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Advances in Aquaculture Vaccines Against Fish Pathogens: Global Status and Current Trends

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ABSTRACT

In recent years, aquaculture has attained a major economic revolution, however, infectious diseases of bacterial, viral, mycotic and parasitic origin are the most significant restrictive agents in the improvement of intensified aquaculture, which has become a fast blooming seafood industry. For environment-friendly aquaculture and human health concerns owing to the rise in incidences of antimicrobial resistant microbes and food safety hazards, the immunoprophylaxis or vaccination strategies are highly effective and economical in protecting the health of fish and aquaculture animals from various infectious agents. Advancements in science have paved newer avenues in both basic and applied research areas for developing and designing novel and effective vaccines, as well as improving existing vaccines for rendering protection from various types of infectious diseases. Current advances in vaccines and vaccinology offer valuable opportunities to discover new vaccine candidates to combat fish pathogens, including mycotic and parasitic agents, for which vaccines are still lacking. This review focuses on the current knowledge, recent advances and future perspectives of vaccines and vaccination in the aquaculture industry, from traditional inactivated and attenuated vaccines to new generation vaccines comprising of recombinant, subunit, vectored, genetically engineered, DNA and peptide vaccines, reverse vaccinology and plant-based edible vaccines, and nanovaccines.

KEYWORDS

Fish; aquaculture; vaccine; infectious diseases

Introduction: Vaccines and their importance in aquaculture

Aquaculture is currently a fastest emerging global food industry. Nevertheless, intercontinental commercial trading and transport of live fishes, their eggs and fish products had increased the risk of global disease transmission in this viable industry due to the various stages of pathogens in fishes (apparently healthy, subclinical or carrier stages) travelling across the countries, which is a major barrier for safe fish production and healthy aquaculture (Khan et al., 2011). One of the major threats to aquaculture is the economical looses imposed by incidences and outbreaks of infectious diseases on account of high mortality in farmed fishes and commercial aquaculture systems. Literature suggested that 54.9% bacterial pathogens, 22.6% viruses, 3.1% mycotic agents, and 19.4% parasitic agents are responsible for periodical disease outbreaks in fish cultures (Dhar et al., 2014). The Office International des Epizooties (OIE)/World Organization for Animal Health has listed certain important diseases including of DNA virus diseases such as epizootic hematopoietic necrosis (EHN), koi herpesvirus disease (KHVD), red sea bream iridovirus disease (RSID), and RNA virus diseases such as infectious hematopoietic necrosis virus (IHNV), infectious salmon anemia virus (ISAV), spring viremia of carp (SVC), and viral hemorrhagic septicemia (VHS) to be the causing major catastrophe for large scale aquaculture industry (Crane and Hyatt, 2011; http://www.oie.int/animal-health-in-the-

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world/oie-listed-diseases-2013/ Llewellyn et al., 2014; Hoseinifar et al., 2014; Thim et al., 2014). The dearth of efficient treatment modules to control viral diseases as well as bacterial infections such as mycobacteriosis (Jacobs et al., 2009), *Piscirickettsia salmonis*, obligate intracellular bacteria have posed a vital demand for developing and implementing appropriate approaches for prevention of these diseases (Wilhelm et al., 2006). Moreover, the fishes are poikilothermal animals hence their vaccination approach needs a special focus on the type of vaccines, fish species to be administered, administration/delivery protocol with specific routes whether immersion, oral or intraperitoneal for enhanced immune response after vaccination (Muktar et al., 2016). Many factors such as intensive aquaculture practices, the introduction of new species, increased trade in ornamental fish, the interaction between wild fish and fish farm, flaring up scenario of emerging and re-emerging diseases and issues with biosecurity gaps along with less knowledge of fish immunology have altogether caused an increase in the losses due to infectious diseases (Adams et al., 2008; Levraud and Boudinot 2009; Hoseinifar et al., 2014). The negative impacts of infectious pathogens have not only demanded developing of various strategies to design newer vaccines to protect aquatic animal health but also expanded the use of antibiotics in the aquaculture for gaining higher fish production and safeguarding aquaculture health; however injudicious and indiscriminate use of antibiotics could make rise problems of developing bacterial resistance, food safety hazards and environmental issues at global levels due to release of antibiotics into the surrounding water during bacterial treatment of diseased fish (Cabello et al., 2016; Hatha et al., 2005; Hoseinifar et al., 2014; Ringø et al., 2014a). Because of increasing antibiotic resistance in fish and its consumer's microflora, significant progress have been made in improving the effectiveness of the existing vaccines as well as developing advanced vaccines and introducing the superior immunomodulatory diet. At the moment, usage of vaccines in aquaculture has shown potential beneficial effects on human health by overcoming the negative effects due to the use of hormones, pharmaceuticals, antibiotics and their residues in human food chain (Meeusen et al., 2007; Ringø et al., 2014b).

Gudding and Van Muiswinkel (2013) in a comprehensive review reported that the early use of fish vaccines was attempted in 1938 by Snieszko and coworkers against *Aeromonas punctata* in carp. They reported induction of protective immunity in fish after injection with killed bacteria in the laboratory (Gudding and Van Muiswinkel, 2013). Snieszko and coworkers did not realize at that time that such immunization approach could be applicable in the future, due to their limited opinion was "*too complicated and time consuming for large scale application in fish farms*." After this innovation, they continued study on vaccination of fish (Snieszko, 1970), application in fish diseases (Snieszko and Hoffman 1978) and fish health management (Snieszko et al., 1980) in the United States. The first report on oral fish immunization was recorded by Duff, who revealed that a diet involving chloroform-killed *Aeromonas salmonicida* in the cutthroat trout enhanced the protection against furunculosis after contact with clinically diseased fish or after challenge by injection inoculation (Duff, 1942). The first U.S. -licensed vaccine against *Yersinia ruckeri* and *Vibrio anguillarum* for reared fish was developed in the 1970s and subsequently introduced into commercial aquaculture in early 1980s (Midtlyng et al., 1996; Shao, 2001). Thereafter since 1990 bacterial vaccines have been used routinely, allowing a reduction of antibiotics application in aquaculture (Brudeseth et al., 2013). Vaccination against Salmon pancreas disease virus (SPDV), also known as salmonid alpha virus (SAV), the causative agent of Pancreas disease (PD) in *Salmo salar* and *Oncorhynchus mykiss*, has been found efficacious because after vaccination the virus shedding was found to be reduced by 80% and 100% viral count in serum and stool, respectively. After vaccination, mortality and outbreak incidences of PD also diminished in vaccinated fishes (Jensen et al., 2012; Skjold et al., 2016; Taksdal et al., 2007). Now, vaccines are accessible for more than 17 fish species and reported to induce protection for more than 22 different kind of bacterial diseases as well as 6 viral diseases in more than 40 countries (Brudeseth et al., 2013).

For strict implementation of vaccination in fish a complete knowledge of fish immune response is required to assess the duration and intensity of protective immunity produced after vaccination in the fish farm. Research studies have reported that innate immunity based on interferon responsive genes (IRGs) for inducing production of interferons plays a primary role in fishes for encountering viral infections. Moreover, IRGs are responsible for significant anti-viral activity and these are conserved set of genes even during the evolution in different fish families including teleosts (Langevin et al., 2013; Verrier et al., 2011; Volff, 2005).

Progress and advancement in the field of immunology, biotechnology, and molecular biology has lead to development of reliable diagnostics for rapid detection of fish pathogens as well as effective and novel vaccines for protecting fish health, which have altogether resulted into a major impact in reducing mortality caused by infectious diseases in fish and aquaculture (Adams et al., 2008; Ballesteros et al., 2014; Dhar et al., 2014; Ji et al.,

2015; Nuñez-Ortiz et al., 2016). Vaccination has always been very successful in reducing the risk of various infectious diseases, but despite extensive research, only few viral vaccines (attenuated, inactivated or recombinant) are available (Salgado-Miranda et al., 2013). Recently, genetic engineering approaches such as production of genetically modified (GM) plants for oral vaccines, transgenic fish, GM feed (based on microorganisms or plants), as well as DNA vaccines have been proposed as novel strategies for controlling infectious diseases of fish (Dunham 2011; FAO and FOODS 2004; Llewellyn et al., 2014). Currently, DNA vaccines or recombinant vaccines approaches against parasitic agents such as *Philasterides dicentrarchi, Cryptobia salmositica*, and *Ichthyophthirius multifiliis* have been reported to be developed. Particularly, a vaccine against the most common infection of fish, White Spot Disease or Ich caused by *Ichthyophthirius multifiliis*, has been found very effective in inducing protective immunity (Jørgensen and Buchmann 2011). Nevertheless, several years ago also the recombinant DNA technology was considered as the best solution for improving vaccines against diseases in fish (Lorenzen, 1999). Immunoproteomic vaccines have also been developed to protect underwater fish culture from Staphylococcosis, characterized by exophthalmia and swollen tail in wild fishes and responsible for high mortality usually. The vaccine is based upon using formalin killed-whole cell and outer membrane proteins of *S. aureus* in concentration of 88 and 55 μg/mL, respectively (Gil et al., 2000; Mumtaj et al., 2016). Until now, a few recombinant vaccines against infectious pancreatic necrosis virus (IPNV), infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), spring viremia of carp virus (SVCV), Salmon Pancreas disease virus, trout sleeping disease alpha virus, and infectious salmon anemia virus (ISAV) are commercially available for fish farmers (Gomez-Casado et al., 2011).

In the following sections, attempts have been made to review current progress and trends in designing and development of effective vaccines, various strategies of vaccinations and advances in fish vaccinology as well as the advantages and disadvantages of different types of vaccines being exploited in aquaculture. The compilation describes research advances from developing inactivated and attenuated vaccines to progress being made for designing new generation vaccines comprising of recombinant vaccines, subunit and vectored vaccines, virus like particles, genetically engineered vaccines, DNA/RNA vaccines, peptide vaccines, reverse vaccinology derived vaccines, plant-based edible vaccines, and nanovaccines. Along with these, advances in vaccine delivery methods including of oral, injectable and immersion vaccination, monovalent and polyvalent vaccine formulations, use of novel adjuvants and potent immune-enhancing/immunomodulatory strategies, large-scale commercialization and marketing avenues have also been presented. Some of the concerned safety issues, side effects and limitations have also been discussed in brief. The information compiled would be helpful for researchers, academicians, fish/aquaculture industry workers/owners/entrepreneurs, market holders and pharmaceutical experts to collaborate jointly for devising a comprehensive health management strategy with development and implementation of effective, reliable, economical, validated, user friendly fish prophylactics, vaccines and vaccination strategies offering wider protection range against a broad spectrum of infectious diseases with suitability for both fish juveniles and adults, having ease of administration and ability to provide long-lasting protective immunity to encourage and support the healthy, profitable, and sustainable commercial aquaculture.

Types of vaccines in aquaculture

Several kinds of vaccines are being used in aquaculture, which are being described in following section and also summarized in Table 1 and Figure 1.

Conventional/traditional vaccines

Inactivated vaccines

Traditionally, inactivated pathogens have been used for vaccination in fish. These are produced by multiplication or replication of the pathogens in large quantities and then subjecting to inactivating agents such as formalin, which kills the entire microorganisms without affecting the induction of protective immunity of the vaccine candidates. The effectuality and biosafety of such vaccines relies on the cultivation conditions such as the kind of the media and range of temperature. Most of the applicability of bacterial vaccines in aquaculture have been recorded as usage with inactivated vaccines obtained from a broth culture of specific strains exposed to subsequent formalin inactivation (Toranzo et al., 2009). Different inactivating agents variably affect the efficacy of inactivated vaccine and duration of protective immunity produced post-vaccination. One study reported that when β-propiolactone (BPL), binary ethylenimine (BEI), formaldehyde and temperature/heat were used as inactivating agents for infectious haematopoietic necrosis virus (IHNV) in rainbow trout *(Oncorhynchus mykiss*), the BPL inactivated IHNV whole virus vaccine illustrated maximum efficacy comparatively (Anderson et al., 2008; Tang et al., 2016). The best results have been obtained with bacterins that comprised of both bacterial cells and

Table 1. Types of vaccines in the aquaculture.

Type of vaccine	Antigens	Name	Fish host	Delivery method	Advantage	Disadvantage	References
Killed or inactivated vaccine	IPNV	Alpha Ject ® 1000	Salmon	IP Injection	Amenable to autogenously	Too costly and less than satisfactory for viruses	(Biering et al., 2004; Adams et al. 2006; Salgado-Miranda et al. 2013, Jang et al. 2014)
	SVCV	Bioveta,	Carp	IP Injection	Easy administration	Adhesions associated with adjutants	
	A. salmonicida, *V. anguillarum*, *V. salmonicida*	MULTIVaC, Microtek,	Salmonid	IP Injection	Safe for use		
Attenuated live vaccine	*R. salmoninarum* (enteric septicemia of catfish disease)	Renogen	Salmon	Immersion	Can replicate, induce cellular and humoral immunity	Safety concerns both in terms of the vaccinated animals and in terms of environmental aspects	(Shoemaker et al., 2009; Buchanan et al. 2006; Sun et al., 2010; Liu et al. 2015; Lawrence et al. 1997; Adams et al., 2008)
		AQUAVAC- ESC	Catfish	Immersion	Do not require an adjuvant	Danger for reversion to virulence	
					Mimic natural infection and immune response	Not good a stimulating innate immunity	
					Amenable to immersion		
Recombinant protein	IPNV/VP2	Microtek	Salmon	IP Injection	Ability to produce sufficient quantities of the protective proteins	Disturbance in glycolysation of the proteins and restoration of the tertiary structure	(de Kinkelin 1994; Dhar and Allnutt 2011,
	SVCV	International Inc.	Carp	IP Injection	Safe and low cost method		Adams et al. 2006; Salgado-Miranda et al. 2013)
	Salmon Rickettsial	Pharos, S. A., Microtek, Bayovac 3.1	Salmonid	IP Injection			
Vector technology	ISA	*In vitro*	Salmon	IP Injection	High levels of heterologous antigen expression in the cytoplasm	Lack of data regarding field performance	(Phenix et al., 2000; Adams and Thompson 2006)
	IHN	*In vitro*	Salmon	IP Injection	Low-level vector protein expression, induction of apoptosis in infected cells		
	IPNV	*In vitro*	Rainbow trout	IP Injection	Biosafety		
				Safety and ease of	production		
Genetically attenuated pathogen	*Aeromonas salmonicida*	Brivax II	Rainbow trout	IP Injection	Induce of cell mediated, humoral, and mucosal immunity	Possibility of back-mutate in attenuated strain to virulent wild type	(Vaughan et al., 1993; Liu et al., 2015,
Live non pathogenic recombinant microorganism	IHNV	*In vitro*	Rainbow trout	IP Injection	Low cost of production	Limiting their potential use as GMO	(Adams et al. 2006; Salgado-Miranda et al. 2013; Gómez et al., 2015)
	VHSV	*In vitro*		IP Injection			
	IPNV	*In vitro*		Oral			
DNA vaccine	IHNV	Aqua Health Ltd,	Salmon	IM Injection	Induce humoral and cellular immunity	Some obstacles limiting the potential uses of DNA vaccines such as:	(LaPatra et al., 2001; Meeusen et al., 2007; Kurath, 2008; Adams et al., 2008; Ballesteros et al., 2014)

	IPNV	Novartis,	Rainbow trout	IM Injection	Possibility for construct a vector encoding several antigens	Some pathogens possess non-protein immunogens	
Pancreatic Disease (PD)	*In vitro*	Rainbow trout	IM Injection	Possibility to create vaccines for targeted diseases	Chance of an immune response against the DNA itself, or the DNA delivery vector		
		In vitro					
Synthetic peptide vaccine	IHNV, Nodavirus, VHS,	*In vitro*	Rainbow trout	IP Injection	Possibility for construct a vector encoding several antigens	Lack of data regarding field performance	(Fridholm et al., 2007; Coeurdacier et al., 2003; Emmenegger et al., 1994; Estepa et al., 1999)
	Rhabdovirus and IPNV						

IP: Intraperitoneal, IM: Intramuscular.

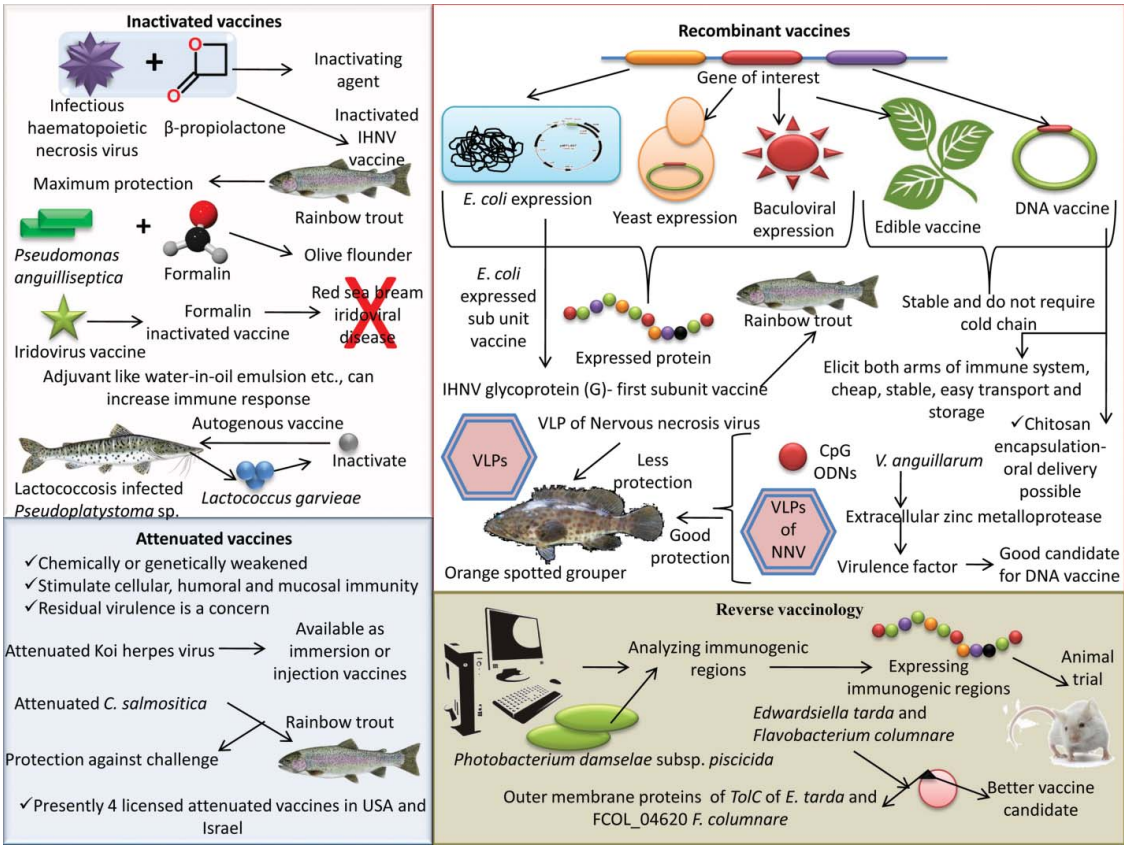

Figure 1. Various vaccine platforms available and their advantages for use in aquaculture.

extracellular products. Bacterins with antigens of gram-negative organisms like *Vibrio anguillarum, Vibrio ordalii, Vibrio salmonicida, Aeromonas salmonicida* subsp. *Salmonicida*, and *Yersinia ruckeri* have been produced by broth fermentation and subsequent inactivation by formalin (Gudding et al., 1999; Shao, 2001; Toranzo et al., 2009). The formalin-killed cells of *Pseudomonas anguilliseptica* could play an important role in immunization of olive flounder against this bacteria (Jang et al., 2014). For viral pathogens, the development of killed vaccines has been too costly and experienced to be less than satisfactory (Nishimura et al., 1985). Also, few of the fish viruses are not easily propagated in cell culture, such as Lymphocystis disease virus of Iridoviridae family, hence development of inactivated vaccines for such viruses still remains a constraint (Lewis and Leong 2004). There is an injectable formalin-inactivated vaccine available to control the red sea bream iridoviral disease (RSIVD), which is a significant cause of mortality among cultured marine fish. This vaccine is commercially accessible in Japan as formalin-inactivated Iridovirus streptococcosis-vibriosis combined vaccine for use in marine fish (Dhar et al., 2014; Nakajima et al., 1999). Also, for some viral pathogen like ISAV, IPNV, SVCV, and salmon alphavirus (SAV), commercial inactivated vaccines are available and licensed to be used in Chile, Finland, Ireland, Norway, Canada and UK (Biering et al., 2004). Studies suggested that inactivated virus vaccine using antigen ALV405 of SAV is capable of protecting the salmonid fishes from infection of Pancreas disease (PD) efficiently either with usage as a single vaccine candidate or as polyvalent vaccine (Jang et al., 2014; Karlsen et al., 2012). Nevertheless, long-lasting protection by the inactivated vaccines can only be obtained when adjuvants are adjuncted to elevate the immunological strength and presentation of the vaccine formulation (Jang et al., 2014).The vaccines for white spot disease which is caused by *I. multifiliis*, contains the inactivated three developmental stages of the parasite: trophonts, tomonts and theronts, consisting of surface immobilization antigens (I-antigens), which has been proven to be efficacious in laboratory experiments (Dickerson and Findly 2014). Nevertheless, vaccination with I-antigens is protective only against *I. multifiliis* expressing the same I-antigens on their surface (Xu et al., 2009). Earlier the salmonid vaccine formulation comprised primarily the water based vehicle, while oil based adjuvants was introduced only early in 1990s which resulted in significant reduction of outbreaks of infectious diseases. The high efficacy and long duration of protection that the water-in-oil emulsion adjuvants induce have been essential for the growth of salmonid aquaculture (Brudeseth

et al., 2013). Inactivated vaccines must be administered by inoculation to obtain protective immunity, but for some viral diseases, it may not be a practical approach because the diseases occur in the early stages of life (Leong et al., 1988). A recent study was conducted with formalin, β-propiolactone (BPL) and heat treatment of Betanodavirus, causal agent of viral encephalopathy and retinopathy (VER) disease in European sea bass. Formalin killed vaccine exhibited greater potency when injected via intraperitoneal route (Nuñez-Ortiz et al., 2016). In another study on IHNV vaccine, researchers have shown the better inactivating effect and immunogenicity with the use of BPL compared to formaldehyde and binary ethylenimine (BEI) (Tang et al., 2016). Killed vaccines are commonly advised to be safe for administration in aquatic animals, however, the insufficient inactivation due to incompetent killing of the vaccine strain sometimes may be problematic and hence delivery of viable disease causative pathogen may occur instead of a potent vaccine candidate. Another problem connected to killed vaccines is the fish adhesions following injection of such vaccines along with the oil-adjuvants, which induces diminished growth in the vaccinated fish and results in loss of quality products due to the side effects of adhesions (Evensen et al., 2005). In some cases, autogenous vaccination seems to control the spread of disease to other fishes in aquaculture. For example, Lactococcosis, which is caused by *Lactococcus garvieae* in *Pseudoplatystoma* sp., outbreak in Brazil has recently been controlled through vaccination with whole-cell inactivated water-based and oil-adjuvant based bacterin autogenous vaccines (Fukushima et al., 2016).

Attenuated vaccines

These vaccines are not inactivated but chemically or genetically weakened; therefore, they are live and induce immune response in the host for a short period of time (Adams et al., 2008). Attenuated vaccines have great potential in aquaculture. The evaluation and application of attenuated or modified live bacterial vaccine in aquaculture was started in the 1990s (Lawrence et al., 1997; Shoemaker et al., 2009; Sun et al., 2010; Thornton et al., 1994). Attenuated vaccines for fish need to undergo severe testing before licensing. Vaccination with an attenuated vaccine is a simulation model of an infection and if vaccinated fish could spread the vaccine strain, a distribution of the antigen in the population would happen over a prolonged period of time. These vaccines have the advantage that they stimulate the cellular immunity significantly. Also, they are capable of stimulating the humoral and mucosal immunity (Clark and Cassidy-Hanley 2005) and typically stimulate a potent and continuous immunity to the related disease. Presently there are only four modified live vaccines that are licensed to be administered in the United States and Israel. These comprise of the vaccine against bacterial kidney disease, enteric septicemia of catfish disease and columnaris disease in the United States (Shoemaker et al., 2009), and one viral vaccine for Koi herpesvirus (KHV) for carp in Israel (Adams et al., 2008). Attenuated viral vaccine against KHV is available with the trade name of KV-3/ Cavoy from KoVax Ltd., Jerusalem, Israel for immersion or injection route and can be used in Israel and USA. Similarly, attenuated viral vaccine against spring viremia of carp virus is also used in China via immersion route (Dhar et al., 2014). In the laboratory, an attenuated strain of *Cryptobia salmositica* was used as a live attenuated vaccine in rainbow trout and protected the fish under *in vitro* challenge condition (Woo and Ardelli 2014). Safety is the major concern of using modified live vaccines. Several research groups have suggested that practical administration of attenuated or avirulent forms of the virus could be undesirable because of the residual virulence in targeted species could spread the virulence in non-target species (Dhar and Allnutt 2011; Salgado-Miranda et al., 2013; Shao, 2001).

Advances in designing and developing vaccines and vaccinations strategies

In conventional/traditional vaccines, the antigens might be weak and could not induce desired protective immune response, there could be risk of reversion to virulence and other limiting factors as discussed in earlier section. These cannot be developed quickly against evolving and emerging pathogens showing antigenic variations, during certain changes in host invasion and events of immune evasion by pathogens, microbes which cannot be grown by *in vitro* propagation and development of these vaccines is a slow and time consuming process, which sometimes poses difficulty in timely countering of emerging and re-emerging pathogens. Therefore, novel methods were needed for discovering newer types of effective vaccines that can be developed from the advances made in genetics, immunology, chemistry, biotechnology and molecular biology (Delany et al., 2014; Effio and Hubbuch 2015; Finco and Rappuoli 2014; Singh et al., 2015). Over the last few years, gene sequences of bacterial, viral and metazoan genomes, combined with the knowledge on gene functions and derived proteins have evolved novel methods for fish vaccination. The following new approaches are being summarized.

Recombinant vaccines

Advent of biotechnology has led to the development of recombinant vaccines where only the immunogenic regions of a pathogen are expressed in heterologous host and are used as vaccine (Adams et al., 2008). Recombinant proteins as vaccine antigens have been demonstrated to give useful protection against various kinds of human and animal pathogens (Diane Williamson et al., 1995; Wilhelm et al., 2006) that have been expressed in *Escherichia coli* (Gilmore et al., 1988; Lorenzen et al., 1993), yeast (Allnutt et al., 2007) and insect cells (Cain et al., 1999; de Kinkelin, 1994) to induce protective immunity against a targeted pathogen. This kind of protein is expressed in prokaryotic (Noonan et al., 1995) or eukaryotic cells (Lecocq-Xhonneux et al., 1994) under strictly controlled laboratory conditions by fermentation methodology. Recognition of the gene sequence of pathogen's protective antigen is important for designing a recombinant protein. This antigen can be inserted into a production host and can be cultured on a large scale; from which the protective antigen is purified and used in vaccine formulation (Adams et al., 2008). There are various kinds of expression systems such as bacteria (Noonan et al., 1995), cell culture (Acosta et al., 2006), yeast (Vakharia, 2008), insect cells (Lecocq-Xhonneux et al., 1994), microalgae as well as transgenic plants (Muktar et al., 2016). The immunity induced by the administration of recombinant antigens produced through fermentation has been found inefficient, perhaps due to poor immunogenicity (Leong et al., 1997; Lorenzen and Olesen 1997). For protein antigen-based vaccine, immuno-proteomics could be an approach of choice (Connolly et al., 2006; Rodríguez-Ortega et al., 2006). Division and characterization of multiplex compound of proteins by two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (2D SDS-PAGE) reveals valuable data about the proteins expression of bacterial pathogens (Chen et al., 2004). Further western blot analysis by using serum of the infected fish, or serum of recovered fish from the disease can identify antigens recognized by infected host immune system (Chen et al., 2004). Therefore, these two approaches can support reorganization of potential candidates for development of effective vaccine (Chakravarti et al., 2000; Mäkelä, 2000). It is practical to investigate the elicited protection by related antigen through fish vaccinating with the antigen and then experimentally infecting fish with live pathogen, which could reveal survival level in vaccinated fish (Irie et al., 2005). Another essential component in the development of vaccine is the capability to produce adequate quantities of defensive proteins required for commercialization of the vaccine. Now-a-days researchers are employing recombinant DNA technology to improve protein-based vaccines due to its economy to cultivate adequate quantities of the immuno-protective antigens (Sun et al., 2009; Wilhelm et al., 2006). These vaccines have vital and potent effects in the industry of aquaculture since they yield an alternative pathway to conventional formalin-killed vaccines. These are also safe and economical in comparison to live attenuated bacteria-based vaccines, which may revert to pathogenic agents (Clark and Cassidy-Hanley 2005). The first subunit vaccine for rainbow trout (*Oncorhynchus mykiss*) was developed by including IHNV glycoprotein (G) expressed in *E. coli* (Gilmore et al., 1988). The company Pharos, S. A., Belgium introduced an intraperitoneal injectable subunit vaccine against carp virus (spring viremia) by including recombinant G protein expressed in baculovirus expression system. In a study carried on VHS virus, it has been observed that the recombinant G protein of VHS virus can induce moderate levels of neutralizing antibody (Lorenzen et al., 1998). Several researcher also have used recombinant protein technology for some pathogens, such as *I. multifiliis* (Dickerson and Findly 2014; He et al., 1997), *A. hydrophila* (Poobalane et al., 2010), grouper nervous necrosis virus (GNNV) (Liu et al., 2006; Tanaka et al., 2001), *Piscirickettsia salmonis* (Wilhelm et al., 2006), grass carp reovirus (He et al., 2011; Lu et al., 2011) to induce protective immune responses against respective viral and bacterial diseases. Also, immunization of tilapia (*Oreochromis* sp.) with the feed-based recombinant vaccine has showed significant ($p < 0.05$) elevation of IgM antibody in mucus, serum and gut lavage fluid samples (Nur-Nazifah et al., 2014). Recombinant vaccine comprising of infectious pancreatic necrosis virus (IPNV) VP2 based vaccine from Microtek International (Canada), and ISAV hemagglutinin-esterase gene based vaccine from Centrovet (Chile) have been successfully developed and used in the market (Frost and Ness 1997).

Two subunit vaccines have been developed against IPNV; one utilizing VP2 and VP3 capsid proteins, marketed as AquaVac and IPN Oral manufactured from Merck Animal Health, New Jersey, USA which can be used in Canada by oral route administration, while the second type of subunit vaccine which is readily injectable via intraperitoneal route, uses VP2 capsid protein and is commercially available as Norvax and Minova-6 from Intervet-International BV, Netherland (Dhar et al., 2014). Bremont (2005) used reverse genetics technology over rhabdo viruses of rainbow trout to design recombinant live viruses with modified viral genome to produce live, safe and economical vaccines.

Vector technology

This method is very similar to recombinant technology but utilizes mainly viral production hosts which express the protein of another pathogen as a vaccine antigen (Adams et al., 2008; Bråve et al., 2007). The capability of viral structural proteins to self-assemble into vectors with nature of native virus have resulted into the improvement of this class of subunit vaccine based on virus-like particles (VLPs) (Dhar and Allnutt 2011). Some expression systems for VLPs include bacteria, yeast, baculovirus/insect cells, and transgenic plants (Dhar and Allnutt 2011). Among these expression systems, the baculovirus/insect cells or insect larvae expression system has proven to be an improvement approach for fast expression of plentiful recombinant proteins and is suggested to be an inexpensive and efficient method for producing heterologous proteins (Adams and Thompson 2006; Hu et al., 2008; Shivappa et al., 2004). The Baculoviruses (family Baculoviridae) are the large double-stranded enveloped viruses consisting of circular DNA genomes of about 80–180 kbp in size and are considered good protein expression systems using insect cell lines. Several research groups have used the baculovirus-expressed antigens and have demonstrated partial to complete protection against IHN (Laurent et al., 1994), VHS (de Kinkelin, 1994), IPNV (Shivappa et al., 2004), and GNNV. The IPNV capsid proteins, namely VP2 and VP3, were expressed separately or as a polyprotein and these expressed proteins formed VLPs similar to original virus thereby inducing stronger immunity (Dadar et al., 2015; McKenna et al., 2001). For IPNV, few researchers have demonstrated that the administration of IPNV-VLPs in rainbow trout elicited immune responses similar to that evoked by live viral infection (Dadar et al., 2015; Martinez-Alonso et al., 2012; Shivappa et al., 2004). Capsid protein VP2 of IPNV based vaccines are already being marketed by three manufacturers namely Norvax (Intervet-International BV, The Netherlands), IPNV (licensed in Chile, Centrovet, Chile), and SRS/IPNV/Vibrio (licensed in Canada and Chile, Microtek International Inc., British Columbia, Canada) (Dhar et al., 2014). Recently, VLPs for Nervous necrosis virus (NNV) has been developed for orange-spotted grouper and the results revealed that VLPs can protect against NNV but the efficacy was less, which has been suggested to be improved by addition of CpG ODNs (Lin et al., 2016).

One of the novel and important expression vectors for delivering heterologous genes is alphavirus expression vector, which is a beneficial tool in vaccine development, and for anti-cancer and gene therapy strategies (Brun et al., 2008; Wolf et al., 2012). Three prototypes of alphavirus-replicon vectors are the Sindbis virus (SINV), the Semliki forest virus (SFV) and the Venezuelan equine encephalitis virus (VEEV) (Olsen et al., 2013; Phenix et al., 2000; Strauss and Strauss 1994). Alphavirus-based replicon immunization strategy would be a protected vaccination approach due to no recombination or spread to other cells occurs after initial replication (Olsen et al., 2013; Wolf et al., 2012). Furthermore, a fascinating property of the particle of alphavirus replicon is the potent ability to improve mucosal immunity, as shown for SFV (Chen et al., 2002b). Alphavirus-replicon plasmids where the virus structural genes of the 3′-ORF have been replaced by gene of interest (GOI) provide a replicon capable of expressing the GOI when introduced into cells (Olsen et al., 2013) and may produce 200,000 RNA copies of GOI RNA molecules (Strauss and Strauss 1994). Vector of SFV was used to demonstrate expression of IPNV antigens which formed VLPs, but never tested as this virus does not replicate in fish (McKenna et al., 2001). In spite of this, salmonid alphavirus (SAV) replicon vectors would be ideal and safe for fish immunization because these vectors are functional in cells from a wide range of animal classes and expresses GOI in the temperature range of 4 to 37°C (Biacchesi, 2011; Olsen et al., 2013). This shows the versatility of the SAV replication machinery and potential use of the SAV replicon as an immunization-vector in aquaculture. For example, Wolf and colleagues reported the administration of a SAV based replicon expressing the ISAV hemagglutinin-esterase for designing candidate vaccine against ISA in Atlantic salmon (*Salmo salar*) (Wolf et al., 2012). Furthermore, as it potential administration of channel catfish virus (CCV) (*Ictalurid herpesvirus 1*) as a vector vaccine has been demonstrated to be useful for developing channel catfish vaccine by inserting the *E. coli* lacZ gene into the CCV genome (Zhang and Hanson 1996).

Genetically attenuated pathogens

Production of live-attenuated vaccine by utilizing genetic engineering is typically done by deletion, disruption or insertion of metabolic pathway or virulence gene that causes an attenuation in pathogen (Meeusen et al., 2007; Shoemaker et al., 2009). The resulting mutant pathogen then serves as an avirulent pathogen induceing a protective immune response, yet it does not cause disease (Adams et al., 2008; Liu et al., 2015; Ma et al., 2010). Immunization with such live-attenuated vaccines unavoidably infers the release of recombinant organisms into the surrounding environment (Vaughan et al., 1993). Related safety concerns to the vaccinated animals as well as for the environmental aspects is likely the major reason for these vaccines not being accepted and or gaining any more attention in aquaculture (Marsden et al., 1998). The

live vaccines against *E. ictaluri* and *F. columnare*, including rifampicin-resistant strains, have been attenuated by serial passages of the virulence wild type parent bacteria on increasing concentrations of the synthetic antibiotic, rifampicin (Klesius, and Shoemaker 1999; Shoemaker et al., 2009; Sommerset et al., 2005). The loss of virulence was linked with genetic alterations in their LPS (Klesius, and Shoemaker 1999; Shoemaker et al., 2009).

Attempt of transposon mutagenesis were employed to produce an O-polysaccharide deficient isolate of *E. ictaluri* to be applicable as a modified live vaccine (Lawrence and Banes 2005; Lawrence et al., 1997). Some researchers revealed that the improvement of an attenuated *E. tarda* vaccine by creating an *E. tarda* mutant (transposon mutagenesis) with low production for siderophore protected tilapia, *Oreochromis niloticus*, upon lethal challenge (Igarashi and Iida 2002). In one of the study, the aroA gene (Brivax II) of *A. salmonicida* was completely deleted, then making it suitable for developing a commercial genetically attenuated vaccine (Marsden et al., 1998). In another study, mini-Tn5 (transposon mutants) promoted growth of protease-deficient *A. hydrophila* and showed utility for use as modified live vaccines in blue gourami (*Trichogaster trichopterus*) (Leong et al., 1997). Nevertheless, the autogenic vaccine strains were not entirely attenuated in blue gourami using this approach. Random transposon (Tn917) mutagenesis and subsequent screening in hybrid striped bass (*Morone chrysops* × *M. saxatilis*) induced a *Streptococcus iniae* with a disrupted phosphoglucomutase gene (Buchanan et al., 2006). It is believed that the phosphoglucomutase enzyme is essential for formation of polysaccharide capsule in bacteria. The presence or absence of capsule has been postulated to be essential for virulence (Barnes et al., 2003; Buchanan et al., 2006). Use of autotrophic mutant is one of the more common strategies that could create attenuated vaccine isolates (Hoiseth and Stocker 1981; Lawrence and Banes 2005; Moral et al., 1998; Temprano et al., 2005; Stocker et al., 1983; Vivas et al., 2004). Unfortunately, immunity of this vaccine persists for a short duration (24–72 hours), and hence cannot induce sufficient immunity in young fish (Chen et al., 2004). Recently, Liu et al. (2015) successfully constructed a live attenuated *Vibrio anguillarum* vaccine without marker gene (or vaccine with unmarked gene deletion) and reported that this vaccine induced both innate and adaptive immune responses via bath vaccination. Marker less deletion system is the novel improving field for expansion of global live vaccine, which has received more attention and a major development aspect in this field. Finally, this approach for vaccine development reliably meet with environmental safety standards (Gómez et al., 2015; Liu et al., 2015).

Nonpathogenic recombinant microorganisms bearing foreign pathogen genes

Live vaccines, in both forms of attenuated pathogens as well as in the form of microbial vectors bearing the components of vaccine such as for *A. salmonicida* (Noonan et al., 1995; Vaughan et al., 1993) possibly promote a potent immune response than non-replicating products (Marsden et al., 1998). A virulent isolate of *A. salmonicida* (A440) that is applicable as a vector for epitopes of IHNV and VHSV can be considered as a live vaccine for rainbow trout (Magnadottir, 2010). Assembling systems for bacterial antigen-delivery to display foreign protein in attenuated *V. anguillarum* for vector vaccine design is a crucial aspect (Xiao et al., 2011; Zhou et al., 2010). In addition to *A. salmonicida*, there are safe bacteria strain namely lactic acid bacteria (LAB) that can be bio-engineered as vectors to deliver and express medical protein and viral antigen (VP2 and VP3 IPNV) in the mucosal immune system (Li-Li et al., 2012) and protect fish from pathogens. This orally applied vaccine has additional advantages, but based on European Union (EU) and other guidelines, such organisms are categorized as genetically modified organisms (GMO), thus limiting their potential application. Despite cost effective protection achieved by live recombinant vaccines against several diseases, such vaccines have not yet been commercialized for the aquaculture industry (Lorenzen, 1999).

Vaccines based on naked DNA (DNA vaccines)

Use of immunogenic genes through DNA or RNA has been considered as the next-generation vaccine approaches of scientific improvement following the prophylactic or therapeutic administration of recombinant proteins (Dhama et al., 2008; Gillund et al., 2008; Heppell and Davis 2000). Vaccination via DNA method with plasmids holding a required antigen of pathogen under the control of eukaryotic promoters has attained wide attention in their utility to promote protective immunity against numerous diseases in fish (Donnelly et al., 1996; Ogas Castells et al., 2015; Robertsen et al., 2016). A plasmid DNA (pDNA) act as a gene delivery vehicle for mammals and fish. The pDNA is raised in microorganisms such as bacteria, purified and dissolved in a saline solution before injection to the host, commonly by an intramuscular injection (IM), and has potent immunization applicability. These vaccines produce a non-specific and early immune response followed by a later, specific immunity, but the exact protective pathways remained unknown in fish (Kurath, 2008; LaPatra et al., 2001). The best usage of DNA vaccines is reflected in gene therapy,

metabolic and inherited disorders and developing prophylactics for various diseases. Moreover, DNA vaccines have been used for the induction of immunity in mammals and fish that are capable to overcome some of the limitation of other modalities of vaccination. Added to this, DNA vaccines are stable and do not need a cold chain (Ballesteros et al., 2014; Meeusen et al., 2007). High levels protection against IHNV and VHSV infections can be evoked by intramuscular injection of viral genes encoding surface glycoproteins (Anderson et al., 1996; Hølvold et al., 2014; Lorenzen et al., 1998; Purcell et al., 2006). An effective immune response has been shown following DNA vaccination by VHSV glycoprotein in rainbow trout (Utke et al., 2007, 2008). In spite of development of effective DNA vaccines against VHS and IHN (Hølvold et al., 2014), some DNA vaccines could not promote significant protection against disease (Kurath and Midtlyng 2005). This phenomenon may be related to the inherent capability of rhabdovirus G proteins to promote antiviral responses while the pDNA involving transgene products of other viruses do not evoke immune responses to the desired levels of protection against the disease (Acosta et al., 2006; Yasuike et al., 2007). Injection of channel catfish and rainbow trout through intramuscular method with DNA vaccines containing plasmid vectors expressing i-antigen genes has been found to induce serum antibodies as identified by western blot technique (Dickerson and Findly 2014; Piazzon et al., 2014). Also, DNA vaccines have been reported to induce different levels of protection against other important viruses such as the IPNV with usage of VP2 gene (Cuesta et al., 2010; de las Heras et al., 2009, 2010). Moreover, *V. anguillarum* is a bacterial pathogen in fish that has extracellular zinc metalloprotease, which is a known virulence factor for this pathogen, but on the other aspect, this toxin has been shown to be a potent candidate antigen for developing a DNA vaccine (Chen et al., 2002a; Denkin and Nelson 2004; Milton et al., 1992; Norqvist et al., 1990; Shao, 2001). The fish receiving DNA vaccine revealed improved immune response to a challenge with live *V. anguillarum* four weeks post-injection, as reported by elevated survival of fish and diminished histopathological changes in vaccinated group as compared to the control group (Yang et al., 2009).

The Norwegian Biotechnology Advisory Board determines animals gene therapy as: "*The intentional transfer of genetic material to somatic cells for purposes other than influencing the immune system*" (Smith and Klinman 2001). Always, gene therapy causes a long-lasting expression of the gene, without immune system evoking. Oppositely, DNA vaccination is known as the designed genetic material to transfer to somatic cells for the aims of manipulating the immune system (Verma and Somia 1997). For DNA vaccination, an expression of gene with short-term duration is adequate for activation of immune response (Davis, 2001). This naked DNA is usually translated into immunogenic protein by the host cell and expressed on the surface of cell. The occurrence of an antigen in connection with surface molecules of host cell will potentially activate an efficient immune response against the related antigen (Adams et al., 2008).

Oral delivery of DNA vaccines has also been developed with encapsulation using suitable carrier. Chitosan encapsulated DNA vaccine against nodavirus (NNV) has been developed for protecting European sea bass juveniles against NNV and improving their survival post infection (Valero et al., 2016).

There are several reasons for investigating the possible advantages and limitations of using DNA vaccines in aquaculture. These include levels of immunological responses after injection with DNA vaccine, the potency of the vaccine with respect to organization, expression and integration as well as diffusion in the environment (Myhr and Dalmo 2005). Site and level of gene expression has been revealed to be associated on the administration volume (Anderson et al., 1996; Heppell et al., 1998), dose (Anderson et al., 1996; Hansen and Strassburger 2000), age (Hansen and Strassburger 2000), and fish size (Heppell et al., 1998; Tonheim et al., 2007). Moreover, variance in levels of stress, conditions of growth and exposure to other pathogens are other factors that influence vaccine efficacy (Lorenzen and LaPatra 2005). Another aspect of DNA vaccine is that vaccinated fish are considered as a genetically modified organism (GMO) in some countries and public health feature of GMOs as food and minor regulatory legislation from the authorities might cause challenges for the aquaculture industry. Few limitations of DNA vaccines comprise of immune tolerance against the expressed antigen, chromosomal integration, risk of autoimmunity, inflammation in the injection site and tissue damage (Hølvold et al., 2014).

The most important benefits of DNA vaccination is that pDNA, such as live or attenuated viruses, efficiently promote humoral and cell-mediated immune reactions. Another benefit comprises of organization of a vector encoding numerous antigens that could be given in a single administration, and thus creating possibility of a vaccine for multiple diseases (LaPatra et al., 2015; Lorenzen et al., 2002). Also, DNA vaccines are relatively cheap, procedure is not much complicated and can be produced via identical production processes (Hølvold et al., 2014). They are very stable in dried powder or in a solution, unlike traditional vaccines that often require storage at appropriate temperature conditions such as cold

environments. Administration of DNA vaccines without adjuvant induce much less direct tissue damage and/or inflammatory reactions compared to traditional oil-adjuvant vaccines (Garver et al., 2005; Kurath et al., 2006), and with no systemic toxicity (Parker et al., 1999). The most significant administration of DNA vaccine approach is the potential for producing vaccines for targeted diseases.

Synthetic peptide vaccine

Synthetic peptides can be used as suitable antigenic site or to serve as a subunit vaccine (Coeurdacier et al., 2003; Tam, 1988). Studies have been carried out by some scientists to find whether synthetic peptides could stimulate antibody production for IHNV, nodavirus, VHS, rhabdovirus and IPNV, birnavirus (Coeurdacier et al., 2003; Emmenegger et al., 1994; Estepa et al., 1999; Fridholm et al., 2007). The results of these studies showed that although vaccinating fish with peptides is possible, but this approach is limited because of the need for greater fundamental knowledge about fish immune mechanisms to different antigens.

Reverse vaccinology

Advancement in biotechnology has led to newer technologies in the recent years bringing into focus latest vaccinology termed as reverse vaccinology. This newer tool aids in designing vaccines against infectious pathogens that are difficult to design and may take years to bring out a successful vaccine. This concept utilizes bio-informatics approach to predict the sequences that are immunogenic. This concept has reduced the time for vaccine production from 5–10 years to 1–2 years (Rappuoli, 2000). Immunogenic regions predicted by software are expressed as recombinant proteins and later these antigens are screened *in vitro* for safety, potency, and immunogenicity testing. Reverse vaccinology has been gaining importance in human and animal health and now this concept has also reached recently to marine species where *Photobacterium damselae* subsp. *piscicida* causing significant problems in aquaculture has been studied for reverse vaccinology (Andreoni et al., 2016). Software aided vaccine designing has been attained for two important intracellular fish pathogens namely *Edwardsiella tarda* and *Flavobacterium columnare* that cause edwardsiellosis and columnaris, respectively (Mahendran et al., 2016). A recent study was conducted using immunoinformatics approach where T-cell epitopes were identified leading to development of novel peptide vaccines. Outer membrane proteins (omp) genes namely *TolC* of *E. tarda* and FCOL_04620 of *F. columnare* were analyzed for their immunogenic potential using software (Mahendran et al., 2016). *Streptococcus agalactiae* causes major problem in aquaculture leading to great economic loss. Recently, major surfome and secretome profile of this pathogen has been analyzed which showed 6 surface-associated and secretory proteins to be good vaccine candidates. By this approach protective antigens of *S. agalactiae* has been identified which will aid in better vaccine design (Li et al., 2016).

Adjuvant technologies

The word adjuvant is derived from the Latin word '*adjuvare*' that means to help (Adams et al., 2008; Anderson, 1992). Adjuvants are chemical or biochemical compounds that help an antigen to induce a protective immune response and are usually mixed and injected with antigen preparation. For two reasons, adjuvants can be used in aquaculture. First, for enhancing the immune response to a vaccine, and second as a stimulator of non-specific defense mechanisms which could induce protection against a wide range of pathogens (Ellis, 1988). Different substances like Freund's complete and incomplete adjuvants, light oils and bacterial lipopolysaccharides have been revealed to promote production of antibodies in fish when mixed to bacterins. Administration of vaccines accompanied by adjuvants such as glucans, alum or mineral oil-like substances caused more effective protection (Leong et al., 1997). In fish vaccinology, adjuvants are currently used in salmon industry and almost all salmon are injected with oil-adjuvanted vaccines. There are many kinds of oil-containing emulsion like water-in-oil (W/O), oil-in-water (O/W), water-in-oil-in-water (W/O/W) and oil-in-water-in-oil (O/W/O) emulsion, and of these W/O is mainly administrated for fish vaccination purposes because of inducing long-term protection (Aucouturier et al., 2001). Antigens contained in a watery suspension are incorporated in oil to produce W/O emulsion, that induce highly effective and long lasting protection due to slow release of antigen in such formulations (Brudeseth et al., 2013). Different oil-adjuvants such as mineral oil have been found to stimulate better and more long-lasting protection than alum and glucan against furunculosis in challenge and field experimental studies (Gudding et al., 1999; Midtlyng et al., 1996). Oil-adjuvants are now extensively used in commercial injectable bacterins. Substances such as beta-1, 3 glucans, chitosan, levamisole can also be used as adjuvant (Mehana et al., 2015; Sirimanapong et al., 2015). The adjutant of β-glucans has been the best and well characterized adjuvant used in oral fish vaccines (Petit and Wiegertjes 2016; Skov et al., 2012). Advantages include easy incorporation into the fish feed and

also it can be used to protect against different antigens as well as environmental stress.

Some specific adjutants would include molecules such as interleukins and heat shock proteins (Adams et al., 2008). Immune stimulating complexes (ISCOMs) and liposomes are also being utilized as effective adjuvants in aquaculture industry. Carp vaccinated against Cypinid Herpesvirus-3 (CyHV-3) using liposome encapsulated vaccine showed good protection while liposome encapsulated *A. hydrophila* showed variable degree of protection (Miyazaki et al., 2008; Yasumoto et al., 2006a, b). Flagellin from *Vibrio* spp. and *Salmonella* spp. can easily survive at the gastric pH and also it can target membrane receptors on the leucocytes stimulating signals on the antigen presenting cells, thereby acts as a good adjuvant (Tafalla et al. 2013). Synthetic ODN containing unmethylated CpG motifs is another interesting choice of adjuvant and can be used along with DNA vaccine in the vector backbone or can be co-administered separately. Motif of CpG triggers TLR9 expressing cells to produce Th1 and proinflammatory cytokines. When used along with the vaccine adjuvants, it boosts macrophages and dendritic cells and both the humoral and cell mediated immune response are generated (Pietretti and Wiegertjes 2014). Motif of CpG, which stimulate TLR9 in human and mice are different (Marshall et al., 2003). The adjuvant effect of CpG motifs is sequence and species dependent (Pietretti and Wiegertjes 2014), so, the CpG ODN may be specifically engineered for each fish species. Hence while administrating same vaccine to different species of fish, it needs incorporation of species specific CpG to be incorporated to exert adjuvant effects.

The use of synthetic oligodeoxynucleotide (ODN) containing CpG DNA motifs as adjuvant has been shown to induce protection against IPNV infections in salmon (Ingerslev et al., 2009). Previous studies have shown that fish reacted to a panel of 31 different CpG ODN, all of which were assembled on phosphorothioate (PS) backbones with no palindromic sequences surrounding the CpG motifs making them fall into B-class of ODN (Anderson, 1997). Since oil-adjuvants were added to the preparations of bacterial vaccine such as different combinations of *V. anguillarum* with other pathogens, such as *V. ordalii, V. salmonicida, Aeromonas salmonicida, Moritella viscosa* and infectious pancreatic necrosis virus, they showed a high degree of protection (Toranzo et al., 2009). To achieve induction of improved immunity of a vaccine through oral route administration it is essential for the antigen to reach hindgut surviving the gastric pH. For the same, liposomes (Anderson et al., 2001), micro/nanoparticles (Lubben et al., 2003) micro/nanoemulsion etc. have been used (Li et al., 2008). Enteric carriers can be used so that the antigens can reach the hindgut along with the added advantage that these carriers are strong inflammatory signals (Embregts and Forlenza 2016). *E. coli* heat-labile enterotoxin (LTB) is one such carrier and hence a study in carp involving Green Fluorescence Protein (GFP) with and without LTB showed that GFP uptake was higher employing LTB (Companjen et al., 2006). Use of exotoxins has also been suggested to be a promising adjuvant option for mucosal delivery of vaccine as these toxins are taken up by activated professional antigen presenting M and dendritic cells of mucus membrane. Such endocytosed antigens are presented by MHC-II molecules and adaptive immune response is induced in the form of activation of both the humoral and cell mediated immune response.

Microparticles based on LPS from meningococcus have recently been evaluated as adjuvant for fish vaccine against *A. hydrophila* in African catfish (Pérez et al., 2013). Molecular adjuvants have been used successfully in human and animal vaccines while their use in aquaculture vaccine has been less reported. Alphavirus replicon has been used in the DNA vaccine of Infectious Salmon Anaemia Virus (ISAV) resulting in good protection upon challenge (Rivas-Aravena et al., 2015). A DNA vector comprising of heat shock protein 70 (HSP70) of *Cryptocaryon irritans* used as a chitosan encapsulated vaccine when fed orally to orange spotted grouper showed effective protection against this parasite. This study revealed HSP 70 to be a good molecular adjuvant along with another finding that DNA vaccine can elicit immunity even when fed orally (Josepriya et al., 2015). Chemokines and cytokines have also been tested for their adjuvant activity in fishes. Recently, IL-8 of channel catfish has been cloned and expressed which was used as an adjuvant along with *Streptococcus iniae* subunit vaccine. Results showed that IL-8 is a good immunopotentiator but further studies are warranted so that this can be used with other vaccines (Wang et al., 2016). Atlantic salmon vaccinated with DNA vaccine against ISAV with IFN plasmid encoding Atlantic salmon type I IFN (*ifna, ifnb*, and *ifnc*) showed good protection, increased antibody level and also improved T and B cell expression in muscles due to influx of leucocytes to the site (Chang et al., 2015). Polyinosinic: polycytidylic acid (poly I:C) is yet another well documented adjuvant used widely in several fish vaccines. Delivery of chitosan-encapsulated poly I:C along with inactivated whole VHSV in zebrafish showed good protection during challenge studies (Kavaliauskis et al., 2015).Various adjuvant technologies used in aquaculture are depicted in Figure 2.

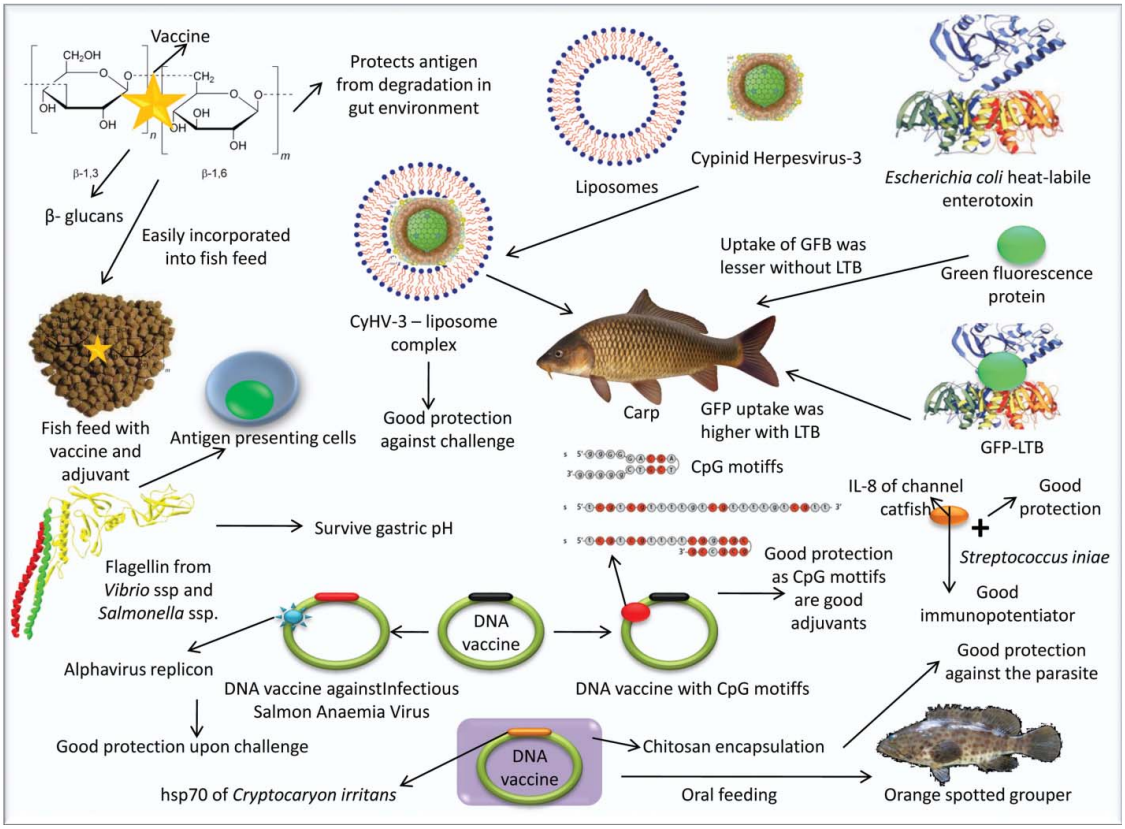

Figure 2. Various adjuvant technologies used in aquaculture.

Monovalent and polyvalent vaccines

The perfect vaccine formulation is a polyvalent vaccine which must protect fish against the majority of important diseases (Busch, 1997; Ma et al., 2010). In recent years, commercial vaccines developed for reared fish, comprise of having two, three, four and five vaccines (Brudeseth et al., 2013; Busch, 1997). Nevertheless, there are evidences that antigenic as well as non-antigenic components of vaccines can interact both synergistically as well as antagonistically, hence proper selection of antigens to be used together in polyvalent vaccine is essential to utilize its full potential and optimum desired immunity (Busch, 1997; Nikoskelainen et al., 2007).

Vaccine delivery methods/routes of administration

Vaccine can be administered to fish through three different routes which include injection (intramuscular or intraperitoneal), immersion (bath or dip-vaccination) and oral route (Adams et al., 2008). The route of vaccine administration may determines the outcome of induced immunological responses and level of protection against pathogens of interest (Palm Jr. et al., 1998). The most effective vaccine delivery method will depend upon the pathogen, route of infection, vaccine production techniques, status of immunological memory, life stage of host/fish, water temperature during the immunization process, workers' understanding of vaccination principles, and labor costs (Yanong and Erlacher-Reid 2012). For achieving adequate and long lasting protection, it may be necessary to administer a specific route of delivery, even multiple applications using different methods. Different routes of immunization and vaccine delivery systems in aquaculture with advantages and disadvantages being used against important pathogens in different types of fishes are presented in Tables 2 and 3, and Figure 3.

Oral vaccination

Immune system of fish is different from mammals as it does not possess payer's patches and lymph nodes as well as from birds in not having bursa of fabricus but have a diffuse gut-associated lymphoid tissue (GALT) (Rombout et al., 2011). Oral administration of antigens does elicit local immunity but has been reported to vary in the efficacy of protection (Maurice et al., 2004; Rombout et al., 2014a, 2014b; Siriyappagouder et al., 2014). Primary vaccination when administered orally does not induce a robust immune response, rather booster oral dose does induce a robust secondary immune response (Ballesteros et al., 2014). Several factors like nature of the antigen,

Table 2. Major advantages and disadvantages influencing the choice of delivery method.

Delivery methods	Vaccination methods	Mode of action	Advantages	Disadvantages	References
Mucosal delivery	Oral vaccination	–Diffused gut-associated lymphoid tissue (GALT) uptake antigen	–Easy to use	–Large quantities of antigen required	(McLean et al., 1999; Vandenberg, 2004; de las Heras et al., 2009; Ballesteros et al., 2012; Ballesteros et al., 2014; Rombout and Kiron 2014)
		–Generation of surface IgA –Local antibody production	–Not time consuming –Moderate cost	–Requires all fish to be fed –Protection generally weak and of short duration	Siriyappagouder et al., 2014
			–Lower stress	–Exact dose of antigen received is difficult to determine	
			–Easiest method for mass vaccination of all sizes of fish –Saves labour –Usually safe–primes mucosal immunity (external surfaces)		
	Anal vaccination	Specific cytotoxicity elicitation in intra epithelial lymphocytes	–Small amount of antigen required	–Repeated vaccination reduce cell mediated toxicity	Sato et al., 2005
			–Antigen specific higher cell mediated immunity	–Rejection of antigen through anal discharges	Sato and Okamoto 2007
			–Involve natural killer and T cells –Better protection than oral immunization		Johnson and Amend 1983 Rombout et al., 2014
	Nanoparticle mediated mucosal vaccination	–Efficient delivery to dendritic cells and macrophage antigen presenting cells	–No discomfort	–Costly	Mody et al., 2015;
		–Particles $> 7\mu$m induce-cell mediated immunity	–Nanoparticle size mimic to the pathogen and efficient delivery to antigen presenting cells	–Toxicity of nanoparticles	Dimier-Poisson et al., 2015
		–Particles $< 4\ \mu$m induce humoral immunity	–Higher cell mediated immune response		Zhao et al., 2014
			–Surface area to volume ratio is high –High diffusion rate –Maximum drug presentation to mucosal surfaces		
Injection vaccination	Intramuscular	–Activates systemic immune response directly	–Longer protection (up to 1 year),	–Unsuitable for small fishes	Vinitantharat et al., 1999; Plant and LaPatra, 2011; Dhar and Allnutt 2011
			–Suitable for large fish (broodstocks)	–Needs sophisticated machinery or highly skilled workforce	
			–Highly efficient in generating both humoral and cellular cytotoxic responses	–Significant handling stress	
			–Multiple antigens from different pathogens can be delivered	–Risk of post vaccination	
			– Minimal wastage of vaccine	Fungal infections and local reactions	
			–With adjuvant good for weal antigens	–Labor and time consuming –Use of anesthetic is required	

(*Continued on next page*)

Table 2. (*Continued*)

Delivery methods	Vaccination methods	Mode of action	Advantages	Disadvantages	References
	Intraperitoneal	–Peritoneal membrane lined with a capillary bed, through which vaccine diffused into the systemic circulation via hepatic portal system.	–Superior level of protection in comparison to immersion vaccination	–Induce latency to eat	Bjørge et al., 2011
	vaccination			–Increased B1 behavior in fish (lying still without any movement)	Evans et al., 2004
Immersion vaccination	Dip vaccination (Exposure to concentrated vaccine for less period of time)	–Solubilized and particulate antigens are adsorbed through specialized cells present in skin and gill epithelium	–Moderate stress for fish,	–Need large amount of vaccine	Vinitantharat et al., 1999; Thune and Plumb 1984; Huising et al., 2003; Brudeseth et al., 2013
	Bath vaccination (Exposure to diluted vaccine for longer period of time)	–Immune response is obtained by both the antibody-secreting	–Lower labour costs,	–Low protection with short duration,	
		Cells and macrophages	Less risk to vaccination team, –Protective immunity for 3–5 months depending on the antigens –Cost effective for small fish, –High efficacy using live, Attenuated vaccines	–Low efficacy for inactivated vaccines, –Cost prohibitive for large fish	

Table 3. Vaccine delivery system used against important pathogens in different types of fishes.

S. No.	Disease/ pathogen	Delivery system	Used in	Remarks	References
1	*A. salmonicida* recombinant A layer proteins	Alginate microparticle	Goldfish (*Carassius auratus*)	After challenge there was no difference in disease susceptibility between the control and treatment groups	Maurice et al. (2004)
2	*Lactococcus garvieae*		Rainbow trout	Study revealed that this technique can be used to boost immunity but not to elicit primary immunity since relative percent survival was 50.	Romalde et al. (2004); Altun et al. (2010)
3	Flavobacterium columnare		Nile tilapia (*Oreochromis niloticus*)	Results were not promising as the immunity level was less	Leal et al. (2010)
4	Infectious pancreatic necrosis virus (IPNV) DNA vaccine		Brown trout (*Salmo trutta*) and rainbow trout	Provided good protection	de Las Heras et al. (2010)
5	Plasmid encoding major capsid protein of LCDV	Chitosan based	Japanese flounder	Expression of the protein was detected in gills, kidney, spleen and intestine	Tian et al. (2008b)
6	*V. anguillarum* outer membrane protein (OMP) 38		Asian sea bass (*Lates calcifer*)	Yielded partial protection against challenge	Kumar et al. (2008)
7	LCDV plasmid DNA vaccine	Poly lactide co-glycolide	Japanese flounder	—	Tian et al. (2008c)
8	*A. salmonicida*		Rohu (*Labeo rohita*)	Intra peritoneal injection stimulated both innate and adaptive immunity	Behera et al. (2010)
9	*L. garvieae*		Rainbow trout	Comparative study between PLGA and alginate showed that PLGA was better than alginate microparticle encapsulation	Altun et al. (2010)

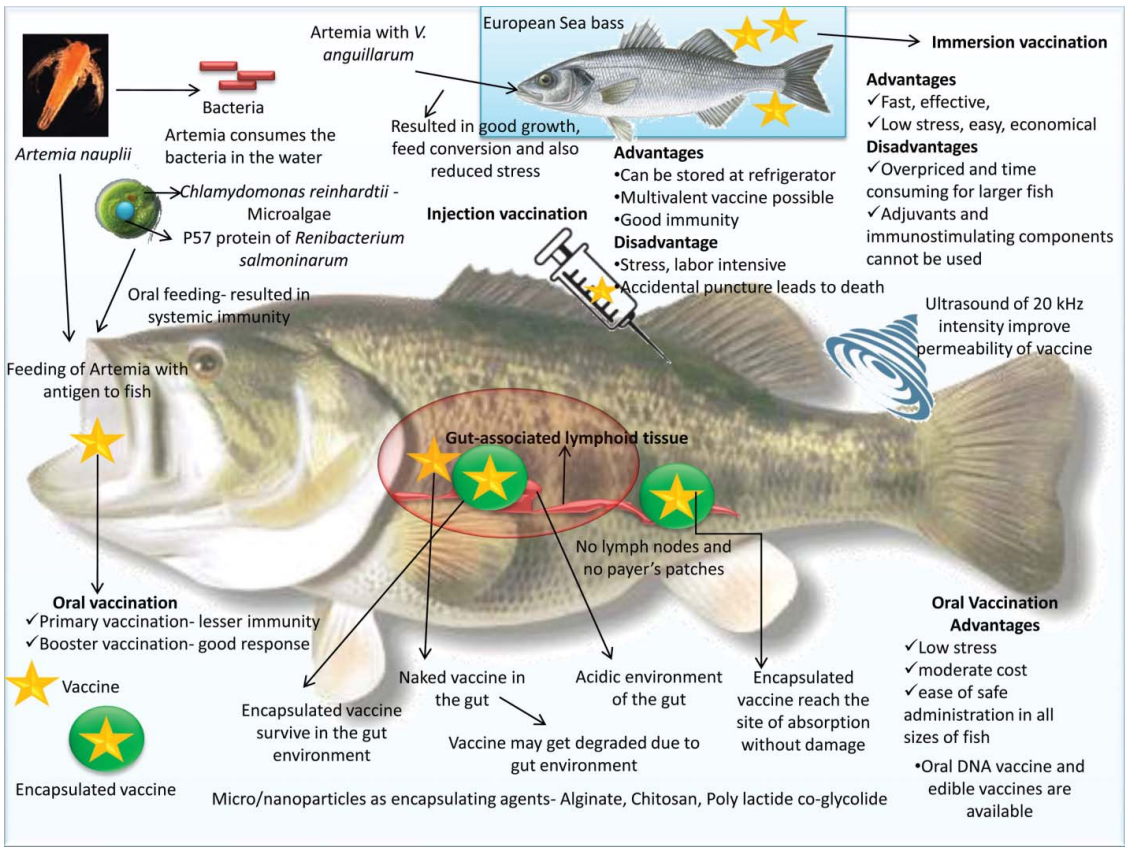

Figure 3. Different routes of immunization in aquaculture with advantages and disadvantages.

dosage regimen and formulation of the vaccine influence the efficacy of oral vaccines (Mutoloki et al., 2015). In this method, antigen can be introduced to fish by direct delivery via the digestive system of the fish of any age. Though immunity level may be higher by injection method but the stress induced by handling of fish can lead to mortality, hence alternative method seems to be oral vaccination feeding. It is the easiest method logistically because feeding is a routine practice in fish farms. In oral vaccination, the vaccine is either mixed with the feed, top dressed on the feed, sprayed over the feed or bio-encapsulated. Delivery of antigen in fish feed offer some advantages like cost effectiveness, ease of safe administration in all sizes/stages of fish, and imposing low stress (Plant and LaPatra 2011). Nevertheless, oral application of vaccines induce low protection levels and relatively short duration of protection which may be due to degradation of the antigens in the gastro-intestinal tract and low transfer rate of the antigens from the intestinal lumen to the immune reactive cells (Brudeseth et al., 2013). Oral vaccines can be administered for primary vaccination or as a booster vaccine to develop protection against long-lasting endemic diseases (Brudeseth et al., 2013). In this method, humoral immunity is not as potent as induced comparatively in injection vaccination (Dhar and Allnutt 2011). Usually the protection of this kind of vaccine is linked to humoral rather than the innate and cellular immune responses (Newaj-Fyzul and Austin 2015).

Currently, several advances have been made in the field of oral vaccination and there are reports of oral delivery of recombinant subunit and attenuated virus vaccines (Adelmann et al., 2008; Lin et al., 2005). To deliver recombinant subunit vaccine, fishes were fed *Artemia nauplii* encapsulated with recombinant bacteria containing the antigen of interest (McLean et al., 1999). Fishes consumed artemia thereby encapsulated vaccine agent is released inside the body (Van Stappen, 1996). *Artemia* sp. containing particular bacteria are usually fed to juvenile fish immersed in water and when these Artemia are fed by fish these antigens can elicit immunity. Encapsulation of *V. anguillarum* inside *Artemia nauplii* when fed to European sea bass (*Dicentrarchus labrax*) resulted in good growth, feed conversion and also reduced stress, but the antibody level was not measured in this study (Chair et al. 1994). Later, a study was conducted to find the fate of bacterin inside *Artemia* spp. Results showed the presence of whole bacterial cells and antigens from the bacterin in individual nauplii by ELISA and immunohistochemistry (Bergh et al., 2001). Hence further studies are warranted to arrive at a solid conclusion regarding the use of *Artemia* spp. as an oral vector for vaccines. *E. coli* expressing *Pseudomonas aeroginosa* was fed to *Artemia* spp., which when later consumed by zebrafish showed that there was 81% protection at 30 days post vaccination (Lin et al., 2005). In the attenuated virus vaccines, the vaccine is usually lyophilized, surrounded by polyethylene glycol (PEG), and extracted under low temperature (Adelmann et al., 2008).

Several biological and synthetic materials are available for oral delivery of antigens into fish to elicit immunity. *Chlamydomonas reinhardtii* is a microalgae that can be easily employed for transformation of foreign genes hence has been used as a successful system for oral delivery of antigens into fish (Siripornadulsil et al., 2007). The protein of *Renibacterium salmoninarum*, p57, expressed in *C. reinhardtii* either as partial antigenic region or as a whole protein, elicited immunity when they were fed orally or by immersion route. Immersion route elicited antibody response mainly in the skin mucosa while oral administration resulted in systemic immunity (Siripornadulsil et al., 2007). This indicates that the microalgae used as a carrier for vaccine antigen resists pH of the gut, hence this microalgae is a suitable candidate for delivery of vaccines in aquaculture. Prevention of antigen from acidic environment of gut is essential to effectively mount a good immune response. Encapsulation of antigens has been attempted using nano or microparticles that can prevent degradation of antigen from acidic environment of the gut (Sinyakov et al., 2006). Nanoparticle encapsulation is a better approach as compared to microparticle encapsulation due to uniformity in size of nanoparticles. Several micro and nanoparticles are used for delivery of vaccines in aquaculture among which alginate microparticles, Chitosan and Poly lactide co-glycolide are being used commonly in aquaculture. Alginate microparticles have been used in the aquaculture industry since 1997 as in *V. anquillarum* vaccine in both carp and rainbow trout (Joosten et al., 1997). Later research studies tested suitability of this alginate microparticle for delivery of vaccine against lymphocystis virus disease (LCDV) in Japanese flounder (*Paralichthys olivaceus*) revealing its promising effectiveness to be used as vehicle for antigen delivery (Tian et al., 2008a). Chitosan has the advantage of mucoadhesive nature thereby increasing the delivery of the attached antigen efficiently. Poly lactide co-glycolide has attracted vaccine industry recently due to its ease of production and cost effectiveness (Takeuchi et al., 1996). Recent studies reported that oral vaccines manufactured with nanoparticles have elicited better immune protection (Adomako et al., 2012). An overview showing the micro or nanoparticle based vaccine delivery in different kinds of fish is presented in Table 3.

Biofilms have also been tried as vaccine delivery system, however, much study is needed in this area. Biofilm of virulent *A. hydrophila* created on a small chitin particle, inactivated by heat and then fed to common carp, catla and rohu revealed that bacteria fed as biofilm elicited higher immune response than bacteria alone thus supporting the use of biofilm vaccines (Azad et al. 1999). It was suggested that *A. hydrophila* stayed longer in the gut due to the presence of glycocalyx present in the biofilm (Azad et al., 2000). Similar studies conducted in walking catfish (*Clarius batrachus*) revealed protection ranging from 93% to 100% when challenged with *A. hydrophila* after immunization with biofilms (Nayak et al., 2004).

Published literature has suggested that there are 30 fish vaccines, however according to a report in 2014, only 17 vaccines have been registered for commercial aquaculture among which only 2 of them are oral vaccines (Mutoloki et al., 2015). Overall, few oral vaccines have been commercialized (Vandenberg, 2004), but recent reports regarding application of either pathogen-coding DNA in trout (Ballesteros et al., 2012, 2014; de las Heras et al., 2009) and Japanese flounder (*Paralichthys olivaceus*) (Tian and Yu 2011) or pathogen-specific recombinant proteins in salmon (Gomez-Casado et al., 2011; Tobar et al., 2011) and determining the relevance of the gut as an immune-competent organ, suggest that fish oral vaccination may be highly promising in the future. But, it is obvious that the production of safe and effective oral vaccines is among one of the most challenging functions of immunologists.

Injection vaccination

Through injectable vaccines, only small identified concentration of antigen can be infused directly into the fish by intraperitoneal (IP) and intramuscular (IM) routes (Plant and LaPatra 2011). The most efficient injectable method of fish immunization is IP and hence many of the current vaccines are predominantly delivered by this route. On the other hand, IM route is preferred for DNA vaccination into fish (Evensen and Leong 2013; Heppell and Davis 2000). This approach is commonly performed manually using a needle or alternatively by devices such as compressed air (Dhar, et al., 2014). The duration of protection in this approach is more prolonged than the immersion approach (Vinitantharat et al., 1999) and vaccine can be concentrated and delivered with compounds such as adjuvants, carriers, bacterial cells, bacterial antigens, etc. which could not be administered by other vaccination routes (Dhar and Allnutt 2011). The antigens can be easily stored at 4°C for injectable vaccines. Another advantage of injection vaccination method is that multiple antigens from different pathogens can be delivered simultaneously in the form of a multivalent vaccine. Nevertheless, this method is not suitable for the fishes weighing less than 5g due to being very labor-intensive, adhesion formation, temporary reduction in feeding, accidental puncture of the intestine, and possible risk of wound at the injection site that may causes secondary infection (Vinitantharat et al., 1999). Moreover, often mortalities have been documented related to the fish handling during injection. The major problem with injected vaccines is that they cannot be economically administrated multiple times in the fish production cycle, and these vaccines cannot be administered in early life stages due to under developed immune system (Dhar and Allnutt 2011).

Immersion vaccination

Immersion vaccines consist of suspension of live attenuated bacteria or live bacterial or vector vaccines. Suspension of formalin inactivated bacterial as well as live bacterial vaccines primarily comprises the commercial immersion vaccines (Brudeseth et al., 2013). Immersion vaccination (short or long bath) is particularly recommended for smaller fishes weighing between 1 and 4 g. This method is rapid, effective, less stressful, convenient and economical to vaccinate fish that require minimal handling stress. It is disadvantageous for large fishes not only due to its time consuming and overpriced affairs but also pose difficulty to use adjuvants and other immune stimulating agents. Immersion vaccination allows direct exposure of antigens to the immune cells located in fish skin and gills. The duration of protection ranges between 3 and 12 months which essentially is not long enough for the culture of some fish species (Vinitantharat et al., 1999) and thus often requires booster vaccination. Several facilitators have been depicted for development of antigen uptake in immersion vaccine such as hyperosmotic dip (Huising et al., 2003; Thune and Plumb 1984), ultrasound mediated uptake (Frenkel et al., 1999) and multiple puncture instrument (Nakanishi et al., 2002). Immersion vaccine is allocated in a diluted solution and some of the vaccine may be wasted during the administration process. Application of ultrasound for delivery of vaccine is a newer concept in aquaculture industry. Sound intensity of 20 kHz can improve the cellular permeability of vaccine. *V. alginolyticus* bacterin delivered by ultrasound method to *Epinephalus awoara* showed that this method produced equal immunity similar to intraperitoneal injection. Another study using ultrasound and hyperosmotic treatments as facilitators of antigen penetration through the skin by bath immersion and as enhancers of the antibody

response in goldfish showed that 5 times lesser dose of bovine serum albumin was required to produce antibodies when compared with simple bath immersion method (Navot et al. 2004). Combination of immersion with puncture method has also been employed to effectively deliver antigens. In this method several small punctures were made in rainbow trout and then the fishes were immersed in water with *S. iniae* which protected 60% of the population from challenge (Nakanishi et al., 2002).

Immersion delivery method does not induce as strong humoral immunity as injection vaccination (Dhar and Allnutt 2011). Although all of methods have different advantages and disadvantages, it is generally approved that only the injection and immersion routes have given enough protection in commercial vaccines. Injection method is obviously the most impressive route, but the side-effects and vaccination cost necessitate to seek another alternative methods (Plant and LaPatra 2011).

Evolution and futuristic vision of fish vaccines

Vaccination plays an influential role in large-scale commercial aquaculture and has been considered as undeniable factor for a successful aquaculture. It has been demonstrated to be cost effective and has resulted to diminish administration of antibiotics. In Norway, the annual administration of antibiotic has reduced from 47 tons to around one ton (Lillehaug et al., 2003; Markestad and Grave 1997). Research in fish immunology and vaccination has progressed tremendously after World War II. Like other vertebrates, fish has a complex immune system comprising of specificity and memory. Teleost fishes have primary and secondary lymphoid organs; however, there are significant variations in the morphological and structural aspects of immune systems between mammals and fishes (Salinas et al., 2007). Innate immune response consists of physical, cellular and humoral factors and involves humoral and cellular molecules in plasma and other body fluids (Uribe et al., 2011). Typical adaptive immune responses of fish are characterized by immunoglobulins, T-cell receptors, cytokines, and major histocompatibility complex molecules (Warr, 1996). The largest lymphoid organs of fish consist of the thymus, spleen and kidney (anterior and middle). Also, fish possess lymphocytes which circulating in the blood as well as in the thymus, kidney, spleen, gills and gut (Secombes, and Belmonte 2016). Furthermore, the significant immune responses of the fish are immunoglobulins which elevate against various pathogenic organisms (Uribe et al., 2011). The fish immune system is similar to the mammalian ones in MHC class I (Hashimoto et al., 1999), TCR (T-cell receptors), and the TCR co-receptor CD8 (Hansen and Strassburger 2000), indicating homologies in the process of antigen presentation. Moreover, the detected lymphocytes of fish are similar to the mammalian T and B cells (Clem et al., 1991).

Live vaccines can activate cellular and humoral immunity without inclusion of adjuvant (Meeusen et al., 2007). Nevertheless, despite the ease of administration, there is potential risk of residual virulence, mutations and environmental contamination. The most important advantage of recombinant expression system is the production of specific antigen when the actual host is difficult to produce or when cultured systems are not available (Adams et al., 2008). The advantage of vector technology is that small protein or peptides are expressed together with a set of host antigens which increase the induced immune reactions (Adams et al., 2008). Also, vector vaccines can be used as live vaccines. Expression of heterologous antigens using SFV expression approach as a kind of vector strategy suggests various kind of advantages over traditional vaccination such as elevated levels of expression of heterologous antigen in the cytoplasm, expression of vector protein at low-level, initiation of infected cells apoptosis (Glasgow et al., 1997), and an elevated level of biosafety. Expression of heterologous antigens results in the generation of strong humoral and cellular immune responses with prolonged memory (McKenna et al., 2001).

Another advantage of genetically attenuated pathogens is that the insertions or deletions of genes are well defined and reversion of this mutant to become virulent is virtually impossible (Adams et al., 2008) and also the capability to induce humoral, cell mediated, as well as mucosal immunity is not diminished (Clark and Cassidy-Hanley 2005). Nevertheless, the disadvantage of this vaccine is that the use of live vaccine for fish may pose a concern for most governments, mostly due to the spread of the vaccine strain through effluents and possibility of back-mutating to the virulent form (Benmansour and De Kinkelin 1996; Sommerset et al., 2005). Use of the genes compared with the proteins could have numerous advantages such as synthesized protein *in situ* from DNA could potentially remain locally or systematically for longer time without causing any related toxicities with high concentrations of intravenously administered proteins. In addition, a synthesized protein from the gene would have eukaryotic post-translational modifications, which can evade one of the important challenges regarding synthesized prokaryotic recombinant proteins. DNA vaccines do not pose risks to safety concerns as with live attenuated vaccines having a likely reversion to virulent forms (Benmansour and De Kinkelin 1996). Other benefit in comparison to alternative vaccination approaches is that pDNA show inherent immunostimulatory capacity owing to the sequences of CpG (sites of

DNA with a cytosine nucleotide linked to a guanine nucleotide by a phosphodiester bond) (Coombes and Mahony 2001).

Some of the advances in vaccines and vaccine delivery systems as discussed below need due attention for exploring their promising potentials to counter infectious diseases of fish.

Mucosal vaccines

Mucosal vaccination in aquaculture has gained a lot of attention in recent decade because of the long duration of protective immunity in vaccinated fish. Nevertheless, some of restriction aspects for designing protective mucosal vaccines for finfish explored has been the absence of protective antigen doses for mucosal vaccines, lack of applicable immunostimulants to elevate the performance of non-replicative mucosal vaccines, decrease of systemic antibodies because of extended exposure to oral vaccination and the absence of predefined relevance of applicable protective immunity in developed mucosal vaccines (Munang'andu et al., 2015). Injectable vaccines have been supposed to be more protective comparatively, hence limiting factors being faced for developing mucosal vaccines in fish need to be given due care for designing effective mucosal vaccines.

Plant-based edible vaccines

The use of conventional killed and live attenuated vaccines seems to be a costly affair and the injectable vaccines and adjuvants are not practical to protect huge population of livestock animals, poultry and fish population (Shahaid and Daniell 2016). To overcome these limitations, plants could provide a smart and economical platform for developing efficient vaccines and their delivery supported with advances in plant biotechnology and genetic engineering which offers plants as good delivery vehicles through which animal and fish vaccines can be developed (Dhama et al., 2013). On one hand, plant vaccines have been found to be cost effective, free of attenuated pathogens, achieve scalable production platforms, does not require cold chain maintenance, and efficient edible vaccines for getting rid of infectious fish diseases for safe and sustainable aquaculture, and on the other hand they could help to meet partial supply of the food demand as vaccinated crop/feed (Clarke et al., 2013; Kolotilin et al., 2014; Shahaid and Daniell 2016). Plant derived vaccines could provide ideal booster vaccines which can reduce requirement of multiple boosters of attenuated bacterial or viral vaccines.

Gut adhesion molecule (LTB) and a viral peptide or green fluorescent protein (GFP) expressed in potato tubers has been able to induce protective antibody response when functionally incorporated into fish feed. Furthermore, LTB has additional effect on the uptake of vaccine and reporter proteins upon mucosal administration in fish (Companjen et al. 2006).

Nanoparticle-based vaccines/nanovaccines (nanodelivery of vaccines)

These vaccines can enhance the targeted and sustained release of vaccine agent in the body, and now nanoparticles are gaining attention of researchers in the field of developing prophylactics and for aquaculture vaccines, though deployment of nanomaterials are in infancy but popularizing. These comprise of dispersion of nano-sized materials (common form/example of nanoparticles include chitosan, alginate, Poly d,l-lactic-co-glycolic acid-PLGA) with specific/defined physical characteristics in which immunostimulants and antigens are incorporated to improve the vaccine delivery in a controlled and targeted manner and increase the intensity of desired immune responses (Ji et al., 2015). Administration of nanoparticle-based vaccines against viral pathogens such as ISAV is a promising field in fish medicine research (Shaalan et al., 2016). This vaccine revealed 77% protection rates against ISAV in Atlantic salmon (Rivas-Aravena et al., 2015). In another study, an oral DNA vaccine in Asian sea bass (*Lates calcarifer*) was designed using chitosan and chitosan/tripoly phosphate nanoparticles which induced immunity against against *V. anguillarum* (Vimal et al., 2012). Further studies are required to utilize the tremendous advances being carried out in the field of nanotechnology regarding various applications of nanoparticles to design newer vaccines and improve vaccine delivery systems for prevention of infectious diseases of fish. Emphasis need to be given for the suitability of the type of biomaterial/nanoparticle to be used, immunostimulant or vaccine candidate to be loaded into the nanoparticles, and how such vaccines would target the fish immune system in an efficient way.

Recent molecular advances for developing vaccines and effective vaccination strategies

Recent molecular advances suggested that immunoprophylaxis approaches which properly stimulate the sensors for viral nucleic acid, high-mobility group box proteins (HMGBs), toll-like receptors (TLRs), pattern recognition receptors (PRRs), retinoic acid inducible gene I- (RIG-I-) like receptors (RLRs) in the grass carp fishes could play significant role for activating the immune system of fish viral disease such as hemorrhagic disease, and can be managed and mitigated for achieving

desired protection levels (Rao and Su 2015). Furthermore, TLRs specifically recognize pathogen associated molecular patterns (PAMPs) in microbes, activate immune signaling cascades and thus promote innate immunity, and recently also have been reported to play roles in adaptive immunity. Thus the incorporation of TLRs as adjuvants and TLR activators in vaccine formulation for use in fish and aquatic animals may provide an effective vaccination approach (Rauta et al., 2014). Various other recent approaches and advances in designing and developing vaccines having potent human and veterinary applications along with gaining insights in fish mucosal immunology and novel biotechnological/molecular tools and techniques need to be explored for their full potential for developing and improving fish vaccines and vaccinology to protect aquaculture industry from harmful pathogens and alleviate economical losses (Delany et al., 2014; Effio and Hubbuch 2015; Finco and Rappuoli 2014; Singh et al., 2015). Some of these include structural vaccinology (SV), marker vaccines, immunomics based vaccines, dendritic cells, and designer cell lines. Differentiating infected from vaccinated individuals (DIVA). Various immunomodulatory approaches including use of molecular adjuvants need priority attention. For vaccines which do not elicit strong immune responses, adjunction with improved adjuvants could play a key role in vaccine development by boosting the level of protection towards a desired level (Pérez et al., 2013). Along with these, vaccination strategies comprising of developing multiple vaccines, prime-boost regimens need to be strengthened. Advances in vaccines could help in meeting the increased demands of effective vaccination and incorporate new antigens during changed antigenic nature of pathogens. Also, the utilization of live vectors such as adenoviruses, or attenuation bacteria, such as *Edwardsiella tarda* or *V. anguillarum*, and the combination of strong mucosal adjuvants such as enterotoxins with weak oral antigens develops a numerous combinations that have not been completely used in development of fish vaccine (Embregts and Forlenza, 2016). Utilizing these advances, requirement of transforming existing vaccines could also be met with a futuristic perspective.

Safety of vaccines

The concept of safety in the fish vaccines is associated with the poor immunogenicity of vaccines which may lead to disease and decreased production in the vaccinated fish. Nevertheless, it is critical that the vaccine strains must not have potential to be released into the environment. Moreover, they must pass safety guidelines which comprise of an experiment using 10 times the immunizing dose (Shoemaker et al., 2009). Killed or inactivated vaccines are commonly investigated to be safe for application in aquatic animals due to the pathogen agents used are killed or inactivated. The main disadvantage of application of modified lives vaccines is their safety concerns.

Also, DNA vaccines may offer a safety advantage in that they only need an immunogenic part of pathogen. The prophylactic possibility of DNA vaccine in farmed fish explores several benefits such as same and low cost production processes, the potential of multiple vaccines co-administration (multivalent), quality of storage because of the elevated chemical stability of plasmid DNA, rapid modification of DNA sequences of vaccine to meet mutants of new pathogen, guarded safety regarding to disease transmission (such as confront with live attenuated vaccines), suitable conformational folding of protein belonging to the pathogen antigen (not always produced with recombinant protein in bacteria), does not require adjuvant administration and improving of immune responses, and efficacy in promoting both humoral and cell-mediated immunity (Adams and Thompson 2006). The immune reaction after DNA vaccination is initiated by antigen presenting cells (APC) such as dendritic cells (DC) in concert with T lymphocytes (Restifo et al., 2000). Professional APC such as DC as well as macrophages have been revealed to contain pDNA after IM administration (Casares et al., 1997; Chattergoon et al., 1998). Also, APC at the administration site may evoke immune cells like naive T-lymphocytes following antigen presentation. Furthermore, APC can also take part in releasing soluble antigens from another transgene producing cell (such as a myocyte), organize it and explore the peptide on cell surface MHC class II molecules (Casares et al., 1997). Moreover, DNA vaccines require no oil-adjuvants that otherwise may result unwanted side effects as reported for polyvalent oil-adjuvanted vaccines. One of the unique features of DNA vaccines is their capability to promote cellular and humoral immune responses (Utke et al., 2007, 2008; Restifo et al., 2000).

Therefore, the therapeutic DNA vaccines may enhance the animal welfare. Moreover, an IHN DNA vaccine (Apex-IHN®) has been authorized for marketing by the Canadian Food Inspection Agency, Veterinary Biologics and Biotechnology Division in 2005 and its has also been approved in USA. It is possible that DNA vaccines will be developed against other viral diseases of fishes to induce protection. One likely candidate is a DNA vaccine against VHS in trout (Lorenzen and LaPatra 2005). Nonetheless, the understanding of possible outcomes induced by escape of transgenic fish and distribution of DNA vaccine and GM feed is limited. For

example, transgenic fish may spawn with wild fish as well as GM feed could spread into the aquatic environment and be used by other marine organisms and human. Horizontal gene transfer may take place from transgenic DNA in vaccines or feed to the recipient genome of micro-organisms or DNA vaccines may be released by faeces to the environment (Myhr and Dalmo 2005).

Side effects of vaccines in fish

Commercially available viral and bacterial fish vaccines are commonly polyvalent vaccines with oil adjuvant systems (Anderson, 1992). The protective effect of vaccine combined with adjuvant is accompanied by side-effects such as intra-abdominal adhesions, inflammation, granulomatous peritonitis and pigmentation near the injection site (Midtlyng et al., 1996) and spinal deformities (Berg et al., 2006). Also, IP administration of oil adjuvant vaccine to Atlantic salmon has been found to cause impaired growth, lower feed intake, difference in weight one year after vaccination and reduced carcass quality (Mutoloki et al., 2004, Sørum and Damsgård 2004). Unfortunately, there is a strong correlation between effect and side-effects of oil-adjuvanted vaccine in Atlantic salmon (Berg et al., 2006). Commonly, it has been revealed that salmon that are vaccinated with commercially available vaccine accompanied with oil-adjuvant showed a lower long term growth than unvaccinated groups (Berg et al., 2007; Midtlyng et al., 1996). Furthermore, it is identified that the risk of adverse intra-abdominal lesions after vaccination with oil-adjuvanted vaccines can be diminished by increasing fish size at vaccination (Berg et al., 2007). Studies also have shown that the inflammatory responses are different within the salmonid species (Mutoloki et al., 2006) and maybe less severe in other species such as sea bass (*Dicentrarchus labrax*), cod (*Gadus morhua*), turbot (*Scophthalmus maximus*), and yellowtail (*Seriola quinqueradiata*) (Brudeseth et al., 2013). The reduction of the side effects of vaccines without compromising long-term protective immunity is a challenging goal for future fish vaccination.

Marketing

Most of the commercial vaccines available for fish vaccination are against bacterial and viral diseases and many such new vaccines are under improvement. Fish vaccination is practiced in the commercial aquaculture for several species such as Atlantic salmon, rainbow trout, sea bass, sea bream (*Sparus aurata*), barramundi (*Lates calcarifer*), tilapia, turbot (*Scophthalmus maximus* L.), yellowtail (*Seriola quinqueradiata*) and gold-striped amberjack (*Seriola dumerili*), striped jack (*Pseudocaranx dentex*) and channel catfish (*Ictalurus punctatus*) (Håstein et al., 2005). The most of the vaccines target salmon and trout and there are extending opportunities in marine fish (Thompson and Adams 2004). Now-a-days, salmonids market utilizes heptavalent vaccines including *Vibrio (Listonella) anguillarum* serotypes O_1 and O_2, *V. salmonicida, Moritella viscosa, A. salmonicida*, ISAV and IPNV antigen (Adams et al., 2008). A recombinant expressed viral protein of IPNV has been improved and used in market for salmon (Frost and Ness 1997). Furthermore, IPNV, IHNV, SVC virus, salmon pancreas disease virus, and ISA virus vaccines are the commercially available viral vaccines in the market (Salgado-Miranda et al., 2013). The commercially available bacterial vaccines in aquaculture are vibriosis (*Listonella anguillarum, V. ordalii*), furunculosis (*A. salmonicida* subsp. *salmonicida*), cold-water vibriosis (*V. salmonicida*), yersiniosis (*Y. ruckeri*), pasteurellosis (*Photobacterium damselae* subsp. *piscicida*), edwardsiellosis (*Edwardsiella ictaluri*), winter ulcer (*Moritella viscosa*), and streptococcosis/lactococcosis (*Streptococcus iniae, Lactococcus garvieae*) (Håstein et al., 2005). Currently, several recombinant vaccines have been licensed for animal health applications. The first licensure DNA vaccine was the IHNV DNA vaccine (Apex-IHN®) against the salmonid rhabdoviruses. It was advanced by Aqua Health Ltd. (an affiliate of Novartis, Charlottetown, Canada) and was licensed for marketing by the Canadian Food Inspection Agency in July 2005 (Gomez-Casado et al., 2011; Meeusen et al., 2007). Also, VHSV DNA vaccine has been successful at the experimental level (Hølvold et al., 2014). Furthermore, three companies have authorized oral vaccine against *L. garvieae* in 100–400 g *Seriola* spp. in Japan. Other commercially available oral vaccines are the ISAV vaccine from Centrovet and the IPNV vaccine from Schering Plough-Intervet (Dhar and Allnutt 2011).

At present, the major producers of fish vaccines are: Agrovet, Aqua Health Ltd., Bioveta, Centrovet, DaeSung Micro. Bio. Lab, Dainippon, Goryo Band P, Green Cross, Hipra, JungAn Vaccine, Komi Pharm, Korea BNP, Kyoritsu, MSD, Nisseiken, Intervet International, Microtech, Novartis Animal Health, Pharmaq, Pfizer, Tecnovax, Recalcine, and Veterquimica (Brudeseth et al., 2013; Salgado-Miranda et al., 2013).

Conclusion and future perspectives

Advances in biotechnology and development of new vaccine against pathogens have made an important contribution in reducing the risk of diseases outbreak and subsequent losses in aquaculture. The progress cause to

recognize protective antigens and make safe and inexpensive manufacture of vaccine. Aquaculture vaccination is becoming a significant section of the health management while it has investigated a cost-effective technology of monitoring significant threatening pathogen. In the other hand, most of the development and efforts in aquatic animal vaccines are still in their infancy and challenges towards multi-component and cost effective vaccination programs are yet to be addressed. Technical scientific, biological as well as limitations control the generation or commercialization of vaccines for all economically significant fish disease. Considering individual fish typically have a low production cost compared to other farmed animals, only low cost vaccine are economic to protect fish against pathogens (Adams et al., 2008). In addition to multivalent vaccine for salmonids, there is a need for additional antigens against ISAV, pancreas disease virus (PDV), VHSV and IHNV for the North Hemisphere (Adams et al., 2008). Besides improvement of recombinant approaches in generation of new vaccines, we require to improve new expression system which produces glycosylation of the proteins and restoration of the tertiary structure (Adams and Thompson 2006). Even though the most antigens are protein based, some of them have polysaccharide based. Some protective immune responses need the inclusion polysaccharide for the induction. These kinds of antigens have been addressed less than protein antigens but it may change the future of the vaccines. Parasitic infection in fish results in losses and a decreased immune response in infected fish. These pathogens have been controlled by chemicals that cause limitation for human consumption so vaccines are needed for these parasites. There have been several attempt to produce vaccines against some fish parasites (Anderson, 1997) but no commercial vaccine is available (Sommerset et al., 2005). In fish, the innate immune system probably plays a key role in the protection against pathogens, therefore more attention should be paid to the innate immune response in connection for immunostimulant and adjuvants (Ringø et al., 2014b; Sommerset et al., 2005). Therefore, researchers and scientists need to achieve better understanding of regulatory and safety issues to inform consumers about the positive effects of the administration of safer and affordable vaccine in aquaculture. In addition, commercial vaccines may or may not be protective, so autogenous vaccine may be required for better protection at a specific facility. Vaccines prepared from a recent disease outbreak and immunization of the other susceptible fish population is an important mode of vaccine in aquaculture in several countries which is stated as autogenous fish vaccine. Several companies also markets autogenous vaccines and Vaxxinova is one such firm that is involved in production of autogenous vaccines against *Pseudomonas fluorescens, Aeromonas salmonicida* and *Flavobacterium psycrophilum*. On the other hand, commercial vaccine development for some aquaculture sectors, such as warm water fish and shrimp are still quite limited and need to be developed. Newly some immunoglobulin isotype has been identified in fishes like IgT in trout, IgZ in zebrafish and carp and IgH in fugu and these sequences does not match with some of the already available databank sequences. Hence studies needs to be targeted towards these newly identified immunoglobulins, their role in immunity and their relatedness with other immunoglobulins so that effective vaccine strategies can be drafted. Overall, acceptable vaccine for aquaculture must have two categories of important characteristic for both farmers and vaccine companies. The first and most essential category contains three important roles:

These vaccines should provide proper immunoprotection against a specific disease in intensive farming conditions; Provide protection of long duration as the animal is most susceptible to disease; Protect against all serotype variants of the disease agent; the second category is value and certainty of the market, better correlation of lab performance to farm performance, ability to measure this in a production setting, and application of useful technology improvement and flexibility in production procedure of a vaccine. Furthermore, more suitable and economical delivery methods need to be developed to vaccinate small fish. It is better to consider vaccination as a part of comprehensive fish health management, and not the only way for a disease problem. The basic information on immunization of fish could be applied for large-scale vaccination in fish and for more progress in this field, it is necessary to have co-operation between more basic and applied science.

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