promoter more suited for lhRNA constructs. This promoter appends 5′ vector sequences and 3′ poly A sequences to the transcript and these are not suitable for shRNA function. Other Pol II promoters suitable for lhRNA expression and active in shrimps are β-actin and SV40 early promoters [61]. Recently, Yazawa et al. [62] characterized the EF-1α gene promoter and Chen et al. [63] characterized the promoter of single whey acidic protein (WAP) domain-containing protein gene from marine shrimps. The expression efficiency of these promoters was found to be comparable to CMVp. The immediate early (ie1) promoter of WSSV has also been studied for expression in shrimps [64, 65].

Plasmid constructs intended for commercial use are best designed keeping regulatory restrictions in mind. Regulations prefer non-antibiotic selection markers over antibiotic resistance markers and a number of them have been developed [66, 67]. As kanamycin resistance is already widespread, the use of this antibiotic is permitted by Centre for Biologics Evaluation and Research (CBER), USA (FAO, 1996).

## 5 Tissue Distribution and Persistence of Plasmid Constructs

It is understood that plasmid DNA injected intramuscularly in fish is first present in the extracellular space at the injection site. Here, it may degrade, enter cells, and/or reach the circulatory system that enables distribution to other tissues (reviewed by Tonheim et al. [68]). Injection of dsRNA in the tail muscle of *Litopenaeus vannamei* was also shown to reach various tissues through circulation [39]. It is also possible that exogenous DNA enters shrimp cells through cell surface receptors such as those reported in nematodes and fruit flies [69, 70]. Rout et al. [71] showed persistence of plasmid DNA in shrimp tissues up to 2 months after injection in the tail muscle. A number of researchers have administered gene-specific siRNA by intramuscular injection and reported silencing of target WSSV genes [37, 55]. The same has been reported for long dsRNA as well [36, 39, 40].

## 6 Concerns Associated with RNAi Vaccines

1. *Off-Target Effects*: If the use of a siRNA/shRNA molecule results in knockdown of additional gene/s other than the targeted one, it is said to have off-target effects. This happens where there is homology between the siRNA sequence and more than one mRNAs. Online software used for the design of RNAi molecules, E-RNAi, does have an option to estimate off-target effects in selected genomes in silico. This option is not available for shrimps yet, although there are a number of fish genomes available. A simple homology search against nucleotide sequences available in the public databases, will help reduce the chances of off-target effects. Those sequences showing very high homology to the host organism should be avoided.
2. *Silencing Efficiency Is Target Gene-Dependent*: The efficiency of the siRNAs that can be designed for a target mRNA depends on the sequence and even when the most efficient siRNAs are selected, the degree of knockdown is variable. Several studies have reported knockdown in the range of 50–90%. According to the current knowledge of siRNA production, the RNAi design software, after screening the entire sequence, selects few appropriate region/s of the target and grades them as per the efficiency of siRNAs they are likely to generate. Both siRNAs and dsRNAs can be designed de novo. A number of options could be tested and compared with regard to knockdown efficiency in in vitro tests.

## 7 Routes of Delivery

***Injection*** This is the most frequently used method for gene knockdown studies at laboratory level. Successful silencing of specific WSSV genes has been reported in shrimps through intramuscular injection of gene-specific siRNA [39, 42], dsRNA [41, 43] and plasmid DNA expressing long hairpin RNA [14, 44]. Chowdhury et al. [72] injected plasmid DNA into immature, mature and berried females and mature

males and mated them with untreated counterparts. Vertical transmission of the plasmid DNA in offspring could be observed in all the groups, except berried females. This can be an ideal strategy to protect the young ones without any stress. However, detailed studies on persistence and pharmacokinetics of the plasmid in the offspring are required from the point of view of protection and biosafety.

***Oral*** In aquaculture, several lab-scale studies have reported the feasibility of DNA vaccine delivery through oral route using chitosan nanoparticles in fish [73]. Chitosan is a biopolymer popularly used for the delivery of nutraceuticals, drugs, hormones and vaccines. It is an excellent delivery vehicle owing to its versatile properties like biocompatibility, biodegradability, membrane forming ability, amenability to surface modification, low toxicity, immunomodulation, membrane adhesiveness, improved stability and enhanced permeability through the epithelial membrane [74]. Kumar et al. [75] reported oral delivery of chitosan–plasmid DNA particles to be a promising method that also induced significant antibody response against OMP38 protein of *Vibrio anguillarum*. Roy et al. [76] reported that oral administration of chitosan-DNA nanoparticles resulted in transduced gene expression in the intestinal epithelium in vivo. However, biosafety of oral vaccines can be a major concern due to contamination of water by unutilized feed and horizontal transmission of genetic elements to other pond microbes and organisms.

***Immersion*** Immersion vaccination is more widely used than oral administration in aquaculture. Though this method, generally, provides protection, the level is often lower compared to vaccination by injection. Immersion vaccination, however, is less stressful, requires less manipulation of the fish and can be used for administration of antigen to very small fish. Immersion has also been used as an effective challenge route for bacterial kidney disease of salmonids as it represents a more natural route of infection than injection [77, 78]. Better uptake of antigen through skin and gills compared to the gut and possible degradation of the antigen/vaccine in the digestive tract makes immersion treatment a viable option to oral delivery [79]. Recent studies have shown that both skin and gills participate in the uptake of vaccine during immersion, with skin playing the more dominant role [80, 81]. Some studies have also reported that oral ingestion is the principal specific route of antigen entry in bath-immunized fish [82].

RNAi vaccines are especially relevant to the control of shrimp viral diseases in light of the fact that specific immune response mechanism is rudimentary in these animals and other approaches have met with little success. Moreover, as these animals are cultured for a limited period of time (about 6 months) before they are harvested, the transience of the treatment is of less concern than in humans and terrestrial animals. It is feasible to work out a strategy of immunizing twice or thrice during the culture period orally or by bath treatment. The off-target effects for shrimps cannot yet be taken into consideration for the design of RNAi molecules because limited shrimp sequences are available in the public domain. However, if an efficient RNAi vaccine

is shown not to hamper the growth rate and general health of the shrimp, it can be said to have served its purpose.

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# Prospects of Vaccination in Crustaceans with Special Reference to Shrimp

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**Abstract**

Among different categories of management options available to combat infectious diseases in shrimp, vaccination is one of the most promising ones. Several vaccination methods such as inactivated (using temperature, gamma irradiation, binary ethylenimine (BEI) and formalin), recombinant and DNA vaccine and RNAi technology have been employed over the years. For white spot syndrome virus (WSSV), many viral genes, especially structural protein VP28, have been targeted for producing different types of vaccines. Many prokaryotic and eukaryotic expression systems, with their own advantages and limitations, have been used for generating recombinant vaccines. DNA vaccine has been suggested to be an ideal approach, considering many advantages including the ability of plasmid DNA to be vertically transmitted from mother to progeny. However, the efficacy of vaccine depends on many host- and pathogen-associated factors. Among various delivery methods, oral vaccination has been suggested as the most appropriate method. Further, the stability of oral vaccines in the gastro-intestinal tract of the animal has been enhanced by encapsulation with chitosan, glucan, liposomes and *Artemia* sp. Use of polyvalent vaccine, vaccine supplemented with nutritional additives, adjuvants and immunostimulants has also been reported to enhance the protective efficacy of vaccine in shrimp.

**Keywords**

Adaptive immunity · Vaccine · Vaccination · Crustaceans · Shrimp

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## 1 Introduction

Farming of crustaceans, dominated by shrimp, is one of the major aquaculture activities practised around the world. However, rapid expansion of shrimp aquaculture resulted in the emergence of various infectious diseases and subsequent collapse of the industry, apart from various environmental stresses. Among various diseases, viral diseases and bacterial diseases pose a threat to the sustainability of the industry. Various management strategies encompassing application of antibiotics, immunostimulants, probiotics, herbal products, phages and vaccines have been adopted over the years for combating various types of diseases. Historically, antibiotics have been widely used for combating bacterial infections in aquaculture. However, indiscriminate use of antibiotics has resulted in the emergence of antibiotic resistant bacteria and deposit of antibiotic residues in animals and culture systems. In this context, prophylactic and therapeutic approaches for managing diseases alternative to antibiotics have been a serious concern for the researchers all over the world. Among all these prophylactic measures, vaccination is one of the cost-effective and environmentally friendly approaches to prevent or minimize the losses due to various infectious diseases [1].

The susceptibility/resistance of shrimp to invading pathogens is greatly influenced by the immune status of the host and, therefore, understanding shrimp immune system is of utmost importance in designing the strategies for the control and management of diseases in shrimp aquaculture. Crustaceans, being invertebrates, do not possess an adaptive immunity and depend entirely on a well-developed innate immunity for protection from pathogens. The innate defence mechanisms in shrimp consist of physical barriers, cellular and humoral components which work jointly for the identification and eradication of all invading pathogens [2]. The entry of pathogens is recognized by the host-associated germline-encoded sensors, known as pattern recognition receptors (PRRs), which detect evolutionarily conserved motifs on the pathogen (called pathogen-associated molecular patterns, PAMPs). The PRRs-PAMPs interaction initiates diverse signalling pathways and activates different transcription factors involved in protective mechanism such as cellular and humoral immune defence mechanism. While cellular components include those reactions that are mediated directly by haemocytes (phagocytosis, encapsulation, nodule formation, apoptosis, etc.), humoral components mainly include prophenoloxidase (proPO) activating system, clotting system and antimicrobial peptides, etc. [3].

In vaccination, biological material (either live/killed pathogen or its antigenic determinant) is administered in order to elicit specific immunity against a particular pathogen and a long-term memory towards the specific antigen. Vaccination requires two basic components: identification of a suitable antigen which is recognized by the host immune system and the elicitation of an optimal level of protective immune response to the specific pathogen [4].

Study on immunization in shrimp against virus was initiated by Venegas et al. [5], who noticed occurrence of certain immune response in *Penaeus japonicus* that had survived previous exposure to WSSV. As the mechanism behind this 'acquired

tolerance' was unknown, the authors named it as 'quasi immune response'. In another study, shrimp exhibited resistance to WSSV about 3 weeks post-infection and haemolymph from the resistant shrimp showed virus neutralizing factors [6]. These results led the researchers to think that some sort of immune memory exists in shrimp and the shrimp could be vaccinated.

Over the years, several vaccination strategies have been adopted to protect shrimp from potential viral and bacterial diseases, including inactivated vaccine, recombinant subunit vaccine, DNA vaccine and RNAi-based vaccine. Most of the vaccine studies have been focused on white spot syndrome virus (WSSV), the most dreadful virus that causes serious impact to the shrimp farming industry.

## 2 Vaccination Trials Against Viruses

### 2.1 Inactivated Vaccine

Inactivated vaccines are usually prepared from a pathogenic microorganism by exposing it to certain physical or chemical agents without losing antigenicity of the pathogen. This process leads to the loss of infectivity or replication capacity of the pathogen. Preparation of inactivated WSSV vaccines through various means including heat [7, 8], binary ethylenimine (BEI) [8], formalin [7, 9–11] or gamma irradiation [12–14] have been reported with varying degrees of success subsequent to WSSV challenge. The description of various inactivating agents is listed in Table 1.

A comprehensive vaccination trial by introducing inactivated WSSV (formalin- or heat-inactivated) in *P. japonicus* prior to WSSV infection has been reported [7]. The formalin-inactivated virus rendered resistance against the virus for shorter duration of time (tenth day post-vaccination). The efficacy of formalin-inactivated WSSV vaccine was found to be enhanced by additional injections with glucan or *V. penaeicida*; however, the RPS did not increase beyond 60% even after three consecutive injections. Heat-inactivated WSSV failed to elicit resistance in shrimp even on tenth day post-vaccination, indicating the heat labile nature of the protective molecules. On the contrary, oral administration of formalin-inactivated virus preparation (IVP) for a period of 7 days provided full protection in *Fenneropenaeus indicus,* following WSSV challenge fifth day and tenth day post-vaccination [9]. However, the surviving shrimp that were challenged with infectious virus beyond 10 days of vaccine administration were found to be PCR-positive for

**Table 1** Details of different agents used for the inactivation of WSSV

| Sl No. | Inactivating agent | Concentration/dose | References |
|---|---|---|---|
| 1 | Gamma irradiation | 14–15 kGy $s^{-1}$ | [14] |
| 2. | Formalin | Overnight incubation in 0.5% formalin | [11] |
| 3. | Heat | 60 °C for 10 min | [7] |
| 4. | BEI | 2 mM | [9] |

WSSV and is an indication of the virus accommodation phenomenon [9]. Although BEI-inactivated WSSV also rendered protective immunity in shrimp (77% RPS), the heat-inactivated WSSV did not result in any protection [8].

## 2.2 Recombinant Vaccine

Recombinant vaccine is produced by inserting the genetic material of the pathogen, which encodes the antigen that elicits protective immune response, and is expressed in a heterologous host [15]. The recombinant viral structural proteins have been targeted for vaccination studies in shrimp because these proteins primarily interact with the host receptors and, thereby, elicit protective immune response. Among the viral structural proteins, envelope proteins such as VP19 [16–23], VP24 [22, 24], VP28 [7, 16–20, 22, 23, 25–42], VP36B [32], VP292 [43] and VP466 [21], tegument proteins such as VP26 [7, 22, 26, 35] and VP39 [33], and nucleocapsid protein, VP15 [22] (Table 2), attracted major attention. Different prokaryotic and eukaryotic expression systems have been adopted for the production of different antigenic proteins for vaccines.

Many of these studies employed *E. coli* to express the recombinant protein with great success. However, bacterial expression system involving *E. coli* poses some disadvantages such as formation of insoluble inclusion bodies and tedious purification steps. Therefore, several alternative and improved delivery vehicles have been employed for vaccine development and met with success. These include both prokaryotic and eukaryotic expression systems such as Gram-positive bacteria (*Brevibacillus brevis* and *Bacillus subtilus*–both vegetative cells and spores), yeast (*Pichia pastoris*), insect cells, silkworm, bacteriophage, baculovirus, plant (*Arabidopsis thaliana*), green algae (*Dunaliella salina* and *Chlamydomonas reinhardtii*) and blue green algae (*Synechocystis* sp. and *Anabaena* sp.). Each of these delivery systems possesses some advantages and disadvantages over others with respect to its effectiveness, stability, bio-safety, cost, time and yield, which limit their utilization in the commercial development of vaccine for shrimp industry. Even though, oral vaccination in shrimp elicits protective immunity, there exists a problem related to the stability of antigens in the gastro-intestinal tract. To tackle this problem, *B. subtilis* recombinant spores have been used as an oral delivery vehicle to protect antigens in the digestive tract [61]. However, further research is indispensable for the optimization of expression or for exploring new expression systems for effective vaccine production.

These vaccine preparations have been delivered by different methods, such as injection, oral and immersion administration, and protective response elicited by them was found to vary greatly. Among all these vaccination approaches, complete protection has been rendered by oral administration of VP26 and VP28 (protection persisted for 10 days), in which oral challenge of WSSV was performed [26]. Similarly, recombinant spores of *B. subtilis* displaying CotC::Vp26 fusion protein on its surface displayed 100% protection in *L. vannamei* [35]. It has been noticed that the recombinant protein expressed in eukaryotic vectors rendered comparatively higher

**Table 2** Chronological record of major vaccination trials in crustaceans

| Shrimp species | Vaccine | Type/mode of application | Response | | References |
|---|---|---|---|---|---|
| | | | Duration | RPS (%)[a] | |
| *Penaeus japonicus* | Live virus | Sub-lethal injection of live virus | 1 month | 67 | [6] |
| | | | 2 months | 54 | |
| | | | 3 months | 6 | |
| *P. japonicus* | Inactivated virus | Injection of heat-inactivated virus | 10 days | 15 | [7] |
| | | | 30 days | 30 | |
| | | Injection of formalin-inactivated virus | 10 days | 55 | |
| | | | 30 days | 5 | |
| | Recombinant protein | Injection of VP26 | 10 days | 57 | |
| | | Injection and booster of VP26 | 10 days | 60 | |
| | | Injection of VP28 | 10 days | 17 | |
| | | Injection and booster of VP28 | 10 days | 95 | |
| *Penaeus monodon* | Recombinant protein (*Escherichia coli*) | Injection of VP19 | 2 days | 33 | [18] |
| | | | 25 days | 57 | |
| | | Injection of VP19 + VP28 | 2 days | 33 | |
| | | | 25 days | 41 | |
| | | Injection of VP28 | 2 days | 44 | |
| | | | 25 days | 21 | |
| *P. monodon* | Recombinant protein (*Escherichia coli*) | Oral vaccination with VP19 | 2 days | 44 | [19] |
| | | | 3 days | 64 | |
| | | Oral vaccination with VP28 | 7 days | 77 | |
| | | | 21 days | 29 | |
| *Penaeus indicus* | Inactivated | Oral vaccination with formalin-inactivated virus | 1 day | 0 | [9] |
| | | | 5 days | 100 | |
| | | | 10 days | 100 | |
| | | | 15 days | 0 | |

(continued)

**Table 2** (continued)

| Shrimp species | Vaccine | Type/mode of application | Response | | References |
|---|---|---|---|---|---|
| | | | Duration | RPS (%)[a] | |
| *Procambarus clarkii* | Recombinant protein (*Pichia pastoris*) | Injection and booster of VP28 (challenged at third day post-vaccination) | 25 days | 91 | [17] |
| | | Injection and booster of VP19 + VP28 (challenged at third day post-vaccination) | | 84 | |
| | | Injection and booster of VP19 (challenged at third day post-vaccination) | | 45 | |
| | | Injection and booster of VP28 (challenged at 21st day post-vaccination) | | 78 | |
| | | Injection and booster of VP19 + VP28 (challenged at 21st day post-vaccination) | | 76 | |
| | | Injection and booster of VP19 (challenged at 21st day post-vaccination) | | 17 | |
| *P. clarkii* | Recombinant protein (*E.coli*) | Oral vaccination of VP19 (challenged at third day post-vaccination) | 25 days | 76 | [16] |
| | | Oral vaccination of VP28 (challenged at third day post-vaccination) | | 43.2 | |
| | | Oral vaccination of VP19 (challenged at 21st day post-vaccination) | | 82 | |
| | | Oral vaccination of VP28 (challenged at 21st day post-vaccination) | | 49 | |
| | | Injection of VP19 (challenged at third day post-vaccination) | | 52.91 | |
| | | Injection of VP28 (challenged at third day post-vaccination) | | 39.6 | |
| | | Injection of VP19 (challenged at 21st day post-vaccination) | | 56.46 | |

| | | | | | |
|---|---|---|---|---|---|
| | | Injection of VP28 (challenged at 21st day post-vaccination) | | 39.83 | |
| | | Immersion of VP19 (challenged at third day post-vaccination) | | 83 | |
| | | Immersion of VP28 (challenged at third day post-vaccination) | | 46.3 | |
| | | Immersion of VP19 (challenged at 21st day post-vaccination) | | 86.3 | |
| | | Immersion of VP28 (challenged at 21st day post-vaccination) | | 46.5 | |
| | | Oral vaccination of VP28 + VP19 (challenged at third day post-vaccination) | | 53.2 | |
| | | Oral vaccination of VP28 + VP19 (challenged at 21st day post-vaccination) | | 51 | |
| | | Injection of VP28 + VP19 (challenged at third day post-vaccination) | | 36.5 | |
| | | Injection of VP28 + VP19 (challenged at 21st day post-vaccination) | | 36.50 | |
| | | Immersion of VP28 + VP19 (challenged at third day post-vaccination) | | 53 | |
| | | Immersion of VP28 + VP19 (challenged at 21st day post-vaccination) | | 56.5 | |
| *P. monodon* | Recombinant protein (*E.coli*) | Injection of VP292 | 30 days | 41 | [43] |
| | | Injection and booster of VP292 | 30 days | 52 | |
| *P. monodon* | DNA vaccine | Injection of VP15 | 25 days | 0 | [44] |
| | | | 50 days | 0 | |
| | | Injection of VP28 | 25 days | 51 | |
| | | | 50 days | 30 | |
| | | Injection of VP35 | 25 days | 0 | |
| | | | 50 days | 0 | |

(continued)

**Table 2** (continued)

| Shrimp species | Vaccine | Type/mode of application | Response | | References |
|---|---|---|---|---|---|
| | | | Duration | RPS (%)[a] | |
| | | Injection of VP281 | 25 days | 46 | |
| | | | 50 days | 34 | |
| *Penaeus chinensis* | Recombinant protein (Baculovirus) | Injection of VP19 | 22 days | 50 | [21] |
| | | Oral vaccination of VP19 | 25 days | 50 | |
| | | Oral vaccination of VP466 | 25 days | 48 | |
| *P. japonicus* | Recombinant protein expressed in *Brevibacillus brevis* | Oral vaccination of VP28 | 3 days | 81 | [25] |
| | | | 2 weeks | 24 | |
| *P. japonicus* | Recombinant protein (*E. coli*) | Oral vaccination of VP26: oral challenge | 10 days | 100 | [26] |
| | | Oral vaccination VP26: injection challenge | 10 days | 61 | |
| | | Oral vaccination VP26: immersion challenge | 10 days | 71 | |
| | | Oral vaccination of VP28: oral challenge | 10 days | 100 | |
| | | Oral vaccination VP28: injection challenge | 10 days | 34 | |
| | | Oral vaccination VP28: immersion challenge | 10 days | 70 | |
| *P. monodon* | DNA vaccine | Injection of VP28 | 7 days | 90 | [45] |
| | | | 14 days | 77 | |
| | | | 21 days | 67 | |
| | | | 30 days | 57 | |
| *P. clarkii* | Recombinant protein (*Bacillus subtilis)* | Injection of VP28 (transgenic spores) | 27 days | 78.3 | [46] |
| | | Injection of VP28 (vegetative cells) | | 51.7 | |
| *Cambrus clarkia* | DNA vaccine (*Salmonella typhimurium*) | Oral vaccination of VP28 (challenged at 7-days post-vaccination) | 15 days | 83.3 | [47] |
| | | Oral vaccination of VP28 (challenged at 15-days post-vaccination) | | 66.7 | |
| | | Oral vaccination of VP28 (challenged at 25-days post-vaccination) | | 56.7 | |

| | | | | | |
|---|---|---|---|---|---|
| *P. monodon* | Recombinant baculovirus with IE1 promoter | Injection and booster of VP28 | 3 days | 86 | [27] |
| | | | 15 days | 74 | |
| *P. japonicus* | DNA vaccine | Injection of VP28 | 7 days | 70 | [48] |
| *P. clarkii* | Inactivated vaccine | Injection with BEI-inactivated WSSV (2 mM) (challenge on seventh day post vaccination) | | 77 | [8] |
| | | Injection with BEI-inactivated WSSV (2 mM) (challenge on 21st day post vaccination) | | 60 | |
| | | Injection with BEI-inactivated WSSV (3 mM) (challenge on seventh day post vaccination) | | 63 | |
| | | Injection with BEI-inactivated WSSV (3 mM) (challenge on 21st day post vaccination) | | 30 | |
| | | Injection with heat inactivated WSSV challenge on seventh day post vaccination) | | 0 | |
| | | Injection with heat inactivated WSSV (challenge on 21st day post vaccination) | | 0 | |
| *P. chinensis* | Recombinant protein (*B. subtilis*) | Oral vaccination of VP28 | 14 days | 83 | [28] |
| *P. japonicus* | Recombinant protein | Oral vaccination with liposome adjuvant of VP28 | 3 days | 68 | [29] |
| | | Oral vaccination with VP28 | 3 days | 50 | |
| *Litopenaeus vannamei* | DNA vaccine | Injection of VP28 | 7 days | 53 | [49] |
| | | | 14 days | 20 | |
| *P. monodon* | Inactivated WSSV vaccine + nutritional additives | Injection of FIV (intramuscular challenge on 3-days post vaccination) | 15 days | 83% (CS) | [10] |
| | | Injection of FIV (intramuscular challenge on 15-days post vaccination) | | 67% (CS) | |
| | | Injection of FIV (intramuscular challenge on 30-days post vaccination) | | 33% (CS) | |

(continued)

**Table 2** (continued)

| Shrimp species | Vaccine | Type/mode of application | Response | | References |
|---|---|---|---|---|---|
| | | | Duration | RPS (%)[a] | |
| | | Immersion of FIV (immersion challenge on 3-days post vaccination) | | 86% (CS) | |
| | | Immersion of FIV (immersion challenge on 45-days post vaccination) | | 66% (CS) | |
| | | Oral vaccination of FIV (immersion challenge on 45-days post vaccination) | | 63.7% (CS) | |
| | | Oral vaccination of FIV (immersion challenge on 60-days post vaccination) | | 8% (CS) | |
| | | Oral administration of FIV | | 60.7% (CS) | |
| | | FIV + wheat grass (oral) | | 72.7% (CS) | |
| | | FIV + MSM (oral) | | 73.3% (CS) | |
| *Cambarus clarkii* | Recombinant protein (*B. subtilis*) | Oral administration of CotB-VP28 (immediate challenge) | 16 days | 37.9 | [50] |
| | | Oral administration of CotC-VP28 (immediate challenge) | | 44.8 | |
| | | Oral administration of CotB-VP28 (challenge at 7-days post-vaccination) | | 46.4 | |
| | | Oral administration of CotC-VP28 (challenge at 7-days post-vaccination) | | 50 | |
| | | Oral administration of CotB-VP28 (challenge at 21-days post-vaccination) | | 30 | |
| | | Oral administration of CotC-VP28 (challenge at 21-days post-vaccination) | | 33.3 | |

| | | | | | |
|---|---|---|---|---|---|
| *P. clarkii* | Recombinant protein (silk worm pupae) | Oral vaccination of VP28 (10 g $kg^{-1}$) | 20 days | 82.2 | [51] |
| | | Oral vaccination of VP28 (50 g $kg^{-1}$) | | 94.4 | |
| | | Oral vaccination of VP28 (100 g $kg^{-1}$) | | 48.2% (CM) | |
| | | Oral vaccination of VP28 (200 g $kg^{-1}$) | | 56.6% (CM) | |
| *L. vannamei* | Recombinant protein | Oral vaccination of VP19 | 21 days | 66.7 (CM) | [20] |
| | | Oral vaccination of VP28 | | 41.7 (CM) | |
| *P. monodon* | Recombinant protein from baculovirus expression system (Bac) | Feeding Bac-VP28 coated feed: Immersion challenge | 3 days | 81 | [30] |
| | | | 15 days | 76 | |
| | | Immersion in Bac-VP28 containing sea water (three times): Immersion challenge | 3 days | 75 | |
| | | | 15 days | 68 | |
| *L. vannamei* | Recombinant protein (*E. coli*) | Injection of VP28 (1 μg) (challenge with $10^3$ or $10^4$ dilutions of WSSV) | 12 days | 100% (CM) | [32] |
| | | Injection of VP28 (5 μg)) (challenge with $10^4$ dilutions of WSSV) | | 0% (CM) | |
| | | Injection of VP28 (5 μg)) (challenge with $10^3$ dilutions of WSSV) | | 100% (CM) | |
| | | Injection of 36B (5 μg)) (challenge with $10^4$ dilutions of WSSV) | | 100% (CM) | |
| *P. clarkii* | Recombinant protein (*E. coli*) | Oral administration of rTAT-VP28 (for 7 days) | | 63.3 | [31] |
| | | Oral administration of rTAT-VP28 (for 14 days) | | 67.8 | |
| | | Oral administration of rVP28 (for 7 days) | | 44.4 | |
| | | Oral administration of rVP28 (for 14 days) | | 53.6 | |

(continued)

**Table 2** (continued)

| Shrimp species | Vaccine | Type/mode of application | Response | | References |
|---|---|---|---|---|---|
| | | | Duration | RPS (%)[a] | |
| *L. vannamei* | Recombinant protein | Oral vaccination of VP28C (challenge on 7-days post feeding) | 14 days | 47.5 | [52] |
| | | Oral vaccination of VP28N (challenge on 7-days post feeding) | | 12.5 | |
| | | Oral vaccination of VP28C (challenge on 14-days post feeding) | | 23.3 | |
| | | Oral vaccination of VP28N (challenge on 14-days post feeding) | | 16.7 | |
| *P. monodon* | DNA vaccine | Intramuscular injection of PmAV chitosan nanoparticles | 15 days | 83.3 | [53] |
| *L. vannamei* | Recombinant protein | Intramuscular and oral administration of rVP39 | 20 days | 60 | [33] |
| *L. vannamei* | Recombinant protein (*B. subtilis*) | Oral administration of CotB-VP28 | 14 days | 35% (CM) | [36] |
| *P. monodon* | Recombinant vaccine (*E.coli*) | Oral administration of VP24 | 15 days | 36% (CM) | [24] |
| *P. japonicus* | Recombinant protein (prepared by cell-free technology) | Injection of VP28 | 25 days | 94 | [54] |
| | | Oral vaccination of VP28 | 20 days | 100 | |
| *Macrobrachium rosenbergii* | DNA vaccine | Oral feeding of chitosan-conjugated DNA vaccine (MrNV) (100 ng $\mu L^{-1}$ concentration) | 20 days | Around 45% | [55] |
| | | Immersion of chitosan-conjugated DNA vaccine (MrNV) (100 ng $\mu L^{-1}$ concentration) | | Around 42% | |
| | | Immersion of chitosan-conjugated DNA vaccine (MrNV) (50 ng $\mu L^{-1}$ concentration) | | Around 40% | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *P. monodon* | Inactivated vaccine (formalin) | Oral administration (challenge on first day post-vaccination) | 8 days | | | 100 | [56] |
| | | Oral administration (challenge on fifth day post-vaccination) | | | | 50 | |
| *L. vannamei* | Inactivated vaccine | Gamma irradiated | 10 days | <0.05 | | 73.33 | [13] |
| | | WSSV vaccine (oral) | | | | | |
| | | GI-WSSV vaccine + GI-V.P (oral) | 2/20 | 10 | 86.66 | 86.66 | |
| *L. vannamei* | *B. subtilis* recombinant spores | Feeding the pelleted feed coated with bacterial spores expressing cotC:VP 26 fusion proteins | 16 days | | | 100 | [35] |
| | | Expressing cotC:VP 28 fusion proteins | | | | 90 | |
| Crayfish | Transgenic *Dunaliella salina* | Lysed cells of transgenic green algae mixed with feed and fed to crayfish | 20 days | | | 59 | [34] |
| *L. vannamei* | Recombinant vaccine (M13 phage) | Injection of VP28 | 5 days | | | 49.99 | [38] |
| *L. vannamei* | Recombinant vaccine (*Anabaena* sp.) | Injection of VP28 | | | | 68 | [57] |
| *P. monodon* | *B. subtilis* recombinant spores | Feeding the pelleted feed coated with bacterial spores expressing cotC:VP 28 fusion proteins | 14 days | | | 75 | [37] |
| *P. monodon* | Recombinant vaccine | Injection of 1XrVP28 | 10 days | | | 67 | [40] |
| | | Injection of 4XrVP28 | | | | 81 | |
| *M.rosenbergii* | Recombinant vaccine (Baculovirus vector) | Oral administration of recombinant bivalent vaccine fused with flagellin 2 | | | | | [58] |
| | | Ac-ie1VP28 | 10 days | | | 28.9% (CM) | |
| | | Ac-ie1VP19 | | | | 34.2% (CM) | |
| | | Ac-VP19-ie1VP28 | | | | 21.2% (CM) | |
| | | Ac-VP28-ie1VP19 | | | | 23.7% (CM) | |

(continued)

**Table 2** (continued)

| Shrimp species | Vaccine | Type/mode of application | Response | | References |
|---|---|---|---|---|---|
| | | | Duration | RPS (%)[a] | |
| | | Ac-VP19-ie1VP28FL2 | | 15.8% (CM) | |
| | | Ac-VP28-ie1VP19FL2 | | 10.5% (CM) | |
| *L. vannamei* | Transgenic *Synechocystis* sp. PCC6803 | Oral administration of VP28 | 7 days | 88.42 | [41] |
| | | | 10 days | 62.5 | |
| *M. rosenbergii* | Recombinant vaccine (Baculovirus vector) | Oral administration of MrNV capsid protein | 30 days post vaccination, | 65 | [59] |
| | | | 60 days of vaccination | 80 | |
| *M. rosenbergii* | DNA vaccine | Oral administration of MrNV-CP-RNA-2- | 20 days post vaccination | 60 | [60] |
| | | | 40 days of vaccination | 80 | |
| *L. vannamei* | Transgenic *Synechococcus* sp. PCC 7942 | Oral administration of VP19 | 10 days | 57.8 | [23] |
| | | Oral administration of VP 28 | | 62.2 | |
| | | Oral administration of VP (19 + 28) | | 71.1 | |
| *L. vannamei* | Transgenic *Chlamydomonas reinhardtii* | Oral vaccination with VP28 | 14 days | 87 | [42] |
| *P. japonicus* | Recombinant vaccine (in *E. coli)* | Injection and booster of VP15 | 20 days | 78 | [22] |
| | | Injection and booster of VP19 | | 27 | |
| | | Injection and booster of VP24 | | 56 | |
| | | Injection and booster of VP26 | | 44 | |
| | | Injection and booster of VP28 | | 50 | |
| | Recombinant vaccine (in silkworm larvae) | Injection of VP15 | 10 days | 45 | |
| | | Injection of VP19 | | 60 | |

[a]Response is calculated as relative percent survival (RPS) = 1 − (mortality in treatment group/mortality in control group) × 100; *CM* cumulative mortality

levels of protection than in *E.coli* expression system [16, 22, 27, 30, 41, 42]. Recently, considering its ability to tolerate extreme conditions in the gastro-intestinal tract of shrimp, transgenic lines of green microalga (*Chlamydomonas reinhardtii*) have been employed as oral delivery system. Oral administration of C. *reinhardtii* harbouring VP28 rendered higher protective efficacy (87%) in shrimp [42]. Administration of many recombinant vaccines enhanced non-specific immunity in different crustaceans, which is enlisted in Table 3.

## 2.3 DNA Vaccine

DNA vaccines comprise an expression vector carrying a gene of interest that codes for a particular antigenic protein, which when expressed in the host and recognized by the host immune system, a strong and long-lasting immune response is induced in the host [64]. DNA vaccine has been gaining considerable attraction owing to the stability of DNA, limited risk, wider range of immune responses, cost-effectiveness and ease of development and production. As plasmid DNA can be vertically transmitted from mother to progeny, DNA vaccine is an ideal strategy for the protection of shrimp from pathogenic microbes [65]. In comparison to the recombinant subunit vaccine, a DNA vaccine rendered prolonged and higher protection [44, 53]. Many WSSV genes have been used for the DNA vaccine preparation in shrimp such as VP28 [44, 45, 47–49, 66], VP15, VP35 and VP281 [44]. Moreover, non-WSSV genes including *Pm*AV [53] and PAP [67] (Table 1) have also been employed. Of all the methods used, DNA vaccines using VP28 have resulted in the highest protection rates [45]. Many previous studies reported enhancement of non-specific immune responses in shrimp following DNA vaccination (Table 3).

## 2.4 RNA Interference (RNAi) Approach

RNA interference (RNAi) is an evolutionarily conserved cellular mechanism that involves post-transcriptional silencing of a specific gene by messenger RNA (mRNA) degradation or translational inhibition in the presence of gene-specific double-stranded RNA (dsRNA) [68]. It is initiated by the processing of dsRNA into 20–30 nucleotide fragments known as short interfering RNAs (siRNAs) by an enzyme, Dicer [69], followed by the incorporation of siRNAs into the RNA-inducing silencing complex (RISC), with the Argonaute (Ago) as the catalytic component. While the antisense strand, called the guide strand, remains with the RISC, the passenger strand is degraded and removed from the complex. Subsequently, the complex binds to the complementary mRNA in the cytoplasm, and results in endonucleolytic cleavage, which leads to post-transcriptional silencing of the target gene [70]. Su et al. were the first to identify and characterise the Dicer gene in shrimp, as they have reported the gene from *P. monodon* [71]. This was followed by other components of RNAi pathway including Drosha, Pasha, TRBP, Dicer1, Dicer2, AGO1, AGO2, AGO3, arsenite resistance gene 2 [72–79].

**Table 3** Summary of non-specific immune responses reported in crustaceans against recombinant vaccines

| Sl No. | Species of animal | Type of vaccine | Parameters studied | References |
|---|---|---|---|---|
| 1 | *Penaeus monodon* | DNA vaccine | Increased PO and SOD activities | [45] |
| 2 | *P. japonicus* | DNA vaccine | Upregulated expression of Rab7, penaeidin, crustin and lysozyme | [48] |
| 3 | *Litopenaeus vannamei* | DNA vaccine | Higher level of lysozyme, alkaline phosphatase, and SOD activities | [49] |
| 4 | *Fenneropenaeus chinensis* | Recombinant vaccine | Increased level of iNOS activity | [28] |
| | | | No variation in PO and SOD activities | |
| | | | Lower level of Caspase3 than that of control | |
| 5 | *P. monodon* | Recombinant vaccine | Significant level of LGBP and STAT expression | [30] |
| 6 | *L. vannamei* | Recombinant vaccine | Changes in the expression levels of STAT, dicer, Argonaute and lysozyme in different tissues | [52] |
| 7 | *L. vannamei* | Recombinant vaccine | – Increased PO and SOD activities in vaccinated groups | [32] |
| | | | – No significant difference in Dscam levels | |
| | | | – Decline in total haemocyte count in vaccinated groups | |
| 8 | *Procambrus clarkii* | Recombinant vaccine | Increased PO and SOD activities | [31] |
| 9 | *L. vannamei* | Recombinant vaccine | Increased PO and SOD activities | [36] |
| 10 | *P. japonicus* | Recombinant vaccine | Upregulated expression of Rab7, penaeidin, crustin and lysozyme | [54] |
| 11 | *L. vannamei* | RNAi | – Increased activities of proPO, superoide anion and SOD | [62] |
| | | | – Upregulated expression of immune related genes such as proPO, cMnSOD, haemocyanin, crustin, BGBP, lipopolysaccharides (LPs], lectin and lysozyme | |
| | | | – No significant changes in the expression of toll receptor and tumour receptor genes | |
| 12 | *P. monodon* | Recombinant vaccine | Upregulation of syntenin and down regulation of STAT, Rab7 and caspase | [24] |
| 13 | *P. monodon* | Formalin-inactivated vaccine | Increased levels of PO, PmRACK, alpha 2-macroglobulin, transglutaminase, crustin, penaeidin, caspase and Rab7 | [56] |

(continued)

**Table 3** (continued)

| Sl No. | Species of animal | Type of vaccine | Parameters studied | References |
|---|---|---|---|---|
| 14 | *P. japonicus* | Recombinant vaccine with adjuvants | Significant up-regulation of Rab7, lysozyme, penaeidin, crustin, Toll and TNF genes | [63] |
| 15 | *L. vannamei* | Recombinant vaccine | No variation in PO and SOD activities | [38] |
| 16 | *P. monodon* | Recombinant vaccine | Significant up-regulation of caspase, penaeidin, crustin, astakine, syntenin, PmRACK, Rab7, STAT and C-type lectin genes | [39] |
| 17 | *P. monodon* | Recombinant vaccine | High PO activity in vaccinated shrimp | [37] |
| 18 | *Macrobrachium rosenbergii* | Recombinant vaccine | Increased expression of antimicrobial peptide, lysozyme, proPO and Toll-like receptor | [59] |
| 19 | *M. rosenbergii* | DNA vaccine | Increased activities of PO, superoxide anion and lysozyme | [60] |
| 20 | *L. vannamei* | Recombinant vaccine | Increased levels of PO, SOD, CAT and LYZ in the muscle and hepatopancreas of vaccinated shrimp | [41] |
| 21 | *L. vannamei* | Recombinant vaccine | Increased levels of PO, SOD, CAT and LYZ in the hepatopancreas of vaccinated shrimp | [23] |

RNAi was found to be involved in antiviral immunity in many organisms including invertebrates, plants, and mammals. The activation of RNAi mechanism is usually performed by administering dsRNA [80–86], small siRNAs [87, 88], short/long-hairpin RNA (shRNA/ lhRNA) [89, 90] and antisense RNA [91–93]. The antiviral mechanism of dsRNA has been reported to function in two ways: sequence-independent antiviral immunity and sequence-specific mechanism. Sequence-independent antiviral immunity in shrimp has been reported where administration of unrelated dsRNA such as duck immunoglobulin υ (Igυ) or green fluorescent protein before virus challenge offered protection [87, 94, 95]. RNAi has been reported to prevent the replication of DNA and RNA viruses by knocking down viral genes or downregulating host genes that help in viral replication process. It has been noticed that silencing of Rab7, significantly reduced the replication of WSSV, yellow head virus (YHV), Laem-Singh virus (LSNV) and Taura syndrome virus (TSV) in infected shrimp [96–98]. RNAi targeting diverse viral genes has also been reported to inhibit the replication of WSSV, YHV, LSNV and TSV [81, 88, 91, 99, 100].

RNAi mechanism has been applied in the immunization of shrimp against potentially dangerous shrimp viruses. For achieving high protective efficacy, choice of proper gene for RNAi-mediated silencing is a key factor. Many studies have shown that dsRNAs targeting both viral genes and host genes elicited varying degree of protective immune responses in shrimp against viral pathogens. Major target

genes of WSSV used in the RNAi vaccination studies include VP9, VP15, VP19, VP28, VP24, thymidylate synthase, ribonucleotide reductase-2, rr1, WSSV477, VP26, WSSV051, ORF89, WSSV191VP39, TK-TMK,VP281, protein kinase, dnapol and icp11 (Table 3). Apart from WSSV, ns1 and vp of *Penaeus monodon densovirus*, RdRp of LSNV and YHV, and protease of YHV have been used for RNAi studies. Non-viral gene such as Rab7 has been reported to protect shrimp against YHV (Table 4).

Antiviral immunity rendered by sequence-specific dsRNA against WSSV has been reported to persist only for short duration, and the antiviral efficacy gradually declined as the time between immunization and WSSV challenge increased [115]. Therefore, strategies for increasing the antiviral effect for extended period is needed. It has been observed that repeated re-infection of vaccinated shrimp prolongs the protective effect (up to 30 days) and considerably minimised shrimp mortality [115]. Continuous administration of dsRNA also displayed to enhance the persistence of antiviral immunity in shrimp [100, 116–118]. Different experimental trials reported on RNAi-based shrimp immunization are summarised in Table 4. It has been found that RNAi approach can be an efficient method for the management of viral disease in shrimp at both hatchery and farm levels. Nevertheless, in most of the RNAi experiments, the protective efficacy has been achieved through injection, which is not possible for mass delivery. Therefore, several authors have made attempts to produce huge amount of RNA molecules and its delivery to large population. These include feeding the pellets coated with inactivated bacteria expressing dsRNA [81, 104, 109, 119] or oral administration of dsRNA conjugated with chitosan nanoparticles [119], liposomes [84], virus-like particles [107], feeding with dsRNA expressed in the microalga *Chlamydomonas reinhardtii* [86], feeding of agar embedded with dsRNA-expressing *E. coli* [106] and bioencapsulated *Artemia* [82], etc.

## 3 Vaccination Trials Against Bacterial Diseases in Shrimps

It has been reported that the vibriosis caused by the opportunistic pathogenic vibrios poses a serious threat to shrimp aquaculture throughout the world leading to massive economic losses [120–123]. Major pathogenic vibrios include *Vibrio harveyi, V. vulnificus, V. parahaemolyticus, V. alginolyticus* and *V. penaeicida* [124, 125]. The initial studies on immunization in shrimp against *Vibrio* sp. was reported by Itami et al. [126], using formalin-inactivated (0.5%) *Vibrio* sp. through diverse delivery methods such as intra-muscular injection, immersion and spray. Interestingly, all the immunization methods rendered more than 50% protection to the vaccinated animals. In another study, larval immunization was conducted in shrimp through oral administration of microencapsulated diets supplemented with different doses of formalin-treated *Vibrio* cells (0.05%, 0.5% and 5%) and it was found that 0.05% supplemented feed yielded maximum protection (39%) [126]. In yet another study, immunization trials on post-larvae of *P. monodon* were performed with either formalin-killed *Vibrio* spp. or beta-glucan or both mixed together as the

**Table 4** Details of the major RNAi trials reported in crustaceans for the management of viruses

| Virus | Shrimp species/ size | Target gene | Delivery method/type of RNAi | Protection | Duration | References |
|---|---|---|---|---|---|---|
| White spot syndrome virus (WSSV) | *Litopenaeus vannamei* (3–4 g) | RR2 | Intramuscular injection of dsRNA | 78% | 10 days | [80] |
| | | DNA polymerase | | 44% | | |
| | | VP28 | | 85% | | |
| WSSV | *P. monodon* (2.5 ± 0.6 g) | VP28 | Intramuscular injection of siRNA | 50% | 13 days | [87] |
| | | VP15 | | 42% | | |
| | | GFP | | 33% | | |
| Yellow head virus (YHV) | *P. monodon* (5.0 g) | Protease | Intramuscular injection of dsRNA (25 μg) | 100% | 10 days | [101] |
| | | GFP | | 50% | | |
| WSSV | *Penaeus chinensis* (5–6 g) | VP28 | Intramuscular injection with long dsRNAs (challenge with $LD_{50}$ of WSSV) | 0% (CM) | 7 days | [102] |
| | | VP281 | | 20% (CM) | | |
| | | Protein kinase | | 6.7% (CM) | | |
| | | GFP | | 13.3% (CM) | | |
| | | VP28 | Intramuscular injection with long dsRNAs (challenge with 2 × $LD_{50}$ of WSSV) | 0% (CM) | | |
| | | VP281 | | 46.7% (CM) | | |
| | | Protein kinase | | 6.7% (CM) | | |
| | | GFP | | 33.3% (CM) | | |
| WSSV | *P. monodon* (8–10 g) | VP28 | Intramuscular injection with dsRNA | 100% | 30 days | [103] |
| WSSV | *P. monodon* (8–10 g) | VP28 | Feed pellets coated with *E.coli* expressing dsRNA | 68% | 30 days | [104] |
| | | | Feed pellet coated with chitosan-dsRNA complex | 37% | | |
| WSSV | *P. monodon* (10–12 g) | VP28 (10 μg) | Intramuscular injection of long-hairpin RNA (lhRNA) | 50% | 20 days | [89] |
| | | VP19 (10 μg) | | 29% | | |
| | | VP28 (25 μg) | | 58% | | |

(continued)

**Table 4** (continued)

| Virus | Shrimp species/ size | Target gene | Delivery method/type of RNAi | Protection | Duration | References |
|---|---|---|---|---|---|---|
| | | VP28 (35 μg) | | 75% | | |
| | | VP28 (50 μg) | | 79% | | |
| WSSV | *P. monodon* (20 g) | VP19, VP28, ICP11 | Intramuscular injection of long-hairpin RNA | | 15 days | [90] |
| | | | T1: ICP11-LH; 35 μg | 67% | | |
| | | | T2: VP28 35 μg | 75% | | |
| | | | T3: VP28 and VP19 17.5 μg each | 75% | | |
| | | | T4: VP28 and VP19 25 μg:10 μg | 58% | | |
| | | | T5: ICP11, VP28 and VP19-11.5 μg each | 58% | | |
| WSSV | *Marsupenaeus japonicus* (10.0 g) | VP28 | Intramuscular injection with β-1,3-D-glucan encapsulated vp28-siRNA particles | Shrimp mortality was significantly delayed | 7 days | [88] |
| WSSV | *P. monodon* (1.5 ± 0.5 g) | vp28 | Intramuscular injection of dsRNA (6 μg/g body weight) | 90% | 14 days | [70] |
| | | vp281 | | 66.7% | | |
| | | rr1 | | 93.3% | | |
| | | dnapol | | 76.6% | | |
| Laem Singh virus (LSNV) | *P. monodon* (10–15 g) | RdRp | Feed pellets coated with *E.coli* expressing dsRNA | Dose 1: 2/10 were LSNV free | | [81] |
| | | | Dose 1: 6 mg dsRNA per kg feed | Dose 2: 6/10 were LSNV free | | |
| | | | Dose 2: 12 mg dsRNA per kg feed | | | |
| LSNV | *P. monodon* (PL1) | RdRp | *Artemia* enriched dsRNA-expressing *E. coli* | Reduction in LSNV copy number noticed | | [82] |
| YHV | *L. vannamei* (3.0 g) | Protease | | | | [105] |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | Intramuscular injection of dsRNA-containing cationic liposomes (2.5 μg/g shrimp) | | | |
| | | | YHV/cationic liposome | 100% (CM) | 10 days | |
| | | | YHV/YHV-Pro-dsRNA | 50% (CM) | 10 days | |
| | | | YHV/YHV-Pro-dsRNA/cationic liposome | 10% (CM) | 10 days | |
| YHV | *L. vannamei* (250–300 mg) | Rab7 of shrimp | Oral feeding of agar embedded with dsRNA-expressing E. coli | 22% (CM) | 6 days | [106] |
| WSSV | *L. vannamei* (8–10 g) | VP28 | IM injection with *E.coli* expressing antisense RNA (10 μg) | 50% | 9 days | [93] |
| | | VP39 | | 60% | | |
| | | WSV477 | | 90% | | |
| | | VP28+ VP39 | | 50% | 10 days | |
| | | VP28+ wsv477 | | 50% | 12 days | |
| | | VP39+ wsv477 | | 50% | 14 days | |
| WSSV | *P. monodon* (25 ± 2 g) | VP24, VP28, thymidylate synthase (TS), ribonucleotide reductase-2 (RR2) | IM injection with *E.coli* expressing antisense RNA under the control of shrimp histone-3 (H3) or penaedin (Pn) promoter | pH 3–VP24: 80%<br>pH 3–P28: 90% | 16 days | [92] |
| | | | VP24 (pH 3–VP24, pPn–VP24), VP28 (pH 3–VP28,pPn–VP28), TS (pH 3–TS, pPn–TS), and RR2 (pH 3–RR2) (25 μg) | pH 3–TS: 80%<br>pH 3–RR2: 70% | | |
| WSSV | *P. monodon* | VP28 | IM injection with dsRNA expressed in virus like particles | 44.5% | 7 days | [107] |
| WSSV | *L. vannamei* (23.3 ± 4.2 g) | VP 28 | IM injection with *E. coli* expressing dsRNA (4 μg) | 10% (CM) | 10 days | [83] |
| | | VP26 | | 21% (CM) | | |
| | | ORF89 | | 10% (CM) | | |
| | | WSSV191 | | 81% (CM) | | |

(continued)

**Table 4** (continued)

| Virus | Shrimp species/ size | Target gene | Delivery method/type of RNAi | Protection | Duration | References |
|---|---|---|---|---|---|---|
| WSSV | *L. vannamei* (PL45) | VP28, VP19, RR2 and TK-TMK | IM injection with *E. coli* expressing dsRNA | 95% | 28 days | [108] |
| WSSV | *L. vannamei* (3–5 g) | VP28, WSSV051 | Feed pellets coated with *E.coli* expressing dsRNA | dsWSSV051: 60% (CM) | 7 days | [104] |
| | | | Oral administration with four doses (1.2 and 2.4 μg dsRNA for single and double dose per meal) | | | |
| | | | 6 mg of WSSV051-dsRNA | | | |
| | | | 6 mg of VP28-dsRNA | | | |
| | | | 6 mg of multi-WSSV dsRNA | | | |
| | | | 12 mg of multi-WSSV dsRNA | | | |
| PmDNV | *P. monodon* (200–300 mg) | ns1+ vp | Feed pellets coated with *E.coli* expressing dsRNA | 88% reductions in PmDNV | | [109] |
| WSSV | *Macrobrachium rosenbergii* | VP9 (ICP11) | Injection of dsRNA | 80% | 25 days | [110] |
| YHV | *L. vannamei* (200–250 mg) | Protease | Intramuscular injection of dsRNA-containing cationic liposome | | | [84] |
| | | Rab7 | Oral feeding of agar embedded with dsRNA-containing cationic liposomes | | | |
| | | | Chol-dsYHV at 0.05 μg dsYHV/g shrimp | | | |
| | | | Chol-dsYHV at 1.25 μg dsRNA/g shrimp | | | |
| WSSV | *L. vannamei* (10.0 g) | Bac-VP28-dsrr2 | Intramuscular injection of dsRNA | 33% (CM) | 14 days | [111] |
| | | Bac-VP28 | | 64% (CM) | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| WSSV | *P. monodon* (7–10 g) | Injection of VP24 | Oral and injection with dsRNA (2.5 μg/g of shrimp) | 50% | 15 days | [85] |
| | | Injection of VP26 | | 60% | | |
| | | Injection of RR1 | | 80% | | |
| | | Injection of WSSV477 | | 90% | | |
| | | Oral feeding of VP24 | | 50% (CM) | | |
| | | Oral feeding of VP26 | | 50% (CM) | | |
| | | Oral feeding of RR1 | | 30% (CM) | | |
| | | Oral feeding of WSSV477 | | 20% (CM) | | |
| WSSV | *L. vannamei* (2.618 ± 0.527 g) | VP28 | IM injection with *E. coli* expressing dsRNA (≥12 μg dsRNA/g) | 97.5% | 10 days | [112] |
| WSSV | *L. vannamei* (5 ± 0.75 g) | ORF89 | IM injection with dsRNA expressed in yeast *Yarrowia lipolytica* (12 μg) | 25% | 30 h | [113] |
| YHV | *L. vannamei* (PL30) | RdRp | Feed pellets with dsRNA expressed in the microalgae *Chlamydomonas reinhardtii* | 50% | 8 days | [86] |
| WSSV | *L. vannamei* (10.5 ± 3.5 g) | VP19 | IM injection with *E. coli* expressing dsRNA | Dose 3 showed significant effect on granular hemocytes | | [114] |
| | | | Dose 1: 0.02 μg; dose 2: 0.2 μg; dose 3: 2 μg | | | |

immunostimulant. Subsequent immersion challenge with *V. alginolyticus* caused enhanced survival in the immunized shrimp in contrast to shrimp in non-vaccinated group and in shrimp which was administered immunostimulant alone, demonstrating a certain role of vibrios in eliciting partial specificity [127]. The difference in the level of immune protection rendered by immersion vaccination versus oral feeding was investigated using formalin-killed *V. harveyi*, pathogenic strain (BP05) and less virulent strain (IN7) by immunizing *Penaeus indicus* larvae [128]. Immersion of shrimp larvae in 0.5% formalin-killed *V. harveyi* ($2.07 \times 10^7$ cells) for 6 h and subsequent challenge with pathogen ($1.24 \times 10^6$ cells/ml) resulted in comparatively higher level of survival (44%) in vaccinated shrimp in contrast to non-vaccinated and challenged shrimp (6%) at 48 h post-immunization. However, significant level of protection was afforded only in virulent strain (BP05 group) and not in less virulent strain (IN7 group) [128]. Interestingly, oral administration with killed, lyophilized *V. harveyi* did not render any protection following challenge with the pathogen [128]. Additionally, cross-protection could be induced in larvae when challenged with other strains of *V. harveyi*, indicating the lack of adaptive immune response in shrimp [128]. On the other hand, haemocytes of crustacean parasite, *Porcellio scaber* (woodlice) showed increased phagocytic activity when encountered with *Bacillus thuringiensis* (previously immune-primed with the same bacterium), in contrast to the non-related bacteria [129]. This observation suggests the existence of immune-specificity in crustaceans in which specific protection is elicited by a single exposure to heat-killed bacteria [129]. Immersion vaccination with either formalin or heat-killed *V. harveyi* in post-larvae (PLs) followed by challenge with the same bacterium at 1 to 3 weeks post-immunization resulted in significant protection in shrimp (more than 80%) in contrast to the controls (35–38%) at 3 weeks post-immunization [130]. Efficacy of several concentrations of formalin-killed vaccine (0.1%, 0.5%, 1%, 5% and 10%) in the PLs of *Fenneropenaeus indicus*, followed by challenge with *V. harveyi* at different time-points (1, 2, 3, 4, 5, 6 and 7 h) was studied in which the highest RPS could be noticed at 1% concentration of vaccine until 5 h [131].

The difference in the pattern of immune response in *L. vannamei* which received either heat-killed or formalin-inactivated *V. alginolyticus* was studied subsequent to *V. alginolyticus* infection [132]. It was observed that the immune response afforded by heat-killed *V. alginolyticus* improved on day 1, in contrast to the late immune activation (fifth day) in shrimp receiving formalin-inactivated *V. alginolyticus*. However, while shrimp which received formalin-inactivated *V. alginolyticus* showed significant protection on seventh day post-infection, only minor protection was displayed by animals that were administered heat-killed *V. alginolyticus.*

Bacterins formulated from different *Vibrio* spp. have been employed to control vibriosis in shrimp culture systems. Patil et al. [133] studied the effectiveness of orally administered formalin-killed vibrio bacterin in the post-larvae of banana shrimp (*Fenneropenaeus merguiensis*) by subsequent exposure to *V. harveyi* and *V. anguillarum*, and found that the group receiving $10^8$ CFU $kg^{-1}$ feed exhibited high average weight gain and survival and low cumulative mortality following

pathogen challenges. Increased survival and production of tiger shrimp in field conditions through orally administered vibrio bacterin has been reported [134].

## 4 Factors Affecting the Efficacy of Vaccination

The effectiveness of a vaccine is dependent on many factors such as mode of administration, the amount of virus used in the challenge and mode of pathogen challenge, dose of vaccine, nature of protein used and life-stage of the animal used for vaccination. In comparison, among the three vaccine delivery methods, injection method has been found to be more effective than oral vaccination. Further, oral vaccination is reported to be better than immersion vaccination. However, injection method has limitations in applying vaccines in the field situation, apart from the fact that effectiveness of the vaccine depends on the dose and number of vaccinations. Considering these facts, development of effective orally administered vaccines will be the most appropriate way in shrimp aquaculture.

The major concern of oral vaccination is the selection of appropriate delivery system for the vaccine and degradability of the vaccine in the gastro-intestinal tract of the animal. The vaccine should be able to withstand the harsh gastric environments of the host and then released at a proper site in the gastro-intestinal tract. Therefore, encapsulation of vaccine using different agents, such as chitosan [45, 53, 55, 119, 135, 136], glucan [88], liposomes [29, 84, 105] and *Artemia* sp. [82], which protect the vaccine under adverse conditions of the gastro-intestinal tract of the shrimp, thereby heightening the survival rate in shrimp following pathogen challenge, has been employed. It was also reported that supplementation of vaccine with different adjuvants, immunostimulants and probiotics enhanced the protective rate in shrimp [10, 11, 13, 63].

It has been demonstrated that booster vaccination of shrimp by different methods led to augmented protection against WSSV [7, 16, 27, 43]. Orally vaccinated shrimp displayed comparatively higher protection rate when challenged by WSSV through oral route, in contrast to the challenge infection through injection and immersion [26]. It has been reported that administration of recombinant multimeric (tetrameric) 4XrVP28 enhanced protection against WSSV compared to the monomeric 1XrVP28 in *P. monodon* [40].

Polyvalent WSSV vaccines have been reported to elicit better protective response in shrimp than corresponding single monovalent vaccine. The survival rate of shrimp vaccinated with the fusion protein rTAT-VP28 was found to be enhanced significantly [31]. Similarly, orally administrated CotC-VP26 recombinant protein vaccine could result in 100% RPS and, therefore, could be used as an ideal vaccine candidate for combating WSSV infection [35]. Moreover, combination of several monovalent vaccines into a single polyvalent vaccine showed better protection rate in shrimp than single monovalent vaccines [16, 17, 137]. In the same way, polyvalent vaccine VP19 + VP28 and VP39 + wsv477 showed better protective response than corresponding monovalent units [23, 58, 94]. Moreover, oral administration of

vaccine with fusion proteins such as TAT and flagellin also enhanced the survival rate in crustaceans [31, 58].

The life-stage of the animal also determines the protective efficacy of vaccination. It has been reported that oral administration of CotC::Vp26 and CotC::Vp28 recombinant spores in the larvae of *L. vannamei* elicited protective immune response with RPS of 100% and 90%, respectively; however, in the adult shrimp vaccine administration resulted in lower RPS [35].

The time of pathogen challenge following vaccine administration also affects the protective efficacy of the vaccine. Many studies have shown that the survival of shrimp gradually reduced as the time of challenge was delayed following vaccination [10, 44, 45, 47, 50, 56]. A study conducted by Amarnath et al. [138] reported that administration of 1 μg dsVP28 /g of shrimp 24–48 h prior to WSSV infection rendered full protection to shrimp; however, no protection was shown when administered at 3, 6, 12 and 18 h before WSSV infection.

## 5 Mechanism of Vaccination in Crustaceans

Limited information is available on the mechanistic basis of the immune responses following vaccination. Major focus in all these vaccination trails was to identify the ideal candidate gene followed by its production and administration. A 'viral accommodation' hypothesis was formulated in shrimps on the basis of multiple viral infection and virus-specific dampened immune response. According to the virus accommodation concept, many invertebrates including crustaceans can adapt to new viral pathogens and become asymptomatic carriers of more than one virus without growth retardation and/or survival threats [139, 140]. Though vaccines are generally considered as one of the most effective methods to prevent viral infections, its effectiveness in crustaceans is questioned as they lack true adaptive immunity with specific memory, and entirely depend on innate immune mechanisms for combating pathogens. However, some form of immune memory, termed 'immune priming' or 'innate immunity with specificity', has been reported from certain invertebrates including shrimp [129, 141] and the same has also been found to be transferred to progeny [142]. Immune priming involves enhancement in the survival of an animal when injected with an inactivated pathogen and subsequently challenged with lethal dose of the same pathogen, [143]. However, the study could not relate this elevated immunity to any of the humoral immunity factors such as antimicrobial peptides and lysozyme. Nevertheless, recent studies showed enhanced phagocytic activity in vaccinated animals, and it is believed to be linked to the specific recognition of the microbial determinants by the haemocytes, and it has been inferred that the mechanism of this recognition depends on the Down syndrome cell adhesion molecule (Dscam) [143]. Dscam possess immunoglobulin-like domain and are capable of generating many isoforms through alternative splicing, sensing diverse pathogens and ligands [144–146]. Different forms of Dscam from crustaceans have been reported, and its role in immune response against different pathogens has been elucidated [147–150].

In the initial studies, the efficacy of vaccination trials has been mainly assessed by observing increased resistance/survival following secondary challenge; however, the underlying mechanism for achieving that enhanced resistance to infection has not been clearly elucidated. It has been suggested that receptor on the shrimp cells could be blocked by the protein vaccines (virus envelop proteins) which prevent the initial attachment and entry of the virus [151]. The mechanism of uptake of vaccine formulation through oral administration was studied by Kulkarni et al. [152], in which formalin-inactivated WSSV and recombinant VP28 were orally intubated and explored by immunohistochemistry employing monoclonal antibodies. The processing of the orally administered antigen was observed in the endo-lysosomal compartments of the gut epithelia leading to the generation of supra-nuclear vacuoles. Furthermore, ample amounts of antigen escaped endo-lysosomal processing and were carried to intercellular space through transcytosis, followed by the simultaneous aggregation of haemocytes in the proximity of transcytosed vaccine suggesting the activation of local immune response. It has been noticed that some proteins were differentially expressed in vaccinated shrimp and were suggested to participate in the protection against WSSV [153, 154]. Many vaccine formulations were found to improve the non-specific immune response of the animals (Table 3). Besides differential expression of the proteins, modulation in the level of expression of some of the important immune genes involved in cellular and humoral immune responses in shrimp has been reported following administration of different vaccine formulations [24, 30, 39, 56, 62] (Table 3).

## 6 Summary

Several vaccination strategies against potential shrimp pathogens have been employed on experimental scale, over the years, using different gene and gene products of pathogens and hosts and have been successful to some extent. These have been recently reviewed by Kulkarni et al. [155]. However, the mechanism underlying the phenomenon of vaccination has not been explained precisely. In the light of these observations, it is indispensable to carry out transcriptome profile of shrimp following administration of vaccines for unravelling the underlying mechanism conferring resistance/protection to shrimp. Research also needs to focus on signalling pathways that activate different components of cellular and humoral immune responses. Moreover, new functional proteins and domains of pathogen as well as host cell receptors that interact with the pathogen need to be identified for developing a potential vaccine candidate. An efficient expression system for vaccine delivery that can withstand adverse condition in the gastro-intestinal tract is a promising approach. Additionally, administration of vaccine as polyvalent vaccine, supplementation with nutritional additives, adjuvants and immunostimulants will definitely enhance the survival rate in the animals. A cost-effective, environmentally friendly vaccine with high efficacy is the need of the hour in combating infectious diseases in shrimp aquaculture.

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# Methods of Vaccine Delivery

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**Abstract**

Vaccination in fish is an important prophylactic measure to prevent disease outbreaks and to reduce loss due to diseases. The route of vaccination greatly influences the immune response and the protection offered. Injectable vaccines produce good systemic immune response. However, it is laborious to inject individual fish and results in handling and vaccination stress. Alternate vaccination routes, such as immersion vaccination and oral vaccination, can be used for mass vaccination without handling the fish. In addition, immersion and oral routes stimulate mucosa-associated lymphoid tissue and induce mucosal immune response thereby preventing pathogen entry through the natural portals of entry. The advantages and disadvantages of different vaccination routes are discussed.

**Keywords**

Vaccine delivery · Injection · Immersion · Oral · Intranasal · Anal intubation · Nanoparticle vaccine

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## 1 Introduction

Vaccination of fish is one of the effective prophylactic measures to reduce incidence of disease outbreaks in aquaculture. Different types of vaccines have been developed and licensed for use in aquaculture. Most of the licenced vaccines which are commercially available are injectable vaccines containing inactivated pathogens or recombinant antigen of the pathogens. The major challenge in vaccinating aquatic animals is the administration of the vaccine to a large population. Fish being an aquatic animal, vaccinating them through injection has many practical difficulties such as netting all the fish from the culture pond, anaesthetizing them, injecting the fish individually, etc. Further, holding the fish for considerable time outside the water and subsequent handling and vaccination cause stress to the fish, resulting in considerable mortality. To overcome these issues, alternate routes of vaccine administration are being increasingly explored.

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 or intramuscular injection, offers the best protection. 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), gill-associated lymphoid tissue (GIALT) and nasopharynx-associated lymphoid tissue (NALT). 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. Following are some of the routes of vaccination used in fish.

## 2 Injection Vaccination

Injectable vaccines can be administered to fish either by intraperitoneal injection or by intramuscular injection at one or multiple sites, usually on the lateral side of the posterior half of the fish. Intraperitoneal injection is the preferred route of vaccination for inactivated vaccines, while intramuscular route is preferred for DNA vaccines. Injection can deliver the required quantity of the vaccine at the desired site without much vaccine loss. Injection vaccines usually produce a higher systemic immune response in terms of specific antibody production than the other routes of immunization [1]. The duration of immunity is also longer in the case of injection [2], 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 injection vaccination [3]. However, injection vaccination causes handling and injection stress to the fish. Hence, it is essential to anaesthetize the fish before vaccination to minimize the stress. Fish can be anaesthetized with benzocaine at a dose of 100 mg $L^{-1}$, tricaine methanesulfonate (MS-222) at 25–250 mg $L^{-1}$ [4], etomidate 2 mg $L^{-1}$ [5], phenoxyethanol 500 μL $L^{-1}$ [6] or clove oil at a dose of 100–120 mg $L^{-1}$

**Fig. 1** Self-refilling automatic syringe for fish vaccination

[7]. However, the anaesthetics have a withdrawal period during which time the fish should not be used for human consumption or enter the food chain. For example, U.S. Food and Drug Administration (FDA) requires a withdrawal time of 21 days for MS-222. Clove oil which contains eugenol as the active ingredient (90–95%), is generally recognized as safe (GRAS) by US FDA and, hence, does not require withdrawal time.

Injection vaccination is labour-intensive and also requires trained manpower for injecting each fish individually. The vaccine can be administered either manually using an automatic syringe (Figs. 1 and 2a, b) which delivers a pre-defined 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 are vaccinated by automatic vaccinators. A trained person can inject about a few 100 fish an hour while an automated system can vaccinate about 7000–9000 fish in an hour [8]. However, the automated system is cost-intensive and requires relatively larger fish for vaccination. This restricts its use to farmed high-value fish which grows to relatively larger size like salmon and rainbow trout. Vaccination using an automated system is less stressful to fishes compared to manual injection [9].

Vaccination causes the release of corticosteroids immediately after vaccination. Increased corticosteroid levels are found to deplete lymphocyte population in circulation and lymphoid organs and have immunosuppressive effects [10]. However, injection vaccination produces a significant immune response which overcomes the stress and immunosuppressive effect. Injection vaccines are normally administered along with adjuvant to increase the immune response by sustained release of the

**Fig. 2** (**a**, **b**) Mass injection vaccination of fish (Olive flounder), conducted by trained professionals. The water level in the tank is reduced and all the fish in the tank are gathered in a caged structure. Each fish is vaccinated using the injection gun and released back into the tank

antigen. However, use of adjuvants has side effects such as development of adhesions at the site of injection between organs and the peritoneal wall [11].

Injectable vaccines 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 does not match with the mammalian immune system. Further, the increase in the antibody affinity observed in mammals is not seen in fishes.

## 3 Immersion Vaccination

Immersion vaccination is the simplest route of vaccine administration. The procedure involves the immersion of 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.

Following immersion vaccination, the antigen enters the body through skin, gills and intestinal tract. In addition to the mucosal immune response, the specific serum antibody levels are also elevated following immersion vaccination. However, the magnitude of elevation of serum antibody level following immersion vaccination is lower than the antibody level attained following injection vaccination. The mucosal antibody levels in skin mucus and gill mucus are elevated following immersion vaccination. 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 in mucosal surfaces and may account for the protection offered in addition to the involvement of cell-mediated immunity. Immunological memory exists in the skin and mucosal immune system, and subsequent booster doses result in increased antibody levels and protection.

Immersion vaccination can be administered by many methods, viz., direct immersion (DI), hyperosmotic infiltration (HI), bath vaccination and flush vaccination [3].

### 3.1 Direct Immersion (DI)

In this method, the fish are transferred and held in a holding tank containing the vaccine for a particular period of time and then shifted to the culture area. This method is ideal for small fish and is practised before stocking them in the culture ponds. The uptake of antigen following immersion vaccination depends on the nature of the vaccine, concentration of the antigen, duration of immersion, pH, osmolality of the water, water temperature and the stress the fish are subjected to. Higher the concentration of the antigen, greater is the uptake and, similarly, longer duration of immersion results in increased uptake of antigen [12]. Hence, the concentration of antigen and duration of immersion have to be optimized for better protection by the vaccine. Further, bigger the size of the vaccinated fish, higher the immune response. The water temperature also affects the antigen uptake and immune response. Higher the temperature, higher the antigen uptake and better the immune response [13].

Nakanishi et al. [14] reported a new immersion delivery method using a multiple puncture instrument while fish were immersed in a vaccine solution containing formalin-killed *Streptococcus iniae*. By this method, it was demonstrated that the antigen uptake at the skin and delivery to the kidney and spleen was increased compared to immersion alone, thereby protecting the fish better when challenged. Fish vaccinated by immersion alone were not protected when challenged with live *S. iniae* intraperitoneally 2 weeks post-vaccination.

Recombinant bacteria expressing immunogenic antigens can also be administered by immersion method. Attenuated strain of *Aeromonas salmonicida* expressing VHSV and IHNV glycoproteins when administered by immersion method could protect rainbow trout against the virulent pathogens [15]. Thus, multivalent vaccines can be easily administered by this route. Immersion vaccination using single-walled carbon nanotubes (SWCNTs) as a delivery vehicle was reported by Gong et al. [16], where grass carp juveniles were vaccinated with SWCNT-conjugated recombinant *Aeromonas hydrophila aer*A by immersion and injection routes. Both routes offered about 80% relative percent survival (RPS) suggesting SWCNT as a promising delivery vehicle for immunizing juvenile fish by immersion method.

## 3.2 Hyperosmotic Infiltration (HI)

In this method, the fish are subjected to a hyperosmotic stress by immersing them briefly in hypertonic solutions 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. Fender and Amend [13] demonstrated that immersing the fish in hyperosmotic solution (5.32% NaCl) along with bovine serum albumin (BSA) resulted in increased uptake of BSA and increased plasma concentration, suggesting hyperosmotic infiltration as a potential means for immersion vaccination and drug delivery for fish. The uptake of BSA was directly proportional to the water temperature ranging from 4 to 20 °C. However, Lio-Po & Wakabayashi [17] did not observe any advantage of hyperosmotic infiltration while vaccinating tilapia with formalin-killed *Edwardsiella tarda*. Contrary to this, the immune response of common carp to *A. salmonicida* bacterin was found to be enhanced following HI method compared to DI [18]. Nevertheless, this method causes stress to the fish and, hence, is not widely practised.

## 3.3 Bath Vaccination

The term bath vaccination is also sometimes used for immersion vaccination and vice versa. This procedure involves lowering the water level in the tank/pond and addition of vaccine to the water. This method can be practised for all sizes of fish. The disadvantage of this method is that it requires large quantities of vaccine. 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 provide better protection than lower dilution and shorter duration [12, 19]. Bath vaccination of orange-spotted grouper (*Epinephelus coioides*) at early larval stage (40 dph) with BEI-inactivated betanodavirus provided 79–95 RPS. Twenty minutes of immersion in vaccine solution was sufficient to produce RPS greater than 75 [20]. Further, nanoencapsulation of the inactivated virus administered by bath vaccination provided better protection with 85% RPS [20].

### 3.4 Flush Vaccination

This method is similar to bath vaccination, except that the water level is not reduced. This method can be followed for all sizes of fish [21]. This method is the least stressful, as fish is not handled and water level is not altered. However, this method is less practised due to the requirement of the large volume of the vaccine.

### 3.5 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 method is usually adopted when fishes are shifted within the rearing facility. Spray vaccination is a fast and efficacious method for administering vibrio bacterin [22]. However, this method is stressful since the fishes have to be netted and carried on a conveyer belt for vaccination [23].

### 3.6 Ultrasound

This is a technique by which fish are immunised by immersing in water containing vaccine and subjected to high-frequency sound waves of about 20 kHz. This enhances the permeability of the cells and uptake of antigen. *Vibrio alginolyticus* bacterin when administered to grouper by ultrasound treatment was found to be equally efficient in protecting fish when challenged, compared with intraperitoneal vaccination [24]. Similar results were obtained when seabream were vaccinated against *V. anguillarum* and *V. alginolyticus* by ultrasound method [25]. This technique requires less concentration of antigen and is said to give protection comparable to injection vaccination [26]. This method is ideal for administering DNA vaccines [27].

## 4 Oral Vaccination

In oral vaccination, the vaccine is usually administered through feed. The vaccine is either pre-mixed with feed while preparing the feed or the feed is coated with the vaccine usually before feeding. The vaccine can also be administered orally by intubation, a technique by which the vaccine dose is directly administered in the pharynx. However, this method 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. Oral administration of vaccine has the advantage that it can be administered to large number of fish simultaneously without handling the fish. This method can be used for all sizes of fish [3]. The disadvantage of oral vaccination is that the quantity of feed consumed by each fish cannot be determined and, hence, the vaccine uptake varies from fish to fish. The antigen may be altered before it reaches the hindgut where it can stimulate the GALT. Novel methods have been devised to administer the vaccine so as to reach the hindgut intact. Some of the methods are as follows:

### 4.1 Biofilm Vaccines

Bacterial biofilms can be formed as well-structured multicellular communities on various inert and biological surfaces by providing a suitable environment [28]. They are encased in glycocalyx matrix which are resistant to several chemical agents like antibiotics, surfactants, antibodies and several enzymes [29]. The property of resistance to several chemical agents and enzymes can be exploited to safeguard the antigen from the digestive enzymes of fish during oral vaccination. Artificially induced biofilms have been evaluated as efficient oral vaccine candidates against several aquatic pathogens to induce mucosal immunity [30–36]. It is also reported that the biofilms thus formed can express several additional proteins, which are antigenic in nature and can induce immunity, when administered orally [37].

### 4.2 Microalgal Delivery of Vaccine

Microalgae are microscopic organisms found primarily in aquatic resources but also found terrestrially. Microalgae are used for the production of biodiesel, biofertilizer, cosmetics, pigments, as feed for animals, and production of pharmaceutical products such as antibacterial, antiviral and anticancer drugs [38]. Microalgae are used as a food source for aquatic animals, especially for the larval stages of fish, shrimp and molluscs. Microalgae is also used for culturing zooplankton like Artemia, rotifer and copepods, which in turn are used as feed for finfish and shellfish larval stages [39]. The potential of microalgae to produce inexpensive recombinant vaccines which can be delivered orally has been reported recently [40]. Bañuelos-Hernández et al. [41] produced an inducible geminiviral vector called Algevir for high-level expression of antigenic proteins in microalgae. Kwon et al. [42] reported the

application of microalgae for the oral delivery of fish vaccines where they expressed recombinant green fluorescent protein (GFP) in *Chlamydomonas reinhardtii* which when administered orally to *Danio rerio* resulted in the uptake of GFP in the intestine. However, no microalgae-based fish vaccines have been reported so far.

### 4.3 Artemia

Brine shrimp (*Artemia* sp.) feed on bacteria and, hence, Artemia immersed in bacterial vaccine solution encapsulates bacteria and can be used to feed larval stages of fish [3]. Vaccinating fish larval stages using Artemia-encapsulated antigen has been reported by many workers [43, 44].

### 4.4 Antigens Expressed in Plants

Large-scale inexpensive vaccines can be produced by adopting genetic engineering, where the antigens are expressed in plants and are used to administer the antigen orally. Several prototype vaccines are available against various antigens engineered using plants and plant products. However, plant-based vaccine research is lagging behind in fish vaccinology. A recent study reported the production and evaluation of protection offered by major capsid protein (MCP) of rock bream iridovirus (RBIV) expressed in rice callus [45]. The rice callus expressing rMCP, lyophilized and incorporated in fish feed, displayed high antibody (IgM) titre and offered significant protection against viral challenge. The advantages of this system include cost-effectiveness, easy scale-up, natural encapsulation of antigens, safety, independent of cold chain and the plant source expressing desired antigen can be used as feed ingredients.

## 5 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, 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. Significant upregulation of IgT transcripts were observed in the gut mucosal tissue and lymphocytes when rainbow trout were vaccinated by anal intubation with inactivated *Flavobacterium psychrophilum* [1]. However, this method has the disadvantage that the fish needs to be handled and anal administration may cause injury if sufficient care is not taken.

## 6 Intranasal Vaccination

Like other mucosal sites, the olfactory organ also has diffused lymphoid cells called nasopharynx-associated lymphoid tissue. Similar to gut and skin, $IgT^+$ cells make up 51.5% of the B lymphocytes while the rest are $IgM^+$ cells. The ratio of IgT/IgM in nasal mucus is 20 times that of serum [46]. The intranasal route can also be used for simultaneous vaccination against many pathogens. Rainbow trout vaccinated intranasally through the nares with live-attenuated viral vaccine against infectious haematopoietic necrosis virus (IHNV) and formalin-killed *Yersinia ruckeri* bacterin separately elicited mucosal and systemic immune response and had protection against respective virulent pathogens equivalent to injection vaccination [47]. Fish can be effectively vaccinated by intranasal route as early as 360° days or 24 days post-hatch [47]. The advantages and disadvantages of different methods of vaccine delivery are presented in Table 1.

**Table 1** Advantages and disadvantages of different routes of fish vaccination

| Vaccination route | Advantages | Disadvantages |
|---|---|---|
| Injection | • Can administer exact quantity of vaccine | • Can be used only for fish above 20 g size |
| | • High systemic immune response | • Causes handling stress |
| | • Economical for large fish | • Results in adhesions |
| | • Use of adjuvants increases the vaccine efficacy | • Not economical for low-value fish |
| | • Protection is for longer period | |
| | • Multivalent vaccines can be administered in single shot | |
| Immersion | • Less handling stress | • Requires large quantity of vaccine |
| | • Can be used to vaccinate fishes of all sizes | • Immune response is less than that of injection vaccination |
| | • Can be used for mass vaccination | |
| | • Can be used to administer multivalent vaccines | |
| Oral | • No handling stress | • Exact dose administered cannot be controlled |
| | • Can be used to vaccinate all size of fishes | • Immune response is less than that of injection vaccination |
| | • Can be used for mass vaccination | • May not be applicable to non-feeding stages of fish |
| Anal intubation | • Can administer exact quantity of vaccine | • Cannot be used for larval stages |
| | • Results in good mucosal immunity | • Causes handling stress |
| | | • May result in mechanical injury |
| Intranasal | • Can administer exact quantity of vaccine | • Cannot be used for larval stages |
| | • Results in good mucosal immunity | • Causes handling stress |
| | | • May result in mechanical injury |

## 7 Nanoparticle-Based Fish Vaccine Delivery

Vaccine delivery systems employing nanoparticles provide good stability to antigens and are very efficient in targeted delivery, while acting as efficient adjuvants. Several nanoparticles are employed in fish vaccine development in all forms of vaccines that are administered orally, intra-peritoneally, intra-muscularly and by immersion methods [48]. Nanoparticles used in vaccine research can be categorised into adjuvants or delivery systems. Those materials which can induce certain immune pathways can be grouped as immune potentiating adjuvants, while those which deliver the antigen to target sites can be grouped as delivery systems [49–51]. Further, these can be grouped as biodegradable or non-biodegradable nanoparticles according to their ability to degrade in the biological systems. Several nanoparticles have been evaluated for their mode of action or efficiency to induce immunity in fish along with a specific antigen. Biopolymers, liposomes, carbon, calcium phosphate, ISCOMs are the most researched materials to develop fish vaccines either for delivery or as adjuvant system. Several advanced materials are available and need to be explored for fish nanovaccines, considering the toxicity of the material, as nanoparticles can cross blood-brain barrier [48]. The status of nanoparticle-based fish vaccine delivery is reviewed by Vinay et al. [48]. Table 2 provides the details of the types of nano-delivery systems in vaccine research with their merits and demerits.

**Table 2** Merits and demerits of nano-delivery systems

| Nanomaterial | Advantages | Disadvantages |
|---|---|---|
| Biopolymers | • Biodegradable and are efficient in targeted antigen delivery | • Premature release of antigens |
| | • Easy to modify and design | • Limited antigen loading |
| | | • Poor encapsulation |
| Inorganic materials | • No premature release of antigens | • Low biodegradability |
| | • Easy to modify and design | |
| | • Very good encapsulation | |
| Lipid materials | • Very good adjuvant property | • Limited antigen load |
| | • Modified liposomes have good gastrointestinal stability | • Poor gastrointestinal stability in native form |
| ISCOMS | • Very good adjuvant property | • No controlled release of antigens as this will not form depot |
| | • Very good encapsulation | |
| Virus like particles | • Good gastrointestinal stability | • Low reproducibility |
| | • Can be modified to mimic natural virus | |
| Emulsions | • Very good adjuvant property | • Poor gastrointestinal stability |
| | • Very good encapsulation | • Premature release of antigens |

## 8 Summary

Injection route is the most commonly used method of vaccine delivery and it gives the best systemic immune response. However, injection route is laborious and stressful to the fish and cannot be practised for smaller fish. Other methods of vaccination like immersion and oral vaccination have the specific advantage that it can be administered with minimal or no handling of fish. However, the level of serum antibodies attained by these routes does not match with the injection route. It is probable that immersion and oral vaccination induce more of mucosal immune response rather than systemic response. Immune response at mucosal surfaces is very important in protecting the fish against pathogenic invasion as these are the sites of natural entry of pathogens. More research on the role of mucosal antibodies such as IgT in protecting the fish against a natural infection and methods of eliciting the mucosal immune response has to be carried out. Nanoparticle-based delivery of vaccines helps in the increased uptake of antigens resulting in better immune response and, hence, needs to be explored further for application in the field of fish vaccinology.

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# Adjuvants for Fish Vaccines

R. P. Raman and Saurav Kumar

**Abstract**

The accelerated incidence of infectious diseases in the aquaculture system is usually associated with high mortality and morbidity rates with increased economic loss to the aqua farmers. This has led to the extensive application of a wide range of chemicals, antibiotics and drugs to mitigate these diseases in aquaculture system. However, unregulated use of these chemicals has become a serious issue to both the host and human health. In this prelude, suitable prophylactic measures like vaccination have become an important strategy in controlling diseases for sustainable aquaculture. Vaccines are of several types and administered in different ways; each has its pros and cons based on their action, persistence and efficacy considering the medium in which host thrives. Therefore, the combination of adjuvants with a potential antigen for vaccination will have several beneficial effects, including prolonging antigen release, aiding in activating and releasing co-stimulatory signals, enhancing local inflammation, and activating lymphocytes proliferation. The most widely used adjuvant is Freund's complete adjuvant (FCA) and others like Freund's incomplete adjuvant, oil emulsions, montanides, minerals, nano/microparticles adjuvants, immune-stimulating complexes lipopeptides and saponin, etc., which have also explored fish vaccination. However, further research is required for the advancement of these adjuvants for use in fish vaccination. This chapter deals with adjuvant types, their mechanism of action, and their role in heightening the immune responses of the vaccinated fish in the aquaculture system.

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**Keywords**

Fish diseases · Vaccines · Adjuvants · Mode of action

## 1 Introduction

Outbreaks of diseases have emerged as the major limiting factor in today's aquaculture, inflicting significant economic losses to aqua-farmers worldwide. Though there are several chemotherapeutants which are used to treat diseases, it is advisable to adopt scientific prophylactic measures to improve the immunity of fish and shrimp through vaccines, immunostimulants and probiotics for sustainable aquaculture. In aquaculture, a vaccine is a suitable prophylactic measure to control the disease based on economic, environmental and ethical considerations. Different types of vaccines using live, attenuated, inactivated or killed, replicating or non-replicating pathogens and their subunits are being considered. Live vaccines are always associated with high risk and, hence, in many countries it is not approved for use in aquaculture. With the advancement in vaccine research, superior new generation vaccines have been developed. However, there are still several areas that need to be optimised such as mass-vaccination through easy routes of immunisation and use of adjuvants for better vaccine efficacy. Therefore, a custom-made adjuvant system along with suitable and potent antigen is desirable to create an effective vaccine. Thus, the role of adjuvants is exceedingly valuable for the activation and promotion of immune responses that will significantly enhance the relative survivability of fish. Against this background, this chapter reviews the various aspects of adjuvants that are required to heighten the immune responses of fish.

## 2 Adjuvants

The word 'adjuvant' originated from Latin word *adjuvare*, meaning to help. Adjuvants are a diverse range of molecules or substances when combined with a potential antigen and administered to the host, to increase the immunogenicity of the antigen. Application of adjuvants helps in lowering the dosage of vaccine given, improving absorption and slowing-down the antigen processing, applying vaccine at low temperature and reducing side effects of antigenic preparations. The use of adjuvants is quite common in human and veterinary vaccines and is becoming increasingly important in aquaculture.

Adjuvants exert their action by the following ways:

- Prolonging antigen release.
- Activating and releasing of co-stimulatory signals.
- Enhancing local inflammation.
- Activating lymphocytes proliferation.

## 2.1 Principle of Adjuvants

Adjuvants help in enhancing the intensity of functional performance of adaptive arm of the immune system of a vaccinated animal. This results in the increase in relative per cent survival in vaccinated animals. Structurally, adjuvants are heterogeneous entities which are able to modulate the essential immunogenicity and enhance the potency of an antigen [1]. Primarily, based on their chemical nature or physical properties, they can be categorised into many classes. The novel classifications of adjuvants are based on the signalling pathway by which the immunological events are persuaded. At present, the signal facilitators and two-signal model have been widely accepted and have become the basis of classification of adjuvants [2] (Fig. 1 and Table 1).

## 2.2 Classification of Adjuvants

The adjuvants via signal 1 aids in better presentation of antigen in terms of concentration, site and time; however, the alternative aim of adjuvants is to activate the specific lymphocytes (both T and B) which can be achieved through signal 2. Signal 1 and signal 2 modulate the antigen for improving its persistence to the immune system, and antigen recognition via co-stimulatory signals, respectively. This will provide an adequate setting for the most effective antigen-specific immune response.

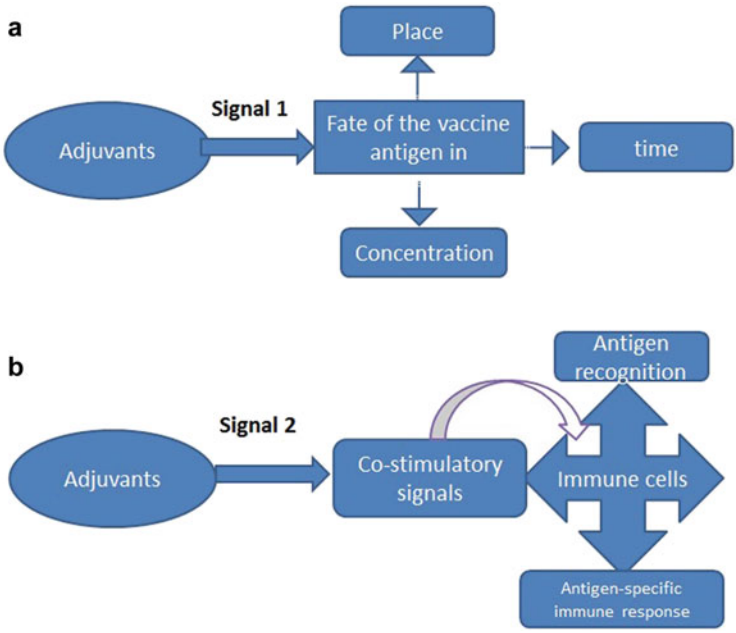


**Fig. 1** (**a**, **b**) Illustration of two signal pathway of adjuvant function

**Table 1** Examples of adjuvants based on their signal activation

| Adjuvant type | Example |
|---|---|
| Signal 1 | Oil emulsions<br>Freund's complete adjuvant (FCA)<br>Freund's incomplete adjuvant (FIA)<br>Montanide<br>Mineral oil<br>Nano/microparticles<br>PLGA particles<br>Immunostimulating complexes (ISCOMs) |
| Signal 2 | Aluminium containing adjuvants<br>ß-glucans-ligands for dectin-1<br>Cytokines<br>Lipopeptides<br>Saponins<br>Toll-like agonist 3: Poly I:C; 5: Flagellin and 9: CpG |

Adjuvants induce pathogen recognition receptors (PRRs) of the innate immune system of fish that are capable of identifying the pathogen-associated molecular patterns (PAMPs) present on different microbes. Recent research on adjuvants has been conducted to explore the important role of different PRR ligands including different PAMPs, and other endogenous TLR ligands including heat shock proteins (hsp) and their ability to persuade targeted Th responses. This recognition is considered significant for induction and downstream activation of distinctive T helper cell subsets. In brief, a combination of vaccine with different adjuvants aids differentiation of Th cells into several T cell lineages, i.e. effector, memory and regulatory Th cells [3, 4].

## 2.3 Mode of Action

Adjuvants can act as immunostimulators and immunopotentiators. They can either be administered in combination with or before or after the immunisation, while some of them can also be given alone. The classical examples of adjuvants administered with antigen are Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), Muramyl dipeptide (MDP), aluminium salts, etc., while glucan is given after and levamisole, BCG, dextran sulphate, etc. are given before to restore the depressed immunity. Some adjuvants may modify the antigen by conformational changes or by altering the net electrical charges of the antigen molecules. These include oily adjuvants or alkyl bases with large hydrophobic moiety. Immunogenic soluble antigens can be made particulate on aggregation and cellular adherence, whereas some are made for adsorption on to the alum and other substances such as micro-silica and polymers, and these become associated with antigen-presenting cells to facilitate the antigen presentation. In some cases, where antigen of low molecular weight (hapten) is not able to elicit an immune response, its conjugation with a carrier, either heavy molecular weight protein or micro-silica carrier or polymer, may

**Table 2** Some of the common adjuvants used in fish and their action

| Enhanced functions | Adjuvants/immunostimulants |
|---|---|
| Phagocytosis | Chitin, chitosan, glucan, levamisole, FK-565, MDP, LPS, FCA, *C. butyricum*, PS-K, PG, lentinan, Vit C, lactoferrin |
| Complement level | Glucan, schizophyllan, Vit C |
| Antibody production | FIA, aluminium salts, FK-565, LPS, glucan |
| Lysozyme production | Glucan, schizophyllan, levamisole |
| Leucocyte migration | Quil A, saponin, glucan |
| Interleukin-1 production | LPS |

Source: Swain et al. [5]

enhance its immunity. Other crucial actions at the level of the host are depot formation for holding the adjuvants for a longer period in order to release slowly; augment the trapping of recirculating lymphocytes at the administration site and lead to more effector cells of the immune system to be exposed to the antigen. Some adjuvants like lipopolysaccharides (LPS), mycobacterial products, vitamin A, silica and saponin may modify the cell membrane of immune cells and facilitate the cell-to-cell contact, thereby eliciting a better immune response. Certain antigens are degraded by the digestive enzymes of fish through oral vaccination. Adjuvants like poly (lactic-co-glycolic acid) (PLGA), Quil A, saponin, cholera toxin beta unit, etc. form a protective coat of the antigen. List of some of the commonly used adjuvants and their actions are given in Table 2.

## 2.4 Signal 1 Type Adjuvants

### 2.4.1 Oil Emulsions

It is always desirable to administer an antigen of higher immunogenicity with release at a slow rate and this can be achieved by using suitable emulsion when the antigen is injected into the host. An emulsion is a complex form where the dispersed phase (liquid) is mixed with the continuous phase (a second liquid) in which the former one is not miscible. The commonest form used in vaccine formulations are water (antigenic media) and oil. In view of side effects of oil-based adjuvants like adhesions and growth reduction in the immunised fish, a recent study using fish (turbot) administered with a *Vibrio anguillarum* vaccine along with oil-based adjuvants showed improved RPS over 90% and the specific immune response (antibody) of water-in-oil (W/O) emulsion inoculated group was higher in contrast to the water-based (WB) vaccine administered group. The findings reveal that Marcol 52, in comparison to other W/O adjuvants (FIA, ISA 763, and ISA 780), is a very much endured oil-based adjuvant for turbot, and could potentially be used as adjuvant for other fish as well [6].

### 2.4.2 Freund's Complete Adjuvant (FCA)

The most widely used adjuvant for experimental trial is Freund's complete adjuvant (FCA) containing inactivated bacterium (Mycobacteria) with mineral oil surfactant. In this method, before injection, a stable W/O emulsion is formed when the antigen in an aqueous solution is mixed with the FCA. A substantial Th1 and Th17 responses via the MyD88 pathway was demonstrated upon vaccination with FCA and antigens. The performance of FCA was tested with 43 kDa outer membrane protein of motile *Aeromonas hydrophila* in blue gourami (*Trichogaster trichopterus*) [7]. Further, the efficacy of FCA was evaluated in coho salmon (*Oncorhynchus kisutch*) and, in this, formalin-killed salmonicida in the absence or presence of FCA was i.p. injected to fish and a higher protection for the FCA-adjuvanted vaccine group [8] was found. In another study, the natural adjuvants induced immunostimulation in fish; however, Zheng et al. [9] found better response with FCA in comparison to astragalus polysaccharide and propolis.

### 2.4.3 Freund's Incomplete Adjuvant (FIA)

The main limitation for the use of FCA is severe inflammatory response in immunised host and, hence, it has been widely replaced by Freund's incomplete adjuvant (FIA) which shows relatively low toxicity. However, FIA elicits relatively lesser immunity and the effects by FIA in Atlantic cod is well documented by Gjessing et al. [10]

The efficacy of FIA with a vaccine against *Edwardsiella tarda* in fish has been determined [11]. Moreover, the expression of vital genes like complement component 3 (C3), tumour necrosis factor-α (TNF-α), interferon γ (IFN-γ), interleukin 6 (IL-6), CD8α, CD40, MHC class I and MHC class II, and Mx in the host was evident upon vaccination with the oil-adjuvanted antigen. However, there was no increase in RPS in fish immunised with multiple doses of formalin-inactivated whole-cell antigen from a virulent strain of *Nocardia seriolae* with FIA [12]. In case of *Aphanomyces invadans*, the vaccinated (fungal extract combined with FIA) group of catla had higher RPS and immune response compared to the control [13].

### 2.4.4 Montanide

Montanide, a mineral oil adjuvant is based on singly or combined mixture of mineral oil and non-mineral oil. Generally, Montanide is a trademark under which various emulsions with specific surfactant chemistry (mannitol oleate) is used [14, 15]. The performance of various grades of Montanide, viz., ISA763A (W/O, non-mineral oil) and ISA711 with vaccine, has been evaluated against a scuticociliate (*Philasterides dicentrarchi*) and *Pseudomonas plecoglossicida,* respectively and found a significant increase in RPS of vaccinated fish in comparison to control [16]. In an investigation, a recombinant S-layer protein of *A. hydrophila* blended with Montanide at a proportion of 30:70 protected *Cyprinus carpio* to a range of 40–75% against six isolates of *A. hydrophila* [17].

### 2.4.5 Other Mineral Oil Adjuvants

Experiments using other mineral oil adjuvants were also reported and showed promising results (Table 3).

### 2.4.6 Nano/Microparticles as Adjuvants

Microparticles can be used as an alternative to oil emulsions and, recently, various researchers have documented the beneficial use of these particles as carriers for vaccine delivery [24]. The interaction between microparticles and antigens can be physical or chemical bond (covalent). Among these two ways of association, the covalent coupling has merit over physical association as it requires a lesser amount of antigen, more efficiently antigen processing and presentation by APCs, and higher storage stability and retention of excess materials. Most importantly, the use of microparticles offers an optimal humoral response even with a very low dose of antigen. Recently, a DNA vaccine (pGPD + IFN) with chitosan nanoparticles (CNPs) for edwardsiellosis was administered through oral, immersion and intramuscular injection (I/M) routes to rohu, *Labeo rohita*. Subsequently, these experimental fish were challenged with $LD_{50}$ ($8.7 \times 10^4$ CFU/fish) of pathogenic *E. tarda* on the 35th day after primary immunisation. The study revealed highest RPS in *L. rohita* through oral mode of vaccination. Besides, non-specific cellular and humoral immune response, viz., NBT, myeloperoxidase activity, serum lysozyme activity and different immune genes (IgHC, iNOS, TLR22, NOD1 and IL-1β) along with Igs levels were elevated. In this way, it was presumed that oral and, to some degree, immersion delivery of nanoparticle-conjugated DNA vaccine can be effective against *E. tarda* in *L. rohita* [25].

**Table 3** Details of mineral oil adjuvants used in various experiments

| Mineral oil adjuvants | Vaccine | Host fish | Response | Reference |
|---|---|---|---|---|
| Oil-adjuvanted (Alpha Ject 5200) | Formalin-inactivated *Moritella viscosa* (poly-valent) | Atlantic salmon | No significant increase in RPS | [18] |
| Commercial oil-adjuvant | Formalin-inactivated *M. viscosa* | Atlantic salmon | Increase protection against vibriosis, coldwater vibriosis and furunculosis | [19] |
| Commercial oil-adjuvant | *V. anguillarum* and *Photobacterium damsela* subsp. *piscicida* | *Dicentrarchus labrax* | Side effects and retardation in growth | [20] |
| Oil-adjuvant | *Aeromonas salmonicida* and *M. viscosa* | Atlantic salmon | Intraperitoneal lesions (typical of granulomas containing large macrophages) | [21] |
| Mineral oil-adjuvant | *V. anguillarum* and atypical *A. salmonicida* | Codfish | Increased protection | [22, 23] |

### 2.4.7 PLGA Particles

There are a few investigations available on the uses of biocompatible and biodegradable polymers as adjuvant in mammals. The foremost mechanism of delivering an antigen from the microspheres is either by dissemination through framework pores or by network degradation. Additionally, the rate of biodegradation of these microspheres can be modulated by changes in polymer organisation and atomic loads. In fish, as a model organism, only limited studies have been carried out on the scope of PLGA as a potential adjuvant and the immune response acquired. In *L. rohita*, significantly higher titres of antibody were found in the PLGA-encapsulated OMP of *A. hydrophila* mixed with FIA at 21 and 42 days post-immunisation [26]. In an experimental trial (for 102 days), oral vaccination against lactococcosis in rainbow trout showed a significantly higher RPS, 63%, despite the fact that booster dose with the same mode of the PLGA offered an RPS of over 60% [27].

### 2.4.8 Immune-Stimulating Complexes (ISCOMs)

Immune-stimulating complexes (ISCOMs) represent a novel approach towards amphipathic antigens to stimulate specific and non-specific immune response [28]. These are non-covalently-bound complex having size ~40 nm diameter of saponin adjuvant Quil-A, cholesterol and amphipathic antigen in a molar proportion of roughly 1:1:1. ISCOMs are used extensively in veterinary vaccines and can be found in various commercialised animal vaccines.

## 2.5 TLR Ligands as Adjuvants

Toll-like receptor (TLR) ligands (agonists) are capable of eliciting strong cell-mediated acquired responses. Teleosts may have approximately two-fold diverse TLRs compared to mammals. Further, intracellular and downstream signalling events of the mammalian and fish TLR pathways show resemblances, though there are distinctions that need to be revealed. In general, a sequence of events could be triggered when TLRs (3, 4, 5, 7, 8, 9 and 11) bind to a ligand that induces the production of IL-12 which favour a Th1 response and also, under certain conditions, the initiation of these TLRs may encourage cross-introduction of antigens to cytotoxic T cell reactions. Specifically, interaction of ligands to TLRs 3, 4, 7 and 9 may trigger type I interferon reactions [29].

### 2.5.1 Aluminium Containing Adjuvants

Aluminium phosphate and aluminium hydroxide are well recognised as potent adjuvants in human vaccines [30]. The merit of using aluminium adjuvants for vaccines is that it is effective at promoting protective humoral immunity via inducing Th2 responses. However, for an immunocompromised host where cell-mediated immunity is required, alum has limited efficacy as an adjuvant. The investigations have uncovered that alum initiates NLRP3 inflammasome and incites necrotic cell deaths that discharge the danger signal in the form of uric acid [31]. Only a few

studies have been reported in fish vaccination trials in which aluminium has been used as an adjuvant. In Atlantic salmon, immunisation against *A. salmonicida* combined with potassium aluminium sulfate was tried, yet not found to have any considerable advantage [32]. However, an aluminium hydroxide-adjuvanted vaccine against *E. tarda* showed improved protection in Japanese flounder [11].

### 2.5.2 ß-Glucans

ß-glucans are one of the most studied molecules that stimulate non-specific immune response both in mammals as well as fishes [33, 34]. The dose-dependent immunostimulatory and adjuvant efficacy of glucan have been investigated through intraperitoneal injection and are found to be effective for short-duration in providing protection against infections. DeBaulney et al. [35] have reported positive effects of combination of the vaccine and ß-glucan with a higher RPS (61%), compared to vaccine alone (RPS 52%). The efficacy of ß-glucan was assessed in fish against *Vibrio damsela* where a mixture of O-antigen of *V. damsela* and ß-glucan was injected i.p. to fish [36]. The enhanced phagocytic index and Igs level were noticed in immunised fish injected with the combination, in contrast to fish injected with ß-glucan or antigen alone [36, 37]. Interestingly, it was found that inactivated (formalin-killed) vaccine of *A. salmonicida* and *Vibrio salmonicida* accompanied by adjuvant ß-glucan resulted in higher protection against furunculosis, in contrast to the group injected without adjuvant [34]. In an experiment, a significant increase in survival rate was reported in coho salmon immunised with bacterin (formalin-treated *A. salmonicida*) with adjuvant ß-glucans like Vitastim-Taito and lentinan in comparison to a group without adjuvant. Besides, a significant rise in agglutinin levels was noticed in a group injected with bacterin alone, while no rise in agglutinin titre was noticed in group which was administered with bacterin and adjuvant [38]. A higher relative per cent survival was found in fish when immunised with a formalin-inactivated *A. hydrophila* combined with ß-glucan in comparison to bacterin alone [39]. The mode of vaccination also influences the performance of vaccine, and it is noticed that instead of bath and oral mode, i.p. immunisation of fish with *A. hydrophila* vaccine along with ß-glucan resulted in higher antibody response [40].

### 2.5.3 Saponins

Saponins are natural bioactive compounds of glycosides of steroid or triterpene. In the mammalian system, these compounds have been widely explored as adjuvants due to their ability to stimulate cell-mediated immunity and trigger both Th1 and Th2 responses. Quil-A (a component of ISCOMs) and their derivatives are most widely used saponins. However, their high cytotoxicity and instability in an aqueous phase make their application in aquaculture challenging. It has been reported that inactivated *E. tarda* (formalin-killed cells) along with curdlan or curdlan with Quil-A saponin, when fed to Japanese flounder, showed a significant increase in protection [39].

### 2.5.4 Polyinosinic Polycytidylic Acid (Poly I:C)

Poly I:C is a double-stranded polyribonucleotide which can mimic viral infection in a biological system. Poly I:C has been extensively used to trigger type I interferon (IFN, cytokines) in an animal model including fish. In general, Poly I:C acts as ligand and binds to TLR3 receptor which induces non-specific antiviral state and the subsequent activation of downstream intracellular signalling events. In a study when fish were administered with Poly I:C before challenging with IHNV, resulted in 100% RPS, besides having significant level of IHNV-specific antibodies in experimental fish [41]. The findings revealed that immunised fish were at an antiviral state due to Poly I:C administration prior to IHNV challenge and the Poly I:C stimulated subsequent specific antibody production. Similarly, *Epinephelus septemfasciatus* showed 90% RPS when immunised with 50 mg Poly I:C $fish^{-1}$ intramuscularly (i.m.) against red-spotted grouper nervous necrosis virus (RGNNV) [42]. Subsequently, 21 days after the primary challenge, the survived fish, (in which a high concentration of specific antibodies was detected), when re-challenged with RGNNV resulted in 100% protection against the virus. Further, the same trials when conducted at field level showed that Poly I:C-adjuvanted vaccine resulted in high RPS (93.7%) in comparison to the experimental control [43].

### 2.5.5 Lipopeptides

Lipopeptides are found to have a potent capacity to elicit both innate and adaptive immune response in mammals. Mycobacteria and mycoplasms are among those microorganisms that are potential sources of lipoproteins and lipopeptides. However, documented literature available on lipopeptides in fish as adjuvants are meagre. In the experimental vaccine development against *A. salmonicida,* efficacy of polar glycopeptidolipids (pGPL-Mc, extracted from *Mycobacterium chelonae*) as an adjuvant was successfully investigated [44]. After 12 weeks of vaccination period, the antibody response of vaccinated fish (bacterin +adjuvant) was reported to be greater than that elicited by a non-adjuvanted bacterin. However, a decreased effector function of these antibodies with side effects to the host was noticed with an increased dose of pGPL-Mc [45].

### 2.5.6 Flagellin—Toll-Like Receptor 5 Agonist

Flagellin is capable to bind TLR5 and induces downstream cascade signalling system resulting in effective T-cells responses. In an experiment involving infection of piscirickettsiosis, a high level of survival was noticed when the fish were vaccinated with the subunit of the flagellum and chaperonins Hsp60 and Hsp70 of *Piscirickettsia salmonis*. Further, it was suggested that better protection in fish could be achieved when more than one recombinant protein antigens are used against this disease [46]. Wangkahart et al. [47] have reported that injection of recombinant flagellin from *Yersinia ruckeri* (YRF) resulted in the activation of inflammatory regulatory molecules. Further, a systemic inflammatory response with important pro-inflammatory cytokines, viz., IL-1β, TNFα, IL-6, and IL-11, etc. along with the secretion of chemokines (CXCL_F4 and CXCL-8) was observed in immunised fish.

### 2.5.7 CpG—Toll-Like Receptor 9 Agonist

Bacterial DNA and particularly manufactured oligodeoxynucleotides (ODNs) conveying unmethylated CpG motifs prompt an immunostimulatory cascade. The response is observed as maturation, differentiation and proliferation of both innate and adaptive effector immune cells, viz., monocytes, macrophages and dendritic cells, B and T lymphocytes, and NK cells. A vaccinated fish group with whole-cell vaccines (*Renibacterium salmoninarum*) with CpG adjuvants showed significant protection against i.p. challenge with *R. salmoninarum*. Under field condition and natural bacterial infection, a mixture of a commercial *R. salmoninarum* vaccine (Renogen) with a CpG adjuvant effectively declined the degree of bacterial antigens in the kidney [48]. In an investigation of furunclosis disease in rainbow trout, the experimental fish were i.m. inoculated with a non-adjuvanted aqueous vaccine (inactivated *A. salmonicida*; Aquavac Furovac 5) alone, or along with three various CpG-ODN, viz., 1982, 2133 and 2143. The vaccinated fish when challenged with virulent *A. salmonicida* had higher percent survival in the group vaccinated with Furovac and CpG-ODN 2143 [49].

### 2.5.8 Cytokines

Although use of cytokines as adjuvants in the mammalian model is well established, lack of information of fish cytokines and its mode of action restricted the scope of these cytokines as an adjuvant in fish. Interferon regulatory factors (IRF-1) is an important element for cytokine signalling and host defence against various infectious agents [50]. Application of IRF-1 in conjunction with a DNA construct as a vaccine (encoding the most important capsid protein MCP) of Red seabream iridovirus (RSIV) resulted in a rise in host-specific antibodies. However, there was no significant difference in the antibody titre between the fish vaccinated with the DNA vaccine alone and along with IRF-1 [51]. Chemo-attractant function of IL-8 during infection has been elucidated in rainbow trout [52]. Considering this, an experimental trial on the use of cytokine IL-8 as an adjuvant along with a vaccine (plasmid coding for the glycoprotein gene of VHSV) was conducted [53, 54]. Elevated levels of IL-1β in the spleen alongside an increased cellular infiltration at the site of inoculation were noticed in vaccinated group (vaccine + IL-8). Remarkably, fish immunised with pIL-$^{8+}$ alone showed distinct manifestation of vital cytokine, viz., TNF-α, IL-11, TGF-β and IL-18 in the spleen.

## 3 Conclusion

Application of adjuvants is vital for enhancing the potency of a vaccine in aquaculture and investigations have proved their promising future as a prophylactic treatment for farmed fish and shrimp. However, these compounds have certain limitations and, hence, have to be used with certain precautions and regulations along with vaccines. The positive aspect of these molecules is in their ability to improve larval rearing in the nursery by activating the innate immunity before the development of competency of the specific immune system for vaccination. Thus,

the research on screening of various novel molecules and testing their efficacy as adjuvants is gaining importance and its application in aquaculture has shown immense potential. However, a great deal of research on this aspect is important to understand the mode of action of these compounds. A considerable investment in R&D is required to conduct the laboratory screening, testing and to make these products commercially viable.

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# Role of Pattern Recognition Receptors and Interferons in Fish Vaccination

Anutosh Paria, Makesh M., and Rajendran K. V.

**Abstract**

The advances in the field of immunology allow the development of novel strategies in vaccination. In this line, the concept of modern vaccinology is continuously evolving in finding more potent antigens as well as non-living highly immunogenic substances used as adjuvants. These adjuvants can specifically activate targeted immune mechanisms to generate the desired immune response against the pathogen in the host. Among several new-age adjuvants, the substances those stimulate pattern-recognition receptors (PRRs), are becoming the method of choice in vaccine development. Hence, employing PRRs agonist as adjuvant, which can simultaneously elicit the immune signalling of different groups of PRRs, can efficiently tailor the potentiality of vaccines. Interferons are one of the effector molecules of PRR signalling, especially following the detection of viral ligands and can seize the spread of invading microbes. Moreover, interferons are functionally described as potent adjuvant in several vaccination programme. As both PRR agonists and interferons are proven to be an efficient choice for adjuvantation in vaccination trials, the strategy of co-application of these molecules along with target antigen would be an

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immunological intervention to manage the disease of economic importance in aquaculture.

**Keywords**

Pattern-recognition receptors · PRR agonists · Interferons · New-age adjuvants · Vaccination · Fish diseases

## 1 Introduction

Innate immunity is pivotal in mounting the first line of defence in teleosts, which has evolved through multiple evolutionarily conserved germline-encoded pattern-recognition receptors (PRRs) for recognizing a wide-array of microbial invaders. The three major classes of the PRRs primarily include toll-like receptors (TLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide binding oligomerization domain (NOD)-like receptors (NLRs). These receptors sense the conserved molecular structures that are essential for the survival of a pathogen known as pathogen-associated molecular patterns (PAMPs). However, as these molecular signatures are present not only in pathogens but also in all microbes, these patterns are otherwise known as microbe-associated molecular patterns (MAMPs). These molecular patterns represent the invariant molecular constituents that are essential for the survival of the microbes. Among the several PAMPs detected by PRRs, some of the important molecules are bacterial structural components, such as lipopolysaccharide (LPS) and peptidoglycans (PGNs), or viral nucleic acids. Upon recognition of various PAMPs, PRRs activate distinct transcriptional signalling programmes that contribute to the efficient removal of the invading pathogen through several mechanisms such as enhanced phagocytic activity, increased production of inflammatory cytokine, activated complement cascade, production of different types of interferons, secretory antimicrobial peptides and stimulation of the adaptive immune system by enhancing the antigen presentation through increased dendritic cell maturation. PRRs organize themselves in different cellular compartments to detect a wide spectrum of molecules exhibited by different microorganisms. The PAMPs on the cell membrane of endosomes or the cell surface are generally detected by TLRs, while RLRs and NLRs are the sentinels for cytosolic PAMPs [1].

Interferons (IFNs) are a large group of multifunctional cytokines, which are produced by vertebrate cells following viral infection and subsequently restrict the spread of virus before mounting a specific antiviral mechanism by the adaptive immune system. Further, these cytokines possess the unique ability to establish an antiviral state not only in infected cells but also in the adjacent uninfected cells through different intracellular signalling cascades leading to the enhanced expression of several IFN-stimulated genes (ISGs). These genes code for a number of enzymes that either limits or completely inhibit viral replication by interfering with different cellular and viral processes, thus ceasing the spread of virus to the

un-infected cells. Moreover, the IFNs play vital roles in adaptive immune system through regulation and activation of cells associated with it [2]. Hence, these molecules are perhaps one of the greatest discoveries (by Isaacs and Lindenmann) in the field of immunology and biomedicine over the past six decades [3]. The IFNs can be classified into three types, i.e., type I, type II and type III IFNs, based on genomic organization, sequence similarity and biological function [4]. Among these, type I and type III IFNs are the typical antiviral cytokines having an important role in innate immunity; however, type I IFNs are expressed in almost all nucleated cells, while type III IFNs have limited distribution only in tissues prone to viral exposure/infection [5]. The type II IFN, otherwise known as IFN-γ, is basically a regulatory cytokine, generally secreted from natural killer cells and T lymphocytes; responsible in modulating innate and T cell-mediated adaptive immunity against viral and intracellular bacterial infections [6]. All three types of IFNs have been identified in different group of animals such as mammals, birds and amphibians, while only type I and type II IFNs have been reported in fish.

The advances in the field of immunology, especially the focussed research in understanding the link between innate and adaptive immunity, allow the development of novel strategies in vaccination. In this line, the concept of modern vaccinology is continuously evolving in finding more potent antigens as well as non-living highly immunogenic substances used as adjuvants for mounting efficient immune response. These adjuvants can specifically activate targeted immune mechanisms to generate the desired immune response against the pathogen in the host. Among several new-age adjuvants, the substances those stimulate PRRs, which are required for the activation of antigen-presenting cells (APCs), are becoming the method of choice in vaccine development. Almost all the major PRRs can be potentially targeted by administrating their specific ligands as adjuvants during the vaccination process [7]. Further, following the recognition of specific ligands, especially viral ligands, PRRs elicit several signalling cascades which lead to downstream induction of different IFNs, in turn curtailing spread of the pathogen. Moreover, it has been evident that IFNs can be promisingly used as adjuvants, which are administered along with antigen in vaccines. The strategy of co-application of IFN adjuvants may aid efficient cellular immune responses to a vaccine [8].

## 2 Major Functional Groups of PRRs in Vertebrates with Emphasis on Teleosts

Functionally, PRRs can be divided into three classes: soluble (secreted) PRRs, endocytic PRRs and signalling PRRs. The soluble PRRs can opsonize pathogens to trigger their complement-dependent destruction and generally include the innate molecules such as collectins, ficolins and pentraxins. The endocytic PRRs such as mannose receptors, scavenger receptors and C-type lectin receptors are generally expressed on cell surface and regulate the recognition and internalization of microbes and/or microbial PAMPs. The third type, i.e., signalling PRRs, are functionally distinct from the other two types and is an extremely important group in

terms of activation of innate immune system and effective pathogen removal. These PRRs follow certain signalling cascades after detecting the PAMPs and generally present as soluble, membrane-bound or cytosolic form. The signalling PRRs are the signature functional group with pivotal immune regulatory ability at both extracellular and intracellular levels. Toll-like receptors (TLRs) are, by far, the best characterized PRR, but two other families of signalling PRRs also have been described in many teleosts: the RLRs, and NLRs [9].

TLRs are a group of transmembrane proteins localized at the cell surface or within phagosome/endosomes that commonly sense the PAMPs associated with different extracellular pathogens. Recognition of the specific PAMP by TLRs leads to activation of different signalling cascades comprising NF-κB-dependent production of innate immune cytokines such as TNFα, IL-6 or IL-12. These cytokines can further recruit inflammatory cells to the site of infection or inflammation, thus mounting an efficient pathogen removal strategy. The RLR family comprises of three members: retinoic acid-inducible gene I (RIG-1), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). These are otherwise known as DExD/H box RNA helicases and potentially detect the cytoplasmic viral RNA. Although RIG-I and MDA5 recognize different kinds of viruses, they follow the same signalling pathway which activates different regulatory genes such as NF-κB, IRF3, and IRF7, thus leading to the production of proinflammatory cytokines and type I IFNs. NLRs are a newly identified cytoplasmic PRR family believed to play a major role in bacterial pathogen detection and removal. The biological function of NLRs is generally exerted through two mechanisms: (1) the activation of NF-κB and MAPK signalling pathway; and (2) the activation of caspase-1, which leads to the secretion of pro-inflammatory cytokine, IL-1β and programmed cell death, thus constituting the basis for inflammasome [10, 11].

## 2.1 TLRs

The TLR was first identified in the fruit fly (*Drosophila melanogaster*) as a molecule required for fungal resistance by Jules Hoffmann and colleagues. Subsequently, first vertebrate homologue of Toll was identified in human (hToll) as an innate immune gene in inducing the production of pro-inflammatory cytokines and other immune regulatory molecules. Since then, different TLRs have been identified in various vertebrate species, and these have been classified into six major TLR families: TLR1, TLR3, TLR4, TLR5, TLR7 and TLR11. It has also been suggested that most of the vertebrate genomes comprise at least one gene belonging to a particular major family of TLR [12].

TLRs, one of the major innate immune sentinels in animals have been identified in every class of the animal kingdom including finfish groups such as cyprinid, salmonid, perciform, pleuronectiform and gadiform [13]. Teleosts possess at least 20 different TLR genes including the typical mammalian classes of TLRs such as TLR1–4, TLR5M, TLR7–9, TLR13, along with fish-specific non-mammalian TLRs including TLR5S, TLR14, TLR16, TLR18–23, TLR25, TLR26 compared to

10 families (TLR1–10) in human and 12 families (TLR1–13, except TLR10) in mouse [11, 14, 15]. The first mammalian TLR homologue of the interleukin-1/TLR family was identified in rainbow trout [16]. Since then, TLRs belonging to different subfamilies have been identified in various teleost species such as fugu or tiger puffer (*Takifugu rubripes*), zebrafish (*Danio rerio*), Japanese flounder (*Paralichthys olivaceus*), rainbow trout (*Oncorhynchus mykiss*), large yellow croaker (*Pseudosciaena crocea*), Asian seabass (*Lates calcarifer*), channel catfish (*Ictalurus punctatus*), Atlantic salmon (*Salmo salar* L.), grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), rare minnow (*Gobiocypris rarus*), orange-spotted grouper (*Epinephelus coioides*), gilthead seabream (*Sparus aurata* L.), rohu (*Labeo rohita*) and catla (*Catla catla*) [9, 17–19].

## 2.2 NLRs

The NLR family of innate immune receptors are the largest known group of cytosolic pattern recognition receptors involved in detecting intracellular PAMPs, which leads to activation of innate immune responses against the invading microorganism [20]. Initially, it has been described as the subset of disease-resistance (R) genes reported in plants responsible for activation of mitogen-activated protein kinase (MAPK) upon detecting the pathogens and eventually leading to cell death [21]. Many researchers termed these R-gene homologues in animals as CATERPILLER, NOD or NOD-LRR [22, 23] and also further described as cytosolic receptors involved in various immune mechanisms such as autoimmunity, immune responses against bacterial and viral invaders and programmed-cell death [24]. Initially, these R-gene homologues were only demonstrated from higher vertebrates but, later on, these genes have been identified in lower vertebrate like teleosts and invertebrate like sea urchin as well [25–27]. NLRs being such a large family of several genes, a proper classification of these genes reported from different lineages has been an area of concern. However, recently five major groups of NLRs have been determined based on the effector domains located on N-terminal of these genes: NLRA/CIITA, NLRB/NAIP, NLRC, NLRP and NLRX/NLRX1. Among these newly classified groups, NLRC subfamily is the largest and the most important group containing NOD1/NLRC1, NOD2/NLRC2, NOD3/NLRC3 and NOD4/NLRC5, whereas NOD5 belongs to NLRX family [28, 29].

The NLR genes are mostly conserved across the animal kingdom, including those identified from mammals and non-mammalian vertebrates like teleosts. In teleosts, many mammalian homologues of NLRs have been identified; however, along with these homologues they also possess an additional fish-specific unique group of NLRs [26]. Different NLR genes belonging to different subfamilies have been identified and characterised recently in many species of teleosts. Among these, NOD1 and NOD2 are widely studied and have been reported from different teleost species inhabiting both marine and freshwater including zebrafish, goldfish (*Carassius auratus* L.), grass carp (*Ctenopharyngodon idella*), rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*), orange-spotted grouper (*Epinephelus coioides*), Nile

tilapia (*Oreochromis niloticus*), catla (*Catla catla*), channel catfish (*Ictalurus punctatus*), miiuy croaker (*Miichthys miiuy*), Japanese pufferfish (*Takifugu rubripes*) and Asian seabass (*Lates calcarifer*) [30–32]. Other NLRs such as NLR-C, is a very unique subfamily, containing a large number of genes having a conserved NACHT domain but an unusual C-terminal domain with multidimensional functional attributes [26]. However, genes grouped in NLR-C family have been characterised only from a few species of teleosts including Japanese flounder, channel catfish and miiuy croaker. Recently, one of the important NLR-C family members with distinct functional ability to sense both bacterial and viral PAMPs, named as NLRC3 has been reported from four teleosts such as miiuy croaker, Asian seabass, blunt snout bream and Nile tilapia [30, 33].

## 2.3 RLRs

RLRs, otherwise known as DExD/H RNA helicases, are a group of cytoplasmic pattern recognition receptors. It has been described that RLRs are an ancient family of PRRs with origin in invertebrates. RLRs identified in invertebrates such as sea anemone and amphioxus along with vertebrates were found to possess conserved domain and genomic architecture [34, 35]. These helicases play a key role in sensing intracellular viral nucleic acids and mount effective immune response mechanisms against invading infectious pathogens. Three proteins constitute the RLR family: RIG-I, which is also called DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (DDX58); melanoma differentiation-associated gene 5 (MDA5), which is also known as interferon induced with helicase C domain 1 (IFIN1); and the laboratory of genetics and physiology 2 gene (LGP2) or DExH (Asp-Glu-X-His) box polypeptide 58 (DHx58) [35]. RIG-I, the most studied RLR representative, is primarily a dsRNA-binding protein which is responsible for triggering type-I IFN induction following detection of the synthetic dsRNA poly I:C [36]. MDA5 was first demonstrated as an interferon-inducible putative RNA helicase in human melanoma cells by Kang et al. [37] having distinct functions such as melanoma growth-suppression and double-stranded RNA-dependent ATPase activity. Later on, the functional aspects of MDA5 were well elucidated in different animal models and found to have an intracellular signal transduction pathway which finally leads to the activation of the IFN-b promoter and mediates type I IFN responses against nucleic acid PAMPs [38, 39]. LGP2, the third member of RLR family is a cytoplasmic DEx (D/H)-box helicase and initially identified as a highly transcribed gene in mammary tumours with the ability to sense RNA. There exists a certain degree of ambiguity regarding the functional aspect of this gene. Initially, the negative regulatory role of LGP2 on RLR-signalling was well evidenced in many experimental set ups [40]. However, recent studies have given a new dimension to the functional role played by LGP2 and a possible positive antiviral innate immune regulation mechanism in vertebrates has been demonstrated [41–43].

RIG-I, the pivotal member of RLR family is present in some of the teleost genomes but not in all teleost species, and this might be due to the species-specific

exclusion of a particular gene or the gene might be present in a diverged form which is no longer recognizable [35]. However, MDA5 and LGP2 appear to be a conserved component of teleost genome. RIG-I, MDA5 and LGP2 have been identified from many teleost species inhabiting freshwater, brackish water and marine environment [9, 11, 35, 44, 45].

## 3 Fish Interferons

### 3.1 Type I IFNs

The IFN was initially discovered in the year 1957 by Alick Isaacs and Jean Lindenmann. However, the fish IFNs were identified almost five decades later in 2003 in three different fishes including zebrafish, Atlantic salmon and green spotted pufferfish. Since then, several fish IFNs are being identified in different freshwater, brackish water and marine finfishes [3]. Although there was an initial speculation regarding the classification of IFNs identified in many teleosts, based on sequence homology and structural features, it was agreed that the fish IFNs be classified under the type I IFNs of mammals with some differences in the IFN receptor [3, 46, 47]. Among type I IFNs, IFN-α and IFN-β are, by far, the two most characterised and almost all the cell types in the body produce both these IFNs, specifically leukocytes are responsible for secretion of products of the IFN-α multigene family, whereas fibroblasts are primarily responsible for synthesizing the products of IFN-β gene [2]. Both these major type I IFN genes share same receptor positioned on cell surface named as type I IFN receptor. However, due to several differences in genomic architecture, evolution and lineage-specific expansion of type I IFNs in different vertebrates, a linear orthologous relationship between fish type I IFNs and those in mammals cannot be established. The fish type I IFNs cannot be classified as IFN-α, IFN-β or any other group similar to mammalian classification, hence efforts are being made by researchers in the field of fish immunology to establish a new fish-specific classification of type I IFN genes [3]. As there are no clear nomenclature system for fish type I IFNs, different researchers have laid out the criteria to classify them differently. As evident in other vertebrate species, the number of type I IFN genes in different fishes varies such as in zebrafish the number is 4, while in Atlantic salmon it is 11 [48, 49]. Initially, the fish type I IFNs have been classified into two groups based on the cysteine residues: group I containing two cysteine (reported in different fish families) and group II having four cysteine residues (reported only in certain families such as cyprinids, salmonids and perciform fish) [47, 50, 51].

### 3.2 Type II IFNs

Generally, type II IFN represented by a single gene named as IFN-γ is a multifunctional cytokine located in chromosome 12 of human along with two other cytokines, namely, IL-22 and IL-26. This IFN can potentially regulate the helper T-cells for

eliminating the intracellular pathogens [52]. Further, it has also been reported that it can play an efficient role in vaccination process by regulating the macrophages, which in turn promotes the antigen presentation [6]. The IFN-γ in fish follows almost similar signalling pathways as in mammals and performs biological functions comparable to that of mammals. Although, type II IFN family is generally represented by a single copy gene, in fish, in addition to the typical IFN-γ mammalian homologue, there exists, another type of IFN-γ. The second IFN-γ in teleosts was initially named as IFN-γ 2 but, later on, to avoid the complexity it has been renamed as IFN-γ related gene (IFN-γ*rel*) [53]. In fish, the first type II IFN has been identified in Japanese pufferfish [54]. Since then, the IFN-γ genes have been identified in many teleosts such as goldfish, ginbuna crucian carp, grass carp, Atlantic cod, Atlantic salmon, Atlantic halibut, black seabream, channel catfish, common carp, medaka, large yellow croaker, rainbow trout, orange-spotted grouper, Japanese flounder, turbot, Nile tilapia, European seabass and Asian seabass [3].

## 4 PRR-Mediated Type I IFN Response in Fish

In mammals, it has been documented that among the PRRs, TLRs and RLRs can potentially activate the type I IFN response. Following recognition of the corresponding ligands by different PRRs, downstream signalling cascades begin, which result in the activation of several key transcription factors known as IRFs. These IRFs eventually kick in the transcription of different type I IFN genes [55, 56]. This phenomenon of PRR-mediated activation of type I IFNs is vital in restricting viral infections and is mostly conserved [57] (Fig. 1).

### 4.1 TLR-Mediated Type I IFN Response

Among the several TLR genes in mammals, TLR3, TLR7, TLR8 and TLR9 typically detect nucleic acids as their ligands and can initiate a type I IFN response [55]. The TLR3 primarily senses double-stranded RNA (dsRNA) and transmits signal through TRIF, which leads to the activation of IRF3 and results in the production of type I IFNs. However, in contrast, TLR7/TLR8 and TLR9 use single-stranded RNA (ssRNA) and cytosine-phosphate-guanosine (CpG)-containing DNA, respectively as their ligands, and follow a MyD88-mediated pathway to activate IRF7-dependent type I IFN response [55].

As mentioned earlier, the number of TLR repertoire is more in fish than in mammals and they also possess some non-mammalian TLRs (TLR5S, 18–23, and 25, 26), apart from the classical mammalian homologues (TLR1–5, and 7–9) [13]. The type I IFN response in fish is largely mediated by TLR3, TLR19 and TLR22 as evidenced from the recent reports from fugu and grass carp [58, 59]. However, the pattern of activation of type I IFN in fish by these TLRs is different because of their localization in cell and the type of ligands they sense. Fish TLR3 is generally observed in endoplasmic reticulum, TLR19 in endosome, whereas TLR22 could

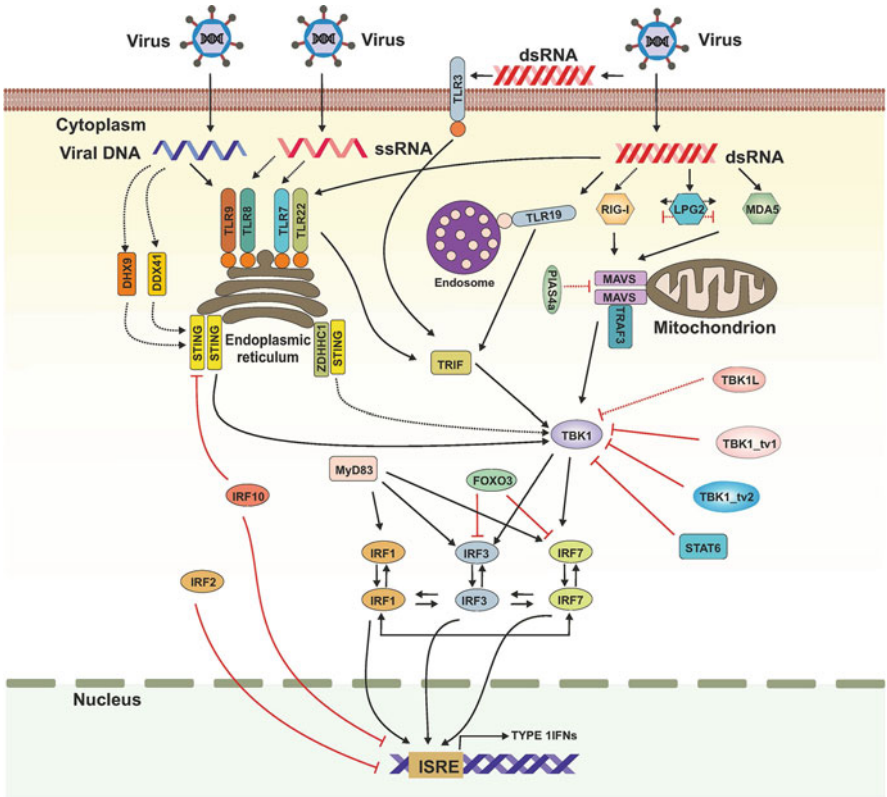


**Fig. 1** PRR-mediated activation and regulation of type I IFN response in fish

only be detected in plasma membrane. The TLR3 in fish preferably senses short dsRNA, compared to the recognition of long dsRNA by TLR22. In mammals, TLR3 is expressed in cytoplasmic compartments as well as on the plasma membrane of fibroblasts; hence, it can be presumed that cell-surface dsRNA recognition of TLR3 in mammals might be functionally substituted by fish-specific TLR, i.e., TLR22 in teleosts. However, the type I IFN induction by these TLRs upon recognition of dsRNA is documented only in a very few teleosts. Moreover, fish TLR9 also can detect CpG-containing DNA as in mammals but it is not clear whether it can elicit similar type I IFN response [60]. The ligand recognition and production of type I IFNs by TLR7 and TLR8 in fish is not well elucidated.

## 4.2 RLR-Mediated Type I IFN Response

All the three members of RLR family are mainly responsible for endosomal viral RNA recognition in animals. The RIG-I mainly recognizes relatively short dsRNA (up to 1 kb) with 5′-triphosphate moiety which results in enhanced interferon activity

[61]. In contrast to RIG-I, MDA5 detects long dsRNA (more than 2 kb) such as poly I:C and RNA molecules with 'non-self'—5′ termini [62]. In contrast to the size-dependent detection of RNA molecules exhibited by RIG-I and MDA5, LGP2 can detect both dsRNA and single-stranded RNA (ssRNA) in a size- and 5′-triphosphate-independent manner [63]. Following detection of viral RNAs, RIG-I and MDA5 can transmit the signal by the interaction of the caspase activation and recruitment domains (CARDs) and the adapter molecule mitochondrial antiviral signalling protein (MAVS) (also known as IPS-1), which leads to recruitment of protein kinase TANK binding kinase 1 (TBK1) and activation of several regulatory genes such as IRF3 and IRF7. The activated IRF3/7 induces the expression of type I IFN genes [56]. However, as LGP2 does not possess any CARD, this signalling mechanism is unlikely, but following ligand detection it can potentially regulate the signalling of RIG-I and MDA5 [42]. The MDA5, LGP2 and other downstream signalling molecules involved in RLR-pathway have been identified in many species of fish, but RIG-I is reported only from a certain group of fishes [35].

RIG-I in fish appears to follow a similar pathway to activate type I IFN genes as in mammals [64]. This mechanism is well demonstrated in EPC cell-line over-expressed with zebrafish RIG-I which can potentially inhibit viral haemorrhagic septicaemia virus (VHSV) or spring viraemia of carp virus (SVCV) infection through activation of type I IFNs and ISGs such as Mx and IRF3/IRF7 [64, 65]. Similarly, CIK cell line over-expressed with grass carp RIG-I or CAB cells transfected with gibel carp RIG-I lead to increased transcription of type I IFNs and their promoters [66, 67]. Even the zebrafish embryos over-expressed with RIG-I could elicit the production of different IFNs and ISGs [68].

Similar to RIG-I, MDA5 in fish can trigger type I IFN-mediated antiviral response. The activation of type I IFN response and ISGs by MDA5 in fish cells was initially demonstrated in rainbow trout and Japanese flounder [69, 70]. The over-expression of orange-spotted grouper MDA5 in GS cells could inhibit red-spotted grouper nervous necrosis virus (RGNNV) and Singapore grouper iridovirus (SGIV) infection through production of IFNs and ISGs [71]. Similarly, over-expression of black carp MDA5 in EPC cell-line is shown to activate type I IFNs, thus inhibiting the SVCV and grass carp reovirus (GCRV) infection [72]. Further, the over-expression of grass carp MDA5 in CIK cells or zebrafish MDA5 in embryos can induce the enhanced transcription of type I IFNs and/or ISGs [67, 68].

As evidenced till now, it appears that fish LGP2 might have a dual role in type I IFN response similar to mammals [42]. The positive roles of LGP2 in inducing the IFNs and ISGs in fish are demonstrated in HINAE cells and RTG2 cells over-expressed with Japanese flounder LGP2 and rainbow trout LGP2, respectively, thus curtailing the infection of VHSV [69, 73]. Likewise, black carp LGP2 in EPC cells is found to inhibit two fish viruses, namely, SVCV and GCRV [74]. However, negative regulatory role of fish LGP2 in type-I IFN response could also be noticed in CAB cells and GS cells transfected with gibel carp LGP2 and grouper LGP2, respectively, thus making the cells more prone to infection with different fish viruses [66, 75]. Hence, there might be different underlying factors which can affect the role played by LGP2 in fish. In this line, the studies on CIK cells over-expressed with

grass carp LGP2 inhibited the expression of type I IFNs and ISGs during initial phase of GCRV infection, but could inhibit the virus replication at a later stage of infection [76, 77]. However, the time-dependant mechanism of type I IFN response was in reverse order in cells transfected with zebrafish LGP2; during early phase of SVCV infection, type I IFN expression was higher but in later stage of infection the type I IFN expression considerably decreased. Further, it has also been reported that zebrafish LGP2 can inversely regulate the type-I IFN response based on the concentration of poly I:C [78]. Hence, the differential functional role played by fish LGP2 in different settings, need to be further evaluated stressing upon the underlying factors controlling the mode of action of LGP2 in type I IFN response in fish.

## 5 PRR Agonists as Adjuvants in Vaccination

Adjuvants administered along with the vaccine can enhance the immune response in various ways, which include the activation of innate immunity and positioning the vaccine (antigen) at specific locations. The new concept in vaccinology targeting regulation of innate immunity by PRRs facilitate the activation and recruitment of specific antigen-presenting cells (APCs) such as dendritic cells, and leads the way through transition from innate immunity to adaptive immunity for priming B and T cell responses, thus achieving the pathogen-specific long-lasting adaptive immune response. Among the adjuvants, PRR agonists are one of the potent candidates due to their targeted activation of innate immunity, which is linked with subsequent adaptive immune mechanisms. Hence, the molecules which stimulate PRRs such as TLR agonists can increase or modulate the immunogenicity of a specific vaccine thus becoming a new choice as adjuvant in the field of vaccination and a number of them have already been approved for human use [7].

TLRs are, by far, the most studied PRR group; hence, most of the PRR agonists permitted for use in human vaccines as adjuvants are TLR agonists. Further, several natural and synthetic homologues of TLR ligands targeting different groups of TLRs are currently being under clinical trial in human vaccination process. One of the most potent adjuvant systems named as adjuvant system 04 (AS04) containing aluminium salt and TLR4-agonist 3-*O*-deacylated-4′-monophosphoryl lipid A (MPLA), which is a derivative of LPS is permitted in two widely used vaccines: Fendrix, the HBV vaccine and Cervarix, the human papillomavirus (HPV-16/18) cervical cancer vaccine [79, 80]. Several other TLR ligands in clinical trial are in the process of making their way into human vaccines as adjuvants. Imiquimod, a synthetic TLR7 ligand has shown the potential against HPV-induced genital warts and skin cancers [81]. Another synthetic molecule, i.e., CPG-oligonucleotides (CpG-ODNs), the ligand of TLR9 is promising as an adjuvant in clinical experiments as it can enhance the immunogenicity of HBV vaccine (Engerix-B) and is found promising in vaccines targeting cancer, infectious diseases and allergies [82, 83]. Further, CpG 7909 along with alhydrogel has been tested in malarial vaccine against *Plasmodium falciparum* [84]. Flagellin, found in bacterial flagella and the ligand for TLR5, has shown promising results to be a candidate in flagellin-adjuvanted recombinant

influenza vaccine [85]. Among the several synthetic dsRNA homologues which can be recognized by RLRs and TLR3, poly I:C is the potent stimulator of type I IFN response, hence considered as a potent molecule for vaccination against viruses. A derivative of poly I:C named as poly-ICLC, stabilized with poly-L-lysine and carboxymethyl cellulose has been utilized as an adjuvant in HIV vaccine [86]. Further, it has been reported that PRR agonists can be potentially used as adjuvant in cancer vaccine. Poly I:C/poly-ICLC, a novel RNA-based PRR agonist (RNAdjuvant®) has potent immunostimulatory effects when used with several cancer vaccines [87].

Despite immense advancement in the field of fish immunology, the use of PRR agonists as potent adjuvant candidate in fish vaccination is still in its infancy. A few preliminary trials have been conducted for evaluating the potential of these agonists against some pathogens of finfish. The adjuvant potential of poly I:C stabilised with chitosan or poly I:C along with CpG oligonucleotides has been evaluated against VHSV infection and salmonid alpha virus, respectively [88, 89]. Bacterial flagellin has recently been evaluated as an adjuvant in fish vaccination against different bacterial fish pathogens [90, 91]. There are numerous reports on the PRR agonists as a potential immunostimulator against many fish pathogens, however, a coordinated approach is required for the use of these agonists as new-age vaccine adjuvants in fish vaccination programmes.

## 6 Fish Type I IFNs as Adjuvant in Vaccination

The use of IFNs in vaccination of higher vertebrates against several viruses has been quite well established and many of them are approved for clinical use [8]. In fish, use of IFNs as potential adjuvants is becoming popular following the encouraging results obtained from different studies. A DNA vaccine formulated against infectious salmon anaemia virus (ISAV) when applied with plasmids encoding different type I IFNs showed enhanced protection against this virus compared to DNA vaccine applied alone [92]. In a study in rainbow trout, the type I IFN used as an adjuvant with DNA vaccine against infectious haematopoietic necrosis virus (IHNV) showed enhanced and persistent Ab-titre, thus leading to the increased rate of survival [93]. A bicistronic plasmid construct expressing the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene of *Edwardsiella tarda* and Interferon gamma gene of *Labeo rohita*, when injected to *Labeo rohita*, resulted in higher relative percent survival compared to injection with monocistronic plasmid construct expressing only GAPDH [94]. Further, DDX41, a potent inducer of IFNs, could also reportedly increase the potency of a DNA vaccine against VHSV in olive flounder [95]. These initial studies on fish IFNs showed that these can be used as potential adjuvants especially in DNA vaccines against fish pathogens. However, more efforts are required to establish this in the field of fish vaccination. Moreover, these adjuvants can also be tested with the already commercially available DNA vaccines such as APEX-IHN®, Elanco and CLYNAV®, for increasing the efficacy of vaccines against the corresponding pathogens.

## 7 Conclusion

The immunoregulatory role played by different PRRs upon detecting PAMPs corresponding to specific pathogen is pivotal in mounting effective antimicrobial response. Further, several PRRs have been demonstrated as the targets for adjuvants in vaccination process. Hence, employing PRRs agonist as adjuvant, which can simultaneously elicit the immune signalling of different groups of PRRs, can efficiently tailor the potentiality of vaccines. Interferons are one of the effector molecules of PRR signalling, especially following the detection of viral ligands and can seize the spread of invading microbes. Moreover, interferons are functionally described as potent adjuvant in several vaccination programme. As both PRR agonists and interferons are proven to be an efficient choice for adjuvantation in vaccination trials, the strategy of co-application of these molecules along with target antigen would be an immunological intervention to manage the disease of economic importance in aquaculture.

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# Evaluating Efficacy of Vaccines in Finfish

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**Abstract**

Vaccination is an important strategy for preventing infectious diseases caused by bacteria and viruses in fish. Worldwide, there are continuous efforts to develop vaccines against bacterial and viral pathogens or improve the protection and duration of immunity of the existing vaccines. However, still many countries including India have only demonstrated development of vaccines under laboratory conditions, but do not have commercial vaccines for application in aquaculture. Trials of experimental vaccines must include data on efficacy of vaccines in terms of eliciting protective immune response in the host. Calculating relative percent survival (RPS) or measuring specific antibody level as a correlate of protection or indirect ELISA for estimating antibody levels in the host after immunization need to be evaluated to demonstrate the efficacy of trial vaccine under laboratory and field conditions.

**Keywords**

Fish · Vaccines · Efficacy · RPS · ELISA

## 1 Introduction

Efficacy of a vaccine is expressed as the percentage reduction of disease in a vaccinated group compared to an unvaccinated group under controlled conditions. Vaccine efficacy is different from vaccine effectiveness. Vaccine effectiveness shows the performance of a vaccine in field conditions, whereas vaccine efficacy

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shows how well a vaccine works in laboratory conditions. Efficacy of a vaccine is used to judge the performance of the vaccine, generally, in terms of eliciting protective immune response in the host. Adaptive immune response/marker that correlates with vaccine protection is termed as a correlate of protection. In other words, a correlate of protection is a biological marker that serves as a cut-off limit at which a vaccine is considered protective. Those vaccines, which do not induce correlate of protection, are not considered optimal. Therefore, finding the correlate of protection for any new vaccine has become the gold standard before being recommended for use [1]. Following methods can be used for determining the efficacy of fish vaccines.

## 2 Calculating Relative Percent Survival (RPS)

The most common method of evaluation of vaccine efficacy is the lethal challenge with virulent organism to cause mortality in vaccinated and control fish. Efficacy is measured as the relative percent survival (RPS) of the vaccinated fish compared with the unvaccinated controls [2].

$$\mathrm{RPS} = 1 - \frac{\%\text{Mortality in vaccinates}}{\%\text{Mortality in control}} \times 100$$

Some of the important inputs needed for calculating RPS are as follows:

1. Vaccinated and control fish.
2. Virulent strain of the pathogen for challenge.
3. A known concentration of the challenge dose: $LD_{50}$ dose could be used for the challenge. Higher doses have also been tested for developing challenge models for many pathogens.
4. Known route of challenge: Generally intraperitoneal route is used for challenge with the virulent organism. However, other routes such as bath and immersion can also be used.
5. Time of challenge after vaccination: To find out the duration of immunity in days after immunization.

Challenge experiment must be done at optimal rearing conditions in order to avoid the undue stress that can change the outcome of the efficacy trial.

**Disadvantages of RPS Approach**

- For many fish pathogens, the challenge models are not standardized and, hence, challenge trials are not reproducible. In such cases, variable results of vaccine efficacy can be expected.
- RPS approach is not suitable for pathogens with low mortality in fish.

For pathogens with low mortality, functional approaches are being used as a correlate of protection after a clear understanding of the pathogenesis and mechanisms of protection. For example, blocking the attachment of infectious salmon anaemia virus to the sialic acid receptor would prevent the establishment of infection [3]. In pancreatic disease, protection can be attained by preventing the establishment of pathological effects in the pancreas and heart. Similarly, the disease caused by infectious pancreatic necrosis virus (IPNV) could be prevented by ensuring that virus replication does not reach the desired concentration ($10^7$), which is a critical determinant of tissue damage in target organs [4]. Therefore, virus concentration in tissue could be used as a correlate of protection in vaccinated individuals. The highly abundant 57 kDa extracellular major soluble antigen (MSA or p57 protein) of *Renibacterium salmoninarum* has been strongly implicated as a principal mediator of immunosuppressive activities in the host. Virulence correlates with MSA protein abundance [5, 6] or functional MSA gene copy number [7, 8] and, hence, can be used as a correlate of vaccine efficacy.

## 3 Measuring Specific Antibody Level as a Correlate of Protection

Most vaccinations in fish are able to induce specific immunoglobulins in the host. Measuring specific serum immunoglobulin levels can be used for assessing protection and efficacy of fish vaccines. Generally, serum antibody levels above a threshold limit are considered protective. However, this needs thorough validation by challenge tests before establishing the cut-off limits. In rainbow trout, it was observed that all fish vaccinated with live-attenuated *Flavobacterium psychrophilum* immersion vaccine developed significantly higher levels of serum antibody titres by 8 weeks, when compared to their respective controls [9]. Atlantic salmon vaccinated against IPNV showed similar antibody levels in vaccinated fish and post-challenge survivors [10]. Protection against disease caused by CyHV-3 is associated with elevation of specific antibodies for 6–9 months against the wild-type virus, showing a correlation between the survival rate and increased titres of anti-CyHV-3 antibodies in the exposed fish [11]. Similarly, high correlation between the increase in antibody levels with reduction of post-challenge mortality is seen in Atlantic salmon vaccinated against furunculosis [12]. Formalin-killed *S. agalactiae* vaccine was able to increase the antibody titre in vaccinated fish compared to control fish [13]. These studies suggest that established threshold units of antibody titres can serve as important correlates of protective immunity in finfish.

In contrast, no correlation between immunoglobulin titres and disease protection after injection vaccination of Nile tilapia fingerlings against *Flavobacterium columnare* was observed [14]. There was no clear correlation between the antibody response to *R. salmoninarum* bacterins and protection from bacterial kidney disease (BKD) [15].

## 4 Indirect ELISA for Estimating Antibody Levels

Antibody levels in the vaccinated fish can be measured with the help of immunoassays. Most common method of estimating serum antibody titres is through indirect ELISA. ELISA is the most widely used immunoassay to detect and measure either antibody or antigen. The assay uses the basic immunology concept of an antigen binding to its specific antibody, which allows detection of very small quantities of antigens such as proteins, peptides, hormones or antibody in a fluid sample. A crucial component of indirect ELISA is the availability of monoclonal/polyclonal based anti-fish species enzyme conjugate. This reagent is not available commercially for Indian fish species and can be obtained from research laboratories of ICAR-Fisheries research institutes.

## 5 Measuring Antigen Dose as Correlate of Protective Immunity

Antigen dose in the vaccine could also be correlated with protective immunity. For this, a threshold unit of antigen dose should be determined that correlates with protective immunity. It has been seen that lower antigen doses of IPNV than the protective threshold unit can cause a significant reduction in protective immunity in fish vaccinated against infectious pancreatic necrosis [4]. Fish vaccinated with live-attenuated *F. psychrophilum* immersion vaccine at higher vaccine doses ($10^{10}$ and $10^{8}$ cfu/mL) showed strong protection for at least 24 weeks with RPS values up to 70%. However, the fish vaccinated with lower doses ($10^{6}$ and $10^{5}$ cfu/mL) had protection only up to 12 weeks and their RPS values dropped to 36% and 34%, respectively by 24 weeks [9]. Novobiocin-resistant *Streptococcus iniae* vaccine had a RPS value of 100% for vaccination dose of above $10^{4}$ cfu/fish [16]. A single intramuscular injection of as little as 1–10 ng of DNA vaccine can significantly protect rainbow trout fry against viral haemorrhagic septicaemia [17, 18]. Oral live-attenuated *Edwardsiella ictaluri* vaccine was tested at four doses between $4 \times 10^{6}$ and $3.2 \times 10^{7}$ viable vaccine cells/g wet feed for protection against enteric septicaemia of catfish in channel catfish. Mortality of channel catfish vaccinated with the lowest dose (26.6%) was significantly higher than fish vaccinated with the highest dose (14.1%) [19]. Therefore, a minimum unit of antigen dose in the vaccine could serve as a correlate of protection.

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# Biosafety and Regulatory Requirements for Vaccines

Sharmila B. Majee

**Abstract**

Biosafety is an important requirement for all laboratories, vaccine production units and aquaculture facilities. Its relevance for framing regulatory protocols under different categories of biosafety has to be emphasized from time to time. Regular monitoring and documentation following Standard Operating Protocols should be within the framework of well established guidelines to maintain uniformity in the functioning of these institutions. Biosafety requirements with respect to living and genetically modified organisms should be formulated keeping in view the impact on preservation of the indigenous populations and is indeed a matter of great concern to biologists, researchers, operating personnel and fish handlers. Imparting skills through training and orientation programmes is the way forward in keeping abreast with the latest developments in biosafety protocols.

**Keywords**

Biosafety levels · Regulatory requirements · Risk groups · Vaccines · Vaccination

## 1 Biosafety in Aquaculture

Biosafety refers to the safe handling of infectious microorganisms, living modified organisms, hazardous biological materials and their containment to safeguard the environment and human health. In light of the fact that most aquatic biological diversity still resides in natural populations; all biotechnologies that have the potential to improve fish production may have an adverse impact on wild aquatic

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resources [1]. In aquaculture sector, there are several pilot projects and research programmes in many parts of the world that are developing commercially important vaccines. The scope of the current biosafety protocols should include vaccines and disease-resistant strains as well and their impact on the natural biodiversity including the wild relatives of domesticated aquatic species.

These biosafety protocols, or similar regulations, should eventually strive to protect these resources while allowing for the development of aquaculture and international trade [2]. Regulatory guidelines should be clearly defined for the use of vaccines in cultured fish populations in order to preserve the natural biodiversity and to preserve certain populations facing extinction [1]. At present, European Union, USA, Canada and Norway have issued guidelines about regulatory requirements for veterinary vaccines that include those for fish as well [3–6]. In India, fish vaccines are still not included in the veterinary supplement 2018 of Indian Pharmacopoeia. However, the Aquaculture Authority is in the process of drafting a specific legislation for disease control within aquaculture facilities in the Compendium on Aquatic Medicines and Animal Health Management [7]. Hence, this chapter will focus on biosafety in relation to regulatory requirements for vaccines.

## 2 Some Common Terms

**Biosafety** In The American Heritage Medical Dictionary, biosafety is defined as 'A set of measures or activity undertaken to ensure the safe handling of biohazardous materials, such as pathogens, biological contaminants and genetically modified organisms, especially to prevent their accidental spread beyond a laboratory or research facility' [8]. As per the Segen's Medical Dictionary, it is defined as 'Any activity intended to safeguard a population from the untoward effects of potentially infectious biological materials or infectious agents, and minimize their environmental impact' [9].

Therefore, guidelines laid down by national level organizations are implemented through institutional bodies that periodically review biosafety norms practiced in the laboratory or production settings to prevent large-scale loss of biological integrity that may otherwise affect the environment and human health [10].

**Veterinary Biological** Substances or derivatives/mixture of animal origin such as helminth, protozoa or microorganism, or any substance of synthetic origin that is manufactured, sold or represented for use in restoring, correcting or modifying organic functions in animals, or products for use in the diagnosis, treatment, mitigation or prevention of a disease, disorder and symptoms, thereof in animals. These veterinary biologicals include vaccines, bacterins, bacterin-toxoids and diagnostic kits [11].

**Production Outline** This can be defined as a detailed description of processes followed while producing a veterinary biologic, and diluents, if any, followed by the tests used to establish its purity, safety, potency and efficacy, and the results of all

such tests including the methods and procedures to be employed in handling, storing, administering and testing a veterinary biological [11].

**Safety** Safety for a veterinary biological can be defined as the freedom from properties or agents causing local or systemic reactions when the biological is used as recommended [11].

**Purity** Purity is defined as the quality of a biological prepared to a final form that is relatively free of extraneous microorganisms and material, as determined by established test methods and approved in the production outline [11].

**Potency** Potency is defined as a measure of the relative strength of a biological that correlates to its immunogenicity/efficacy when tested by established methods as documented in the production outline [11].

**Efficacy** Efficacy is defined as a measure of the specific protective response to the biological when used at the recommended dose [11].

## 3 Committees Involved in Biosafety

In India, vaccines and other recombinant products are regulated by the 'Rules for the manufacture, use/import/export and storage of hazardous microorganisms/Genetically Modified Organisms (GMO's) or cells, 1989' (Rules, 1989) notified by the Ministry of Environment and Forests (MoEF), GOI under the Environment Act (1986) [10]. These rules are implemented by Ministry of Environment and Forests (MoEF), Department of Biotechnology (DBT), Ministry of Science and Technology (MoST) and state governments through six competent authorities notified under the Rules, as follows:

1. Recombinant DNA Advisory Committee (RDAC)
2. Institutional Biosafety Committee (IBSC)
3. Review Committee on Genetic Manipulation (RCGM)
4. Genetic Engineering Appraisal Committee (GEAC)
5. State Biotechnology Co-ordination Committee (SBCC)
6. District Level Committee (DLC)

***Functions of each committee*** RDAC is mainly an advisory body whereas IBSC, RCGM and GEAC have a regulatory function. SBCC and DLC are responsible for monitoring the activities related to GMO's in the state/district levels, respectively. RDAC, RCGM and GEAC are constituted at the central level by DBT and MoEF.

***Role of IBSC*** An IBSC should be constituted by organizations engaged in research, handling and production activities related to GMO's and is a statutory committee that operates as a liaisoning body between DBT and the institution and should

co-ordinate with SBCC and DLC wherever necessary. Therefore, it has a pivotal role within an organization for implementation of a biosafety regulatory framework.

## 4 Classification of Biosafety Levels and Risk Groups

In this section, the major characteristics of the four biosafety levels, definition of 'risk group' with examples, and how risk groups are used in conjunction with risk assessment to set biosafety levels, are discussed. A biosafety level is the assignment of agent based on risk assessment, depending on the type of agent and the conditions of use requiring professional judgment, especially in the case of unknown pathogens. Hence, the biosafety levels are classified into four groups based on the risk assessment [10].

1. **Biosafety Level 1 (BSL 1):** This type of facility is suitable for work involving well-characterized agents not known to consistently cause disease in immuno-competent adult humans. They pose a minimal potential hazard to laboratory personnel and the environment. The laboratories are not necessarily separated from the general traffic patterns in the building. Work is typically conducted on open benchtops using standard microbiological practices. Special containment equipment or facility design is not required. However, laboratory personnel must have specific training in the procedures conducted in the laboratory and must be supervised by a scientist with training in microbiology or a related science [10].
2. **Biosafety Level 2 (BSL 2):** It builds upon BSL 1 and has additional safety features over the basic set up. It is suitable for work involving agents that pose moderate hazards to personnel and the environment. The laboratory personnel should have undergone specific training in handling pathogenic agents and be supervised by scientists competent in handling infectious agents and associated procedures. There is restricted access to the laboratory when work is being conducted in the facility. Procedures involving or creating infectious aerosols or splashes are conducted in biological safety cabinets (BSCs) [10].
3. **Biosafety Level 3 (BSL 3):** It is applicable to clinical, diagnostic, teaching, research or production facilities where work is performed with indigenous or exotic agents that may cause serious or potentially lethal diseases through inhalation route of exposure. Laboratory personnel must receive specific training in handling pathogenic and potentially lethal agents. Such work should be supervised by scientists competent in handling infectious agents and associated procedures. The work should be conducted in Class III biosafety cabinets placed in rooms with airlock systems with HEPA filters, to prevent the infectious agents escaping from the facility by placing air showers at the entrance and airlocks thereafter at each stage inside the facility. Personnel should wear additional protective gear such as N95 masks, goggles, protective gowns for respiratory and personal protection as determined by risk assessment. As discussed above, a BSL 3 laboratory has special engineering design features, primarily among them being a directional airflow maintained by air pressure and interlocking doors [10].

4. **Biosafety Level 4 (BSL 4):** Such a facility is required for work with dangerous and exotic pathogens that pose a high individual risk of life threatening diseases, aerosol transmission or a related agent with unknown risk of transmission. Agents with a close or identical antigenic relationship to agents requiring BSL 4 containment must be handled at this level until sufficient data are obtained either to confirm continued work at this level or re-designate the level. The laboratory staff should have specific and thorough training in handling extremely hazardous infectious agents. Laboratory staff must understand the primary and secondary containment functions of standard and special practices, containment equipment and laboratory design characteristics. All laboratory staff and supervisors must be made competent in handling agents and procedures requiring BSL 4 containment. Access to the laboratory for designated personnel is controlled by the laboratory supervisor in accordance with the institutional policies. In this type of facility, there are two separate entities that provide absolute separation of the worker from the infectious agents, namely, the suit and the biosafety cabinet. Hence there are two distinct laboratories, namely, suit laboratory and cabinet laboratory [10].
5. According to the biosafety levels, the infectious agents are divided into four major risk groups [10]:
   (a) **Risk Group 1:** These agents do not pose any individual and community risk. It defines a microorganism that is unlikely to cause human or animal disease.
   (b) **Risk Group 2:** These agents pose a moderate individual risk and low community risk. It defines a pathogen that causes human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. The agent may cause serious infection in a particular species but can be controlled by effective treatments, and preventive measures are available in order to limit the risk of spread.
   (c) **Risk Group 3:** These agents pose a high individual risk but low community risk. It defines a pathogen that usually causes serious human or animal disease but does not ordinarily spread to other species. Effective treatment and preventive measures are available to control their spread.
   (d) **Risk Group 4:** These agents pose high individual and community risk. It can be defined as a pathogen causing serious human or animal disease readily transmitted from one individual to another. Effective treatment and preventive measures are usually not available for their control or limit their spread.

## 5 Biosafety Requirements for Vaccines

Most of the pathogens of fish can be classified in risk groups 1 and 2. Few may belong to risk group 3. However, handling of any microorganism, virus or recombinants should be carried out following biosafety protocols during handling and preparation of vaccines that can be divided into four phases [3].

Some of the common steps in vaccine preparation [5, 12, 13] include:

- Isolation and identification of the causative agent and preparation of master seed stock and working seed
- Culturing of the microorganism itself or the target antigen in some other way using biotechnological tools
- In case of inactivated vaccines, inactivation steps using chemical or physical agents to kill the microorganism
- Confirmatory tests to prove that the vaccine is free of extraneous agents.
- Confirmation that the vaccine is safe for the target species using safety studies following approved protocols
- Confirmation that the vaccine is effective in preventing and reducing the disease in the target species of fish
- Adequate sterility and quality control checks, including vaccine formulation in appropriate diluents, carrier with or without adjuvant and in a package to facilitate storage

In order to meet all the regulatory requirements for safety, quality and efficacy, the development process needs to generate a complete dossier to satisfy the accessors and in case of rDNA vaccines, the dossier should go through RCGM [10].

Three 'R's in the approaches are recommended in the production and quality control of vaccines [3] and that include Replacement, Reduction and Refinement.

**Replacement:** Includes methods which permit a given purpose to be achieved without conducting experiments or other scientific procedures on animals.

**Reduction:** Includes methods for obtaining comparable levels of information from the use of fewer animals in scientific procedures, or for obtaining more information from the same number of animals.

**Refinement:** Includes methods which alleviate or minimise potential pain, suffering or distress, and which enhance animal well-being during the safety and efficacy studies [3].

## 6 Autogenous Vaccines and Regulatory Issues

Autogenous vaccines are recommended for important diseases of sporadic nature that do not have commercially available vaccines in place. These vaccines are custom-made and prepared from a pathogen isolated during a sporadic outbreak or a specific epidemic as compared to commercially available vaccines that are prepared from standardized cultures. Some companies can provide autogenous vaccines for certain diseases. Hence regulations governing the use of autogenous vaccines vary among countries. In USA, autogenous vaccines are prepared from culture of microorganisms that have been inactivated or are non-toxic. Further, the product should be prepared for use only under the direction of a veterinarian and under a veterinarian-client relationship or such products may be prepared for use under the direction of a person with appropriate expertise in specialized situations such as aquaculture, if approved by USDA [4, 14]. Autogenous vaccines should be

prepared using seed organisms isolated from sick or dead fish, to be used in the population of origin or adjacent populations after due permissions [15]. Other regulatory requirements include information on the designated facility, specifying the precautions that will be taken to prevent contamination of licensed products [5, 13].

## 7 Regulatory Requirements for Licensing of Vaccines

These requirements include full information on the original organism used as master seed, details of various aspects of in-process controls to the final batch release tests, quality control test data, results of the batch-safety test performed on all the batches of the vaccine produced for sale. The batch-safety test should have been carried out on a target species for the first five to ten batches of vaccine produced [5, 13]. These quality control checks are designed to ensure batch-to-batch consistency in the production of vaccines that begin as cultivation of a living microorganism including slight variations in how the microorganism is grown, replicated and standardized to obtain the final product making each vaccine a unique development [3, 5, 13].

At present, there is no regulatory authority for fish vaccines in India, and standard guidelines for fish vaccines are yet to be established [7] and incorporated in the Indian Pharmacopoeia. Unlike USA, Canada and Norway, where strict guidelines for product licenses and permits are in place, the fish vaccines are classified as veterinary biologicals, and licenses are issued by Veterinary Biologics and Biotechnology Section (VBBS) [5].

As per Canadian regulatory guidelines [11], veterinary biologicals can be classified based on the qualitative risk assessment as follows:

1. **Class I (low risk):** includes inactivated viral/bacterial vaccines (conventional or rDNA), viral/bacterial subunit vaccines (conventional or rDNA), cytokines and monoclonal antibody (hybridoma) products and modified live conventional or gene deleted vaccines.
2. **Class II (high risk):** includes vaccines using live vector to carry recombinant derived foreign genes and live organisms modified by introduction of foreign DNA.
3. **Unknown Risk:** Plasmid DNA vaccines are a novel group of vaccines that are presently undergoing evaluation by VBBS for risk classification. The novel biology of plasmid DNA vaccines poses several regulatory challenges, particularly in the evaluation of safety and potency. Safety concerns focus on the potential for integration, tumour formation, replication activity and germ line transmission.

For most vaccines, a Biosafety level 3 containment facility having biosafety cabinets and other primary devices are required for all activities to be carried out without creating aerosols. Any spillage is considered as hazardous and disposed as per biosafety norms. Protective clothing, access control through directed airflow

using airlock systems provide sterility as well as containment of the potential pathogen.

## 8 Operator Safety During Injection Vaccination of Fish

There are a number of reports of adverse health effects of vaccinators after accidental self-injection of fish vaccine during injection of fish [16]. A reaction is considered serious if it led to absence from work for more than 10 min. Most of the self-injections occurred exclusively on fingers and hands. The reactions to injuries can be differentiated into four types:

1. The most common reaction is mild localized pain, oedema and sometimes infiltration induced by superficial stabs from the tip of the needle of syringes.
2. Depending on the dose and type of vaccine injected, reactions may spread to whole hand or parts of the underarm. In some cases, fever with lymphangitis and swollen axillary glands was reported. In cases where there was accidental injection of vaccine containing aluminium hydroxide and mineral oil as adjuvant, the affected finger had to be amputated.
3. Injectors also experience lymphatic reactions along with influenza-like syndrome with fatigue, dizziness, headache, fever and muscle ache lasting 2–12 h.
4. Perhaps, the most severe manifestation is the form of anaphylactic reaction. Within minutes after self-injection, symptoms like tachycardia, breathing difficulties, nausea and loss of consciousness appear [16].

Overall, the risk of self-injection seemed to be associated with lack of experience and awareness, as experienced vaccinators seldom self-inject themselves compared to seasonal inexperienced workers. Medical treatment includes mainly non-steroid anti-inflammatory drugs indicated for some days and, in case of anaphylactic reactions, adrenaline is recommended. Local doctors should be acquainted with the characteristics of such injuries or local reactions and the treatment for the same for timely intervention whenever necessary [16].

Devices to protect the fingers against self-injections have been developed. A double bow is attached to the tip of the syringe on both sides of the needle, allowing the fish to be supported during the injection, and at the same time shielding the tip of the needle [12]. This has been extensively used in Norwegian aquaculture [17] that includes risk assessment and risk mitigation measures, whereby the number of reports of self-injection drastically reduced. However, although use of automatic injection devices and immersion or oral administration of vaccines are being adopted, self-injection still remains a potential hazard for inadequately equipped and untrained aquaculture workers especially in unorganized sectors [12, 16].

## 9 Conclusion

In the Indian context, biosafety and regulatory requirements for fish vaccines are still in the nascent stage. So far, no Standard Operating Protocols (SOP) or product information regarding bacterial or viral vaccines of fish are available in the Indian Pharmacopoeia. Hence, the biosafety requirements for veterinary vaccines are to be used as a guideline at present, as most countries classify the fish vaccines as veterinary biologicals. What is more important is that the safeguards to be used in the injectable fish vaccines are overlooked, leaving much to be desired as far as biosafety and regulatory issues are concerned. However, research on recombinant vaccines is being undertaken worldwide, as these vaccines can be administered by immersion technique or oral routes. Nevertheless, the evaluation of their biosafety is under consideration by international bodies as these vaccinated fish will enter the human food chain. Moreover, protection of the wild aquatic species also comes under the scope of biosafety in aquaculture. These facts further reiterate the important role of biosafety and regulatory bodies in fish vaccines and aquaculture. Therefore, we must recognize that in the formulation of biosafety policy and regulations for living modified organisms, the characteristics of the organisms and of potentially accessible environments are more important considerations than the processes used to produce those organisms.

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# Adverse Effects of Fish Vaccines

Gayatri Tripathi and K. Dhamotharan

**Abstract**

Fish diseases remain to be a serious economic threat in commercial aquaculture across the world, despite several innovative approaches to novel therapy. Although antibiotics or chemotherapeutics are used to treat fish diseases, they exhibit some obvious disadvantages, such as drug resistance and safety concerns. Vaccination is regarded as an effective prophylactic measure to prevent a wide range of bacterial and viral infections in aquaculture. Fish immunization has been practiced for more than 50 years and is well recognised as an efficient means of avoiding a variety of infectious diseases of fishes. It can activate the specific and non-specific immune systems of fishes and provide long-term protection. Though vaccination is vital for preventing disease outbreaks and reducing antibiotic usage, it may also have adverse effects on the vaccinated animal, environment or the consumers of vaccinated fishes. It varies with the type of vaccine, mode of administration, environmental parameters, size, stress and immunity of fishes, etc. Inflammation, abdominal lesions, growth retardation, spinal deformities and systemic and autoimmunity development are the major adverse effects associated with injection vaccination in fishes. Apart from this, adjuvants used along with vaccines are also reported to induce granuloma in all the vital organs. Midtlyng and Speilberg scales are used as a standard method for measuring the severity of the lesions developed during fish vaccination. Vaccination is undoubtedly the most effective intervention for disease prevention and control. However, the negative consequences must be considered when creating alternative safe techniques. The present chapter deals with the major adverse effects associated with fish vaccination and their significance in vaccine development.

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**Keywords**

Fish vaccination · Immunization · Adverse effects of vaccines · Histology · Vaccine failure · Midtlyng and Speilberg scoring

## 1 Introduction

Vaccination is the process of developing immunity to a particular disease by administering disease-specific antigens. Globally, vaccines have long been known to reduce the mortality rates in humans and animals. Vaccines are variously categorized as first, second and third generation vaccines. Conventional vaccines are grouped in the category of first generation vaccines. These require whole microorganisms for their preparation; whereas the second generation vaccines utilize the recombinant protein components. The third generation vaccines are also known as DNA vaccines, and they use the recombinant technology employing genetic engineering of bacterial plasmids.

Vaccines are vital as a preventive medicine to minimize the losses due to infectious diseases in aquaculture and to improve the welfare of farmed fishes. Intensive use of antibiotics in the aquatic ecosystem has raised concerns among fish consumers and also resulted in the development of antibiotic-resistant pathogens. An inactivated vaccine against *Yersinia ruckeri* was the first fish vaccine to be licensed in 1976 in the USA. Since then, many vaccines have been developed and licensed worldwide in order to control and combat the disease problems in aquaculture. Fish vaccines against many bacterial and viral diseases are available commercially in many countries [1]. The use of vaccines has tremendously reduced the application of antimicrobial drugs in aquaculture, particularly in the Norwegian aquaculture industry [2]. An ideal vaccine should be safe (without adverse/side effects), easy to administer, efficacious (efficient to protect the animal from diseases), stable and cheap [3]. Generally, vaccines have no hazardous risks but they do have some adverse effects. In aquaculture, injection of multivalent vaccines is a common method for preventing disease outbreaks. Many studies have found that the adverse effects of fish vaccines are mainly related to the intraperitoneal administration of oil-adjuvanted vaccines.

Vaccines may have adverse effects on the vaccinated animal, environment or the consumers of vaccinated fishes (Fig. 1). Furthermore, contaminated vaccines or unhygienic procedures adopted for injection may introduce unwanted microbes into the fishes. In aquaculture, the losses due to vaccination methods have been observed to be highest in direct immersion method, followed by injection and prolonged bathing [4].

Fish vaccination involves greater ecological risks than in terrestrial animals due to environmental complexities. Pre-licensure and post-licensure vaccine safety, food safety and maximum residual limits of the vaccine in the animals should be evaluated for licensing of fish vaccines. Though vaccination is important to prevent

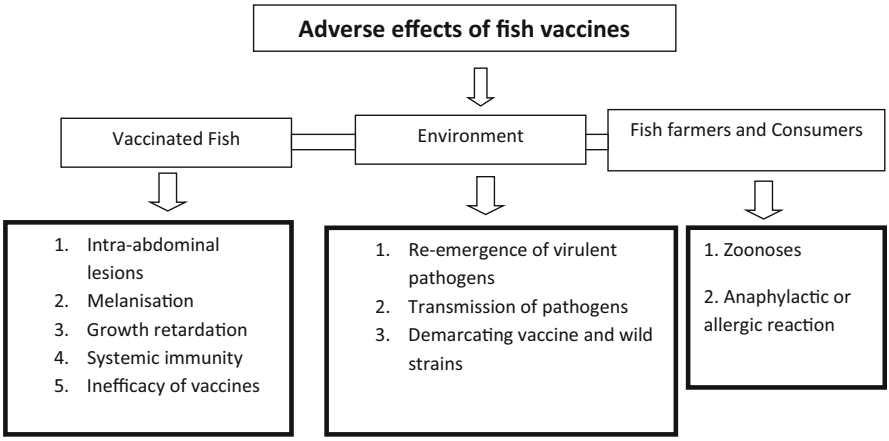


**Fig. 1** Adverse effects associated with fish vaccines

disease outbreak and to reduce the use of antibiotics, the associated adverse effects cannot be neglected for developing alternative safe methods [5].

## 2 Adverse Effects of Different Types of Fish Vaccines

### 2.1 Live-Attenuated Fish Vaccines

Live-attenuated vaccines are produced by different methods like serial passaging of pathogens through cell cultures, chemical or physical mutagenesis and genetic modifications [6]. The main advantage of live vaccines is that they are able to infect and multiply inside the host without causing clinical disease and are capable of eliciting both adaptive and innate immunity. Live-attenuated vaccines are efficient against many fish diseases as they provoke cell and antibody-mediated immunity.

Safety concerns affiliated with live-attenuated vaccines are reversion from avirulent to virulent strain, risk of transmission of pathogens, release of vaccine strain into the environment and risk of infecting other animals or humans. Live attenuated vaccines are not pathogenic to targeted species but may pose risk to other non-targeted aquatic organisms [7]. The attenuated vaccines must allow serological differentiation from infected animals, and the vaccine strain should also be traceable in the environment [8]. These safety issues restrict the wide use of live attenuated vaccines in the aquatic ecosystem. Nevertheless, attenuated vaccines are commercially available and used in aquaculture without any report of adverse effects [2].

## 2.2 Inactivated/Killed Vaccines

Inactivated vaccines consist of pathogens or products that are killed or inactivated by various chemicals (formaldehyde or β-propiolactone or binary ethylenimine or urea or tri-*N*-butyl phosphate) or by physical (ultraviolet [UV] light, heat, and sonication) methods. During inactivation, care is taken not to alter their immunogenicity; hence the inactivated pathogen is still capable of eliciting immune responses [3]. Inactivated/killed vaccines against several fish pathogens are commercially available in many countries and are the most common types of vaccines used in aquaculture [7, 9].

Generally, inactivated vaccines are injected as monovalent or multivalent vaccines and have to be evaluated for possible risk of causing clinical infections in fishes. Killed/inactivated vaccines have a possible risk of delivering viable pathogens due to inadequate killing or due to contamination of vaccines with extraneous infectious agents [3]. Improper inactivation would lead to a lack of antigen recognition by the fishes. Moreover, the presence of various chemical residues in vaccines that were used for inactivation/killing needs to be assessed for its safety and persistence. In this vaccination method, booster doses are required to increase the protective efficacy, and this requirement of repeated administration of vaccines by injection method inflicts enormous stress and adverse effects on the fishes. Inactivated vaccines are usually injected intraperitoneally as oil or water adjuvanted emulsion. Most of the reported adverse effects using inactivated vaccines are mainly due to intraperitoneal injection of various adjuvants (refer Sect. 3.3). For this reason, the adverse effects of intraperitoneal injection of oil-adjuvanted inactivated vaccines should be evaluated before authorization, marketing and field application.

## 2.3 Subunit Vaccine

Subunit vaccine consists of recombinant proteins that are part of the microbes which can induce protective immunity. Inconsistent efficacy and lack of humoral responses are reported in subunit vaccinated fishes [10]. Purifying the proteins for subunit vaccination may reduce the immunogenicity and this may demand the need for an immunogenic carrier protein or adjuvant. The use of adjuvants further increases the adverse effects.

## 2.4 DNA Vaccine

In DNA vaccination, bacterial plasmid DNA encoding antigen of a fish pathogen is injected into the animal for protecting it from diseases. APEX-IHN DNA vaccine manufactured by Vical-Aqua Health Ltd. of Canada (Novartis) is the first DNA vaccine for use in aquaculture [11].

The adverse effects of DNA vaccines include possible integration of DNA within the genome of the vaccinated animal, inducing an anti-DNA immune response, autoimmunity, immune tolerance, injection site inflammation, tissue destruction and other wild bacteria taking up the plasmid DNA [12]. In some countries, DNA-vaccinated animals are referred to as a genetically modified organism (GMO) [13]. The risk assessment for all the above aspects has to be conducted for the approval of any DNA vaccine, besides assessing the fate of plasmid DNA (stability, persistence and integration) in the vaccinated fishes and assessing the post-consumption effect (if any), when the DNA vaccinated fishes are eaten by predatory fishes or by humans.

## 3 Adverse Effects of Different Vaccination Methods

Three methods—injection, immersion and oral—are commonly used for vaccine administration in aquaculture, with each having its own advantages and disadvantages. The adverse effects associated with each of these methods are discussed below:

### 3.1 Immersion Vaccination

The different types of immersion vaccination methods used in aquaculture include hyperosmotic infiltration (HI), direct immersion (DI) (bath, dip and flush), spray, ultrasound immersion and puncture immersion. Besides its inconsistent efficacy, the immersion vaccination method is wasteful as it requires large quantities of the vaccine. This method may not be applicable to all types of vaccines. The spray method is found to be more stressful than DI methods [14].

### 3.2 Oral Vaccination

Oral vaccination is the most suitable method for aquaculture as it involves no stress to the fishes and less labour cost [15]. Noted adverse effects of oral vaccination methods include inconsistent efficacy and a considerable waste of vaccine than ingested. The duration of protection is less and fishes may develop immunosuppression in oral vaccination methods [16]. To increase the efficacy, different encapsulation methods like alginate, PLGA, chitosan, liposomes, silver or gold nanoparticles are utilized. Use of silver or other metal nanoparticles are toxic to the environment and to the fishes [17].

## 3.3 Injection Vaccination

Injection vaccination is the most efficacious method of vaccination. Fishes are vaccinated either intraperitoneally or intramuscularly as oil-adjuvanted vaccines to protect them from different bacterial or viral diseases. In this method, handling, anaesthetization and injection are stressful to the fishes and it is a labour-intensive method. Commercially available fish vaccines contain oil-based adjuvants which can protect the animal for a longer duration but severe adverse effects are reported with the use of different adjuvants and intraperitoneal injections in several fishes. The main adverse effects associated with the injection vaccination method are grouped into three categories: (a) inflammation and abdominal lesions, (b) growth retardation and spinal deformities and (c) systemic autoimmunity [18–20].

### 3.3.1 Inflammation and Abdominal Lesions

Antigen injected intraperitoneally with oil adjuvants acts as inflammation stimulant, initially attracting acute inflammatory reaction components—lymphocytes, polymorphonucleocytes (PMN), eosinophilic granulocytes/mast cells and, later, macrophages. This stimulation persists at the injection site for many months [21]. Following injection, oil-adjuvanted vaccines develop into cell vaccine mass (CVM) which is found to have a blood vessel-like connection between internal organs. Cell vaccine mass originally comprises neutrophils and macrophages but eventually develops into granulomas inside the peritoneal cavity in advanced stages [22]. Lesions were observed in many vaccinated fishes [23, 24] around the pyloric caeca and spleen, near the urinary bladder and oesophagus and intra-abdominal lesions in pancreas, pyloric caeca and the spleen [21]. Ocular inflammation and occlusion of uveal vessels are also found in vaccinated fish [25].

The adjuvants aid in the formation of depots of antigens at the injection site and slow release of these antigens [26]. After the acute injection site inflammatory responses, these develop into chronic inflammatory reactions characterized by the presence of visible adhesion between the organs and between organs and the abdominal wall. Granulomas, the hallmark of chronic inflammatory reaction, develop at later stages that are characterized by the presence of large numbers of macrophages, apoptotic neutrophils [22, 27, 28], lymphocytes and multinucleate cells [29, 30].

Two most commonly observed adverse effects associated with injection vaccination methods are adhesions and melanin formation. Adhesions are caused by the production of fibrinous strands [31] which surround the pancreas and pyloric caeca [28]. In addition, the formation of cell vaccine mass (CVM), viscosity and adherence of oil itself contribute to the development of internal adhesions [22]. Melanin pigmentation is a routinely observed adverse effect of the oil-adjuvanted vaccine in many fishes [31–33]. Initially, the adverse reactions increase for a period of time and then the reactions subside until the antigen is fully removed by competent cells [33]. Among different types of vaccines, the highest adverse reactions were observed in oil-adjuvanted multivalent vaccines by many researchers [27, 29].

### 3.3.2 Histological Changes in Organs

The oil-adjuvanted vaccines induce granulomatous peritonitis, oedema in splenic mesoderm [27], white military spots on liver [28], petechial haemorrhages in liver, abscess near lateral line, thickening of splenic ellipsoids, mild disruption of splenic structure, fibrin in liver, recruitment of melanomacrophage centres in anterior kidney [34], granulomas in liver, spleen, heart and kidney and hyperplastic white pulp of spleen [28].

### 3.3.3 Scoring of Adverse Effects of Vaccines

In fish vaccination, different scoring methods are used to evaluate the vaccine-induced adverse effects. Midtlyng and Speilberg score (0–6) based on visual appearance of the abdominal cavity is used as a standard method for measuring the local reactions due to intraperitoneal injections of oil-adjuvanted vaccines (Table 1) [23], and this scoring is modified into Visual Analogue Scales (VAS) for continuous scoring [35]. Vaccination-induced melanisation is evaluated using visual observation on a 0–3 scale: 0—no melanin; 1—pinpoints or small spots; 2—considerable

**Table 1** Midtlyng and Speilberg scoring of adverse effects [23]

| Score | Visual appearance of abdominal cavity | Severity of lesions |
|---|---|---|
| 0 | No visible lesions | None |
| 1 | Very slight adhesions, most frequently localised—close to the injection site. Unlikely to be noticed during evisceration | No or minor opacity of peritoneum after evisceration |
| 2 | Minor adhesions, which may connect colon, spleen or caudal pyloric caeca to the abdominal wall may be noticed during evisceration | Only opacity of peritoneum remaining after manually disconnecting the adhesions |
| 3 | Moderate adhesions including more cranial parts of the abdominal cavity, partly involving pyloric caeca, the liver or ventricle, connecting them to the abdominal wall may be noticed during evisceration | Minor visible lesions after evisceration, which may be removed manually |
| 4 | Major adhesions with granuloma, extensively interconnecting internal organs, which thereby appear as one unit may be noticed during evisceration | Moderate lesions which may be hard to remove manually |
| 5 | Extensive lesions affecting nearly every internal organ in the abdominal cavity. In large areas, the peritoneum is thickened and opaque, and the fillet may carry focal, prominent and/or heavily pigmented lesions or granulomas | Leaving visible damage to the carcass after evisceration and removal of lesions |
| 6 | Even more pronounced (than the lesions described above), often with considerable amounts of melanin. Viscera unremovable without damage to fillet integrity | Leaving major damage to the carcass |

amount of melanin; 3—melanin covering large areas of the abdominal wall/abdominal organs [36].

The inflammation and granuloma are assessed on a 0–2 scale where 0—none, 1—few, and 2—a large number of granulomas [33]. The spinal deformity is scored by macroscopic observation and palpation on a 0–3 scale, in which 0—no deformity, 1—palpable deformity recognizable by fish health professionals, 2—medium deformity recognizable by laymen and 3—high degree of deformity [35].

### 3.3.4 Growth Retardation and Spinal Deformities

Growth reduction due to spine deformities [35], visible vertebral changes in 10% fishes [28], reduced interest in feed, behavioural changes like decreased activity [31], weight reduction up to 20% [37] and growth reduction up to 8% [32] are observed as vaccine-associated adverse effects. Vaccine-induced abdominal lesions and adhesions also affect the normal functioning and metabolism of different organs, and may reduce appetite and damage the digestive function [38].

### 3.3.5 Systemic Autoimmunity

Autoimmunity refers to a system of the immune response against its own cells or tissues. The systemic autoimmunity following vaccination was first reported in fishes by Koppang et al. [39]. Presence of autoantibodies, thrombosis and chronic granulomatous inflammation in liver and immune-complex glomerulonephritis were observed in vaccinated fish [39]. Further, the presence of rheumatoid factor, self-reacting anti-nuclear and cytoplasmic antibodies and immune-complex-mediated glomerulonephritis, after the intraperitoneal injection of the killed multivalent mineral oil-adjuvanted vaccine confirmed this condition as vaccine-induced systemic autoimmunity [28].

## 4 Risks to Fish Farmers and Consumers

Fish farmers or technicians involved in vaccinating the fishes may be exposed to fish vaccines by accidental self-injection. During fish vaccination, vaccinators reported one to more than 50 stabs or self-injection. Even though self-injection of fish vaccines has low health effects, two cases of hospitalisation were reported because of anaphylactic/allergic reaction after accidental self-vaccination [40]. Hence, the studies on adverse reactions in humans during accidental injection also need to be taken into consideration prior to vaccine approval.

## 5 Vaccine Failures

There are many reasons for the failure of vaccines such as poor storage condition, immunizing unhealthy and immune-compromised fishes, loss of immunogenicity of antigens in a killed vaccine and use of antimicrobials along with live-attenuated vaccines [41]. Overexposure of fishes to various stressors, vaccinating unhealthy

fishes [2], not following the recommended vaccination protocol in dilution, species, life stage and administration methods, etc. may result in vaccine failures [42].

## 6 Measures to Reduce the Adverse Effects

The adverse effects (internal adhesions and melanisation) caused by the parenteral vaccination of oil-adjuvanted vaccines on breeding and seed production, fish welfare and marketing of fishes can be overcome by the use of novel methods of vaccination and adjuvants. Development of adverse reactions can be reduced by increasing the fish size at vaccination [38], selective breeding method [43], use of alternate adjuvants like water-based adjuvants, liposomes, etc. In safety testing, the following parameters are assessed: the presence of viable pathogens in inactivated vaccines, contamination of vaccines by extraneous pathogens or chemicals and residual level of formaldehyde in formalin-inactivated bacterial vaccines [44–46]. Novel strategies are required to develop the most effective and efficacious oral vaccines for aquaculture. The hurdles in the development and licensing of DNA vaccines can be overcome by addressing all the research gaps in development and application.

## 7 Future Perspective

The world fish farming sector has transformed from an extensive to intensive industrial-scale production system. Global food fish production has reached an all-time high of 178.5 mmt in 2018 with an average annual growth rate of 5%. Global per capita fish consumption has increased from 9.0 kg in the 1960s to 20.5 kg in 2018, mainly due to the increase in aquaculture production [47]. Diseases are the primary constraint hindering further growth and sustainability of the sector. In recent decades, the enormous expansion of aquaculture practices including intensive stocking and transboundary movement of aquatic animals has paved the way for the emergence of many new pathogens [2]. The outbreak of certain diseases such as white spot syndrome virus (WSSV), early mortality syndrome, hepatopancreatic microsporidiosis (EHP), infectious salmon anaemia (ISA), tilapia lake virus (TiLV) and spring viremia of carp (SVC) has caused catastrophic losses in aquaculture [7]. ISA caused an economic loss of approximately US $1.0 billion from 2007 to 2011 [48], which could have been saved by the use of appropriate vaccines.

The emergence of new pathogens and detrimental use of antimicrobial drugs have to be addressed to sustain aquaculture production. Fish vaccines play a major role in maintaining the farmed fish welfare and reducing the economic losses by preventing disease outbreak in aquaculture. Vaccination is undoubtedly the most effective intervention for disease prevention and control. However, there is still a need for improving hazardless vaccine delivery methods in aquaculture.

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