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Fish and Shellfish Immunology 83 (2018) 84–95

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Cyprinid viral diseases and vaccine development

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ARTICLE INFO

Keywords:
Cyprinid
Viral disease
Vaccine

ABSTRACT

In the past decades, global freshwater fish production has been rapidly growing, while cyprinid takes the largest portion. Along with the rapid rise of novel forms of intensive aquaculture, increased global aquatic animal movement and various anthropogenic stress to aquatic ecosystems during the past century, freshwater fish farming industry encounter the emergence and breakout of many diseases, especially viral diseases. Because of the ability to safely and effectively prevent aquaculture diseases, vaccines have become the mainstream technology for prevention and control of aquatic diseases in the world. In this review, authors summarized six major cyprinid viral diseases, including koi herpesvirus disease (KHVD), spring viraemia of carp (SVC), grass carp hemorrhagic disease (GCHD), koi sleepy disease (KSD), carp pox disease (CPD) and herpesviral haematopoietic necrosis (HPHN). The present review described the characteristics of these diseases from epidemiology, pathology, etiology and diagnostics. Furthermore, the development of specific vaccines respective to these diseases is stated according to preparation methods and immunization approaches. It is hoped that the review could contribute to aquaculture in prevention and controlling of cyprinid viral diseases, and serve the healthy and sustainable development of aquaculture industry.

1. Introduction

Aquaculture is an ancient practice and it is believed to date back to China at least 4000 years ago. There are also references of fish ponds in The Old Testament and in Egyptian hieroglyphics of the Middle Kingdom. Fish farms were common in Europe in Roman times and a recent study of land forms in the Bolivian Amazon has revealed a complex array of fish weirs that pre-date the Hispanic era [1,2]. Despite its ancient origin, aquaculture remained a low-level farming activity until the mid-20th century when experimental husbandry practices for salmon, trout and a mass of tropical fish and shrimp species were developed. Nowadays, aquaculture is commended to be a major global industry, with a total annual production exceeding 80 million ton and estimated value of almost 230 billion US dollar (Food and Agriculture Organization of the United Nations, FAO, 2016). In the past three decades, aquaculture industry has made impressive progress and constitutes high quality protein for world population accounting for nearly 50% of the global food fish supply in 2016. In addition to being an important form of nutrition, aquaculture is also a critical form of employment (FAO, 2018).

Aquaculture is a highly dynamic production and is characterized by enormous diversity in both the farmed species range and the production systems feature. Over 350 different species of aquatic animals are cultured in global in freshwater, brackishwater and marine environments. In 2016, the yield of freshwater fish production reached 46.6 million ton (FAO, 2016). Carps (*Cyprinidae*) make up almost 65% of the global farmed freshwater fish production and are an important source of food in China and India where 72.8% and 13.8%, respectively, of farmed cyprinid are produced (FAO, 2016). Economic aquaculture species belonging to cyprinid principally include grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), crucian carp (*Carassius auratus*), black carp (*Mylopharyngodon piceus*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*) and blunt snout bream (*Megalobrama amblycephala*), etc. Meanwhile, in the international ornamental fish trade, more than 90% of the freshwater fishes are farm bred, and about 4000 species of freshwater ornamental fishes are traded every year [3]. Goldfish (*Carassius auratus auratus*) and koi (*Cyprinus carpio haematopterus*) are the most extensively traded fish species [4].

Importantly, the global fish trade relocates large quantities of live fish species between countries, and therefore it can be a potential source for spread of exotic pathogens, particularly viral pathogens, which have been associated with high morbidity and mortality of farmed fishes [5,6]. In the past century, the rise of new forms of intensive aquaculture, the increasing global movement of aquatic animals

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https://doi.org/10.1016/j.fsi.2018.09.003
Received 15 July 2018; Received in revised form 31 August 2018; Accepted 5 September 2018
Available online 07 September 2018
1050-4648/

and their products, and various artificial pressures to aquatic ecosystems have led to the emergence and outbreak of numbers of diseases in fishes. The global expansion of fish farming industry and the consequent improvement in fish health monitoring have led to discovery of several new scientific viruses, which are almost endemic in local population and overflow opportunistically to infect fishes in aquaculture facilities, while other well-known fish viruses also cause significant losses in aquaculture.

Diseases caused by various fish viruses, bacteria, parasites and other pathogens pose great hazards to aquaculture industry and threat to food safety. As people pay more and more attention to the quality and safety of aquatic products and environmental pollution, the utilization of various chemical drugs to prevent diseases in aquaculture animals has been increasingly questioned. At present, prevention and control of aquaculture diseases mainly include methods of drug control and immune prevention. The potential risks of drug resistance, allergic reactions and poisoning reactions caused by drug residues have a serious impact on the environment, farmed animals and consumption of cultured products. Immunoprevention can not only improve the specific immunity of fish, but also can enhance the ability to resist stress, meeting the requirements of no environment pollution and no residues in aquatic products. Due to its ability to safely and effectively prevent aquaculture diseases, vaccine has become the mainstream technology for prevention and control of aquatic diseases in the world. Vaccination has also become the normative production standard for international modern aquaculture. In the late 1990s, the commercialization of fish vaccines developed rapidly and as a result, there are 38 global approvals in 2003, over 100 in 2006, and over 140 in 2012 according to incomplete statistics [7].

In the following sections, authors summarized the major emerging viral diseases that cause major losses in cyprinid fish farming, expanding in host or geographic scope due to the risk of spreading through the commercial trade of finfish. The respective pathogen-targeted vaccines were described subsequently. It is hoped that the information provided in this review could contribute to cyprinid farming industry decreasing the economic loss caused by viral disease and improving the quality of aquatic products.

2. Cyprinid viral diseases

Six cyprinid viral diseases are summarized in Table 1.

2.1. Koi herpesvirus disease

Koi herpesvirus disease (KHVD) caused by koi herpesvirus (KHV) [8] is a listed notifiable disease to the International Office of Epizootics (OIE, 2018), spreading to most regions around the world due to the global fish trade and international ornamental koi shows [9,10] (FAO, 2018). First detected in the late 1990s, KHVD has been found in different continents (Europa, Asia, North America and Africa), leading to serious worldwide financial losses in common carp and koi culture industries. Once fish are infected, the virus could persists for some period of time in a latent or carrier state without obvious clinical signs [11]. Molecular analysis shows that little variation among isolates is found, as it might be expected for a virus that is being rapidly disseminated by the global movement of infected fish [12].

KHVD is relatively host-specific, while only common carp and its ornamental subspecies, koi [13], are involved in the explosive losses reported globally [8]. In addition to its negative economical and societal impacts, KHVD has also a negative environmental impact by affecting wild populations of carp. In addition, hybrids of common carp and goldfish show partly susceptibility to KHV infection, however the mortality rate is observed to be rather limited [14]. Cohabitation experiments indicate that some carp species such as goldfish, common bream (*Abramis brama*), silver carp and grass carp, can carry KHV asymptomatically and transmit it to wild carp [15–19]. KHVD is

Table 1
Cyprinid viral diseases.

Disease	Pathogen	Genome	Taxonomic classification[a]	Year emerged	Major influenced species	Known geographic distribution	OIE listed[b]
Koi herpesvirus disease	Koi herpesvirus (KHV); Cyprinid herpesvirus-3 (CyHV-3)	dsDNA	*Herpesvirales Alloherpesviridae, Cyprinivirus*	1997 [8]	Koi; Common carp	Asia; Europe; North America; Africa	Yes
Spring virenis of carp	Spring virenis of carp virus (SVCV)	(−) ssRNA	*Mononegavirales, Rhabdoviridae, Vesiculovirus*	1971 [64]	Common carp; Grass carp; Bighead carp; Silver carp; Goldfish	Asia; Europe; North America; South America	Yes
Grass carp hemorrhagic disease	Grass carp reovirus (GCRV)	dsRNA	*Reoviridae Spinareovirinae Aquareovirus*	1954 [98]	Grass carp; Black carp;	Asia; Europe; Africa	No
Koi sleepy disease	Carp edema virus (CEV)	dsDNA	*Poxviridae*	1974 [28]	Common carp; Koi	Asia; Europe; North America; South America	No
Carp pox disease	Carp pox herpesvirus (CPHV); Cyprinid herpesvirus-1 (CyHV-1)	dsDNA	*Herpesvirales Alloherpesviridae, Cyprinivirus*	1563 [1]	Common carp; Crucian carp; Orfe	Asia; Europe	No
Herpesviral haematopoietic necrosis	Herpesviral haematopoietic necrosis virus (HVHNV); Cyprinid herpesvirus-2 (CyHV-2)	dsDNA	*Herpesvirales Alloherpesviridae, Cyprinivirus*	1995 [146]	Goldfish; Crucian carp and its common varient	Asia; Australia	No

Note.
[a] ICTV, 2017.
[b] OIE, 2018.

seasonal, occurring when water temperature is between 18 °C and 28 °C. It is highly contagious and extremely virulent with mortality rate up to 80%–100%. Fish infected with KHV and kept at 23–28 °C die between 5 and 22 dpi (days post infection) with a peak of mortality between 8 and 12 dpi [8,20,21]. Carp of all ages, from juveniles upwards, are affected by KHV, but younger fish (1–3 months, 2.5–6 g) seem to be more susceptible to infection than mature fish (1 year old, nearly 230 g) [22].

Loss of osmoregulation of the gill, gut and kidney contributes to mortality during acute infection with KHV [23]. After infection, the initial clinical signs appear at 2–3 dpi, when fish become lethargic, lose appetite and lie at the bottom of the tank with the dorsal. In ponds, infected fish usually gasp at the surface of water gathering close to the water inlet or sides of the pond. Gill necrosis coupled with extensive discoloration and increased mucus secretion appear as early as 3 dpi. The skin exhibits different clinical signs depending on the stage of the infection. Hyperemia particularly at the base of the fins and on the abdomen and pale, irregular patches on the skin associated with mucus hyper-secretion often appear at the beginning of infection. Then, dead epithelium peels away lacking of mucus cover. Appearance of epidermis surface with a sandpaper-like texture and herpetic lesions are observed at the following infection condition [24]. In the later stages of infection, fin erosion and bilateral enophthalmia are observed. Some fish show neurologic signs, getting disoriented and losing equilibrium in the final stage of disease [8,22,25–28].

The enveloped virion of KHV is formally classified as the species cyprnid herpesvirus 3 (CyHV-3). CyHV-3 is a member of genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales* [29]. The *Alloherpesviridae* is a newly designated family which regroups herpesviruses infecting fish and amphibians [30], being divided into four genera: *Cyprinivirus*, *Ictalurivirus*, *Salmonivirus* and *Batrachovirus* [29]. The genus *Cyprinivirus* contains viruses that infect common carp (cyprinid herpesvirus, CyHV-1 and CyHV-3), goldfish (cyprinid herpesvirus 2, CyHV-2) and freshwater eel (anguillid herpesvirus 1, AngHV-1) (International Committee on Taxonomy of Viruses, ICTV, 2017). The genome of CyHV-3 is a typical herpesvirus containing a 295 kb, linear, double stranded DNA molecule consisting of a large central portion flanked by two 22 kb repeat regions [31]. So far, it is the largest genome among all sequenced herpesviruses [32].

Various CyHV-3 diagnostic methods have been developed based on detection of infectious particles, viral DNA, transcripts or antigens. A series of molecular techniques for detection of viral DNA fragments has been developed, such as DNA hybridization, PCR, nested PCR, one-tube semi-nested PCR, semi-quantitative PCR, real-time TaqMan PCR, and loop-mediated isothermal amplification [33]. CyHV-3 can be detected in environmental water, infected fish tissues and cell cultures by real-time PCR after viral concentration [34,35], in carp serum by ELISA aiming at the specific anti-CyHV-3 antibodies [33], in tissues and touch imprints of organs from infected fish by immunohistochemistry, immunofluorescence assays [36] and monoclonal antibodies against CyHV-3 ORF68 without cross-reaction against CyHV-1 and CyHV-2 [37]. A CyHV-3-detection kit (The FASTest® Koi HV kit) allows detection of CyHV-3 in gill swabs in just 15 min [38].

CyHV-3 also attracts originally fundamental researches. It is phylogenetically distant from the vast majority of studied herpesviruses, thereby providing an original field of research. As the genome sequence published, it is revealed to be a fascinating virus with unique properties in the *Herpesvirales*, such as an extremely large genome, a high number of genes which are not homologous to known viral sequences and genes that are normally found exclusively in the *Poxviridae* [31]. Meanwhile, CyHV-3 genome analysis also reveals several genes encoding proteins potentially involved in immune evasion mechanisms [31].

Immune system of vertebrate recognizes virus particles by pattern recognition receptors (PRRs), which senses particular protein ligand and virus nucleic acids [39]. PRRs detect viral pathogen associated molecular patterns (PAMPs) to trigger intracellular innate immune pathways and subsequent adaptive immune responses by producing cytokines, chemokines, T-cell stimulatory factors, etc [40]. Mammalian cells have the capacity to detect viral proteins using a series of receptors, such as cell surface toll-like receptor 2 (TLR2) [41] and a multiple set of cytoplasm receptors to viral DNA including a DNA dependent activator of interferon regulatory factors (DAI) [42], the interferon-inducible protein 16 (IFI16) [43] and RNA polymerase III [44]. In teleost, endosome TLR9 has been identified in numbers of carp species [45–47], but further information on receptors for viral DNA remains unclear and the recognition mechanism of CyHV-3 by cyprinid cells need further research. The recognition of viral molecular patterns leads to the production of type I interferon (IFN−I), which is crucial in antiviral response to many virus infections [48–50]. IFNs bind to specific cell-surface receptors, then trigger the JAK/STAT signal transduction pathway and subsequently activate interferon stimulated genes (ISGs), which encode for a wide array of proteins with antiviral properties [51]. In carp cells, CyHV-3 can be interfered by the activation of IFN-I. poly(I:C) stimulation in fibroblastic carp cells induces the expression of IFN-I, limiting CyHV-3 infectivity and replication, delaying the spreading of CyHV-3 *in vitro* [52]. In carp infected by CyHV-3, IFN-I response is detected in skin at 24 h post infection [53] and subsequently in spleen [52]. IFN-I system triggers the expression of a quantity of genes encoding antiviral proteins, including grass carp reovirus-induced genes 1 (GIG-1) protein, ubiquitin like protein (ISG15) and ubiquitin specific protease 18 (USP18), inhibiting viral replication directly [52]. Furthermore, in CyHV-3 infected carp, genes encoding chemotactic cytokines of CC family are up-regulated [54]. Additional observation indicated that the induced inflammation may derive from the rapid prominent leukocytosis in the blood cycle of carps during the initial two days after CyHV-3 infection. Complement system also participate in the reaction against CyHV-3 displayed as pathway triggering and complement hemolytic activity increasing in blood plasma [52]. The activation of the complement system leads to neutralisation of viruses, phagocytosis of coated virus particles or elimination of virus infected cells by lysis. In conclusion, innate immune system including IFN-I, inflammation and complement are induced by CyHV-3 infection, while the importance for resisting the infection still needs to be addressed in greater detail.

2.2. Spring viraermia of carp

Spring viraemia of carp (SVC) is one of the ten piscine viruses listed by the OIE (OIE, 2018) as a notable animal disease. It is caused by a fish rhabdovirus, spring viraemia of carp virus (SVCV). However, unlike infectious haematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV), SVCV is related to genus *Vesiculovirus* of the family *Rhabdoviridae*, order *Mononegavirales* [55]. It exhibits an enveloped, bullet-shaped virion and measures ~80–180 nm in length and 60–90 nm in diameter [55,56]. It has a negative-sense, ssRNA genome of ~11 kb, containing five ORFs encoding five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA dependent RNA polymerase (L). The five viral genes are organized in the typical order of rhabdoviruses: 3′—N—P–M–G–L-5’ [57–59]. Genotypes of SVCV isolates from various locations form four major genetic clades [60]. SVCV isolates of the recent emergence and geographic range expansion link to the spread of the virus within China where common carp are reared in large numbers for food and koi are reared for export [61]. It is responsible for the highly contagious SVC, especially in common carp [55,62,63].

SVCV was first detected in Yugoslavia in 1971 [64]. It was initially believed to be endemic among common carp in Eastern and Western Europe, and subsequently reported in the Americas [61,65] and Asia [55,58,66]. SVCV infection has now emerged in many European countries, including the UK, Denmark, Germany, the Netherlands, Austria, Spain, France, the Czech Republic, Georgia, Belarus, Moldova, Ukraine and Russia associated with numerous financial losses in

common carp and koi [60,61,67–70]. These outbreaks occur in both farmed and wild fishes suggesting the expansion of influenced range. The emergence of SVC in region where is free of SVC, such as North America, Asia and in portions of Europe, appears to be the consequence of both improved surveillance and the large volume of ornamental fish global shipment.

Outbreaks of SVC usually occur in spring, which is why the disease is called so [71]. Studies in carp have shown that few adult fish are infected when the water temperature is over 17 °C, but juveniles can be infected even at 22 °C − 23 °C [71]. SVCV infection is highly lethal in young fish, with mortality rate up to 90% [63]. Other risk factors associated with morbidity and mortality include fish density, geographical location, fish species and the immune status of susceptible fish [71]. SVCV infection can spread by fomites and parasitic in vertebrates. Natural SVCV infections have been reported in other cyprinid fish, including goldfish, koi, silver carp, crucian carp, bighead carp, grass carp, tench (*Tinca tinca*) and orfe (*Leuciscus idus*) [55,62,72]. Experimental infections have been reported in zebrafish (*Danio rerio*), golden shiner (*Notemigonus crysoleucas*), roach (*Rutilus rutilus*), guppy (*Lebistes reticulatus*), pumpkinseed (*Lepsomis gibbosus*), northern pike (*Esox lucius*), fathead minnow (*Pimephales promelas*), emerald shiner (*Notropis atherinoides*) and white sucker (*Catostomus commersonii*) [55,73–75].

SVCV infection is generally associated with non-specific symptoms, such as exophthalmia, abdominal distension and oedema of the vent region [55]. Petechial hemorrhages can be seen on the skin, gills, eyes and internal organs, particularly on the walls of the swim bladder [55,76]. Other lesions may include degenerated gill lamellae, oedematous internal organs, swollen and coarse-textured spleen, hepatic necrosis, enteritis, and pericarditis [75,77]. The histological changes associated with hepatopancreas may range from perivasculitis to panvasculitis with a higher degree of oedematization and loss of structure of blood vessels walls. The hepatopancreas parenchyma show hyperaemia, multifocal necrosis and adipose degeneration [77], whilst the spleen is often hyperaemic, showing a co-significant hyperplasia of the reticuloendothelium [55]. Multifocal necrosis and non-purulent inflammation often occur in the pancreas of affected fish, and the heart shows pericarditis and discontinuous myodegeneration [75]. The visceral and parietal serosa of the peritoneum show peritonitis. In the intestine, perivasculitis with subsequent atrophy of the villi is often observed [55,75]. The epithelial layer of the swim bladder changes into a discontinuous multilayer and hemorrhages are commonly observed in the submucosa [55].

As SVCV has a significant impact on carp aquaculture, its rapid detection and identification are crucial for effective control of the disease. Conventional serological techniques include the virus neutralisation test, immunoperoxidase assay, indirect immunofluorescence assay and ELISA. However, these techniques are laborious and time consuming [78–81]. Moreover, the indirect immunofluorescence assay and ELISA appear to cross-react with other rhabdoviruses [80], leading to possible false-positive diagnoses. mAbs are also useful tools for detecting SVCV and studying the function of virus-specific proteins [82,83]. Recently, a single-chain fragment variable antibody against SVCV has been developed using phage display technology and employed for rapid detection of SVCV [84]. This antibody reacts specifically with SVCV, not cross-react with other viruses. Thus, this approach provides the basis for establishing simple and cost-effective way for the development of immunological detection assays for SVCV. Various PCR-based assays have also been used to detect SVCV owing to their high sensitivity. These include reverse transcription (RT)−PCR combined with nested PCR [85], multiplex real-time quantitative RT-PCR [86] and one-step TaqMan real-time quantitative RT-PCR [87]. These assays have clearly improved the specificity and sensitivity of detection [88]. Although PCR is generally considered impractical for routine diagnostics in the field owing to the need for specialized instruments, skilled operators and isolation of RNA extracts, a recent report described an improved RT-PCR assay that is able to accurately detect SVCV directly from fish tissues, indicating the potential application of this technology for SVCV detection in infected fish in the field [89]. A loop-mediated isothermal amplification (LAMP) assay has been increasingly used for the detection of viruses [90–93]. RT-LAMP is a simple and effective technique to rapidly amplify specific nucleic acid sequences under isothermal conditions. Moreover, it requires uncomplicated and inexpensive equipment easily manipulated at fish farms. This assay has been successfully applied for disease control in aquaculture [94,95]. Two studies have used RT-LAMP to detect SVCV based on nucleotide sequences of the G and M genes [96,97].

2.3. Grass carp hemorrhagic disease

In 1950s, Chinese experts found that sick grass carp suffer from bleeding symptoms, suspecting that the hemorrhage disease was caused by virus [98]. Grass carp hemorrhagic disease caused by grass carp reovirus (GCRV) leads to billions of RMB yuan of economic loss every year [99]. The disease is identified as a viral etiology in 1978 and is the first fish viral disease studied in China [100]. The mortality rate of grass carp can reach more than 90% at the fingerling stage, and it can also infect other species, such as black carp, making these fish die of bleeding symptoms [101].

The prevalence of GCRV has a typical seasonality. The high incidence period of GCRV is mainly in summer when water temperature is 25 °C-30 °C. The main reason is that the optimum temperature of the virus polymerase is 28 °C. In many high density grass carp pools, when the temperature of the water exceeds 24 °C, the virus rapidly proliferates and may result in serious dysfunction of the fish and death. When the water temperature is lower than 20 °C, the virus proliferation will be inhibited, and even lose their infectivity.

The clinical signs are organ hemorrhages showing spots or plate forms, in combination of some or all of the following signs: exophthalmia, body darkening, hemorrhage of the mouth cavity, hemorrhagic or pale gills, and hemorrhage at the base of fins and branchiostegites. Internal hemorrhage may occur throughout the musculature, hepatopancreas, spleen, kidney, and intestines. Present researches classify the disease into three types according to clinical signs: red muscles, enteritis, or red fin and branchiostegite [102].

GCRV belongs to group C, *Aquareovirus* genus in the *Spinareovirinae* subfamily, *Reoviridae* family. It is the first fish virus isolated in mainland China and is also known as the most virulent virus in *Aquareovirus* [103]. The genome of GCRV consists of 11 segments of dsRNA, encoding 12 proteins, including seven structural proteins and five nonstructural proteins [104,105]. To date, more than thirty GCRV strains have been isolated from infected grass carp in global. Based on the difference in VP6 genome constitution, GCRV could be mainly clustered into three types, and the representative strains of three types are GCRV-873 (type I), GD108 (type II), and GCRV104 (type III), respectively [104–106]. As a consequence of the fast evolution, identities of amino acid sequences among types are less than 30% [104–106]. Among the three types, GCRV type II is considered to be the most pathogenic and prevalent type currently in China and may be closer to *Orthoreovirus* than any other known species of *Aquareovirus* showed by phylogenetic analysis [105,107].

The virus identification by RT-PCR are the main method of surveillance and diagnosis of GCRV infection. Primary diagnosis in fish farms is based on typical external and internal clinical signs, especially the “red muscles” that appears in some sick fish. Epidemiological investigations are usually carried out in summer when it is easy to detect the virus from the carrier or sick fish. The virus can be identified by ELISA or RT-PCR.

At present, the antiviral immune responses against GCRV in grass carp are broadly researched. As a dsRNA virus, GCRV activates the similar PRR-PAMP recognition mechanism of innate immune system, being sensed by different receptors. Cytoplasm viral infection is

primarily detected by RIG-I-like receptors (RLRs), a PRR family consisting of retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetic and physiology 2 (LGP2) [108]. RIG-I preferentially binds to relatively short 5′-phosphorylated dsRNA, while MDA5 binds to long dsRNA [109,110]. By GCRV or poly(I:C) challenge, mRNA level of RIG-I is up-regulated in CIK cells [111]. CPE assay and viral titter reveal the significant antiviral activity of full-length RIG-I in response to GCRV infection. Meanwhile, the expression levels of these three genes are significantly induced in spleen and hepatopancreas tissues after GCRV infection [111–114]. LGP2 is found as a negative regulator for both RIG-I and MDA5 in resting state or early stage of GCRV infection. TLR signaling pathways also participate in the immune responses to GCRV infection. Up-regulations of TLR3, TLR7, TLR8, TLR19, TLR22 and other TLRs are observed post poly(I:C) or GCRV challenge [115–119]. The enhancement of NOD1 and NOD2 also occur in grass carp post GCRV infection [120,121]. All the members of HMGB family can respond to poly(I:C) and GCRV challenge in grass carp, delaying the CPE in CIK cells, meanwhile modulating RLR and TLR pathway genes [122–126]. Similar to mammals, fish IFN antiviral responses are initiated through the pattern recognition of virus component by TLRs and RLRs [127]. Although antiviral immune researches in teleost have achieved great progresses and several fish specific immune genes have been identified, studies on the regulation mechanisms of fish antiviral immune signaling pathways remain far behind those in mammals.

2.4. Koi sleepy disease

Koi sleepy disease (KSD) is an emerging problem harmful to koi. It was first detected in Japan in 1974 [128,129]. After its initial detection in koi populations in Japan, the disease was limited to Japan until 2013 when it was reported in both koi and common carp in numerous European countries, including Germany, England, Austria, Poland, France and the Netherlands [130–133]. In addition, it also spreads to Brazil, the United States, China and India [134–136]. The disease is exclusively described in common carp and koi breeding facilities.

KSD shows clinical symptoms when the water temperature is 15 °C-25 °C [129,137]. Clinical signs are seen in extreme association with stressful farm conditions, often occurring when fish are transferred from earthen to concrete ponds, causing a mortality of up to 80–100% and prevalence to 87.5%, especially in juvenile koi [137]. The typical appearance, lying on the bottom of the ponds, leads to the name KSD. Clinical signs are lethargy, skin hemorrhages with oedema in the underlying tissues, enophthalmos and pale swollen gills [5,129,137]. In addition, gill hyperplasia and necrosis as well as lamellae clubbing, leading to dyspnoea and hypoxia are also seen in infected fish [5,128,137].

Carp edema virus (CEV), a double-stranded DNA virus, belonging to the family *Poxviridae*, is the causative agent of KSD, based on transmission electron microscopy [129,137]. This mulberry-like enveloped member of the pox virus group has been identified in gill cells from diseased fish, and further morphological characterization is provided by electron microscopy [5,128,137]. Sequencing the known DNA fragment encoding the core protein P4a from infected fish in different locations in Europe and Asia shows a 6%–10% of genetic diversity. It is classified into three genetic genogroups, called I, IIa and IIb [138,139]. Viruses from genogroup I usually present in farmed carp, while CEV from genogroup IIa mainly in diseased koi [133,138]. Infection experiments with both genogroups in koi and farmed carp confirmed biological differences between virus isolates [140]. The genetic diversity and the observed differences in biology reveal that the virus has been present in the European carp population for a prolonged period and infections are diagnosed until recently.

Similarities of pathognomonic signs of viruses affecting carp in spring, such as CEV, KHV and SVCV [6,130], highlight the necessity for exploiting specific diagnostic procedures. Although the disease has been successfully transmitted, using filtrated suspensions of gill homogenates obtained from sick fish [5], the virus has yet to be isolated successfully and grown in cell culture. KSD diagnosis relies on detection of viral DNA by PCR [129,133,141]. In the present reports, the diagnosis relied on clinical findings is confirmed by molecular detection of an undefined segment of the viral genome. A nested PCR protocol is initially designed in Tokyo University of Fisheries [129], recently is modified in CEFAS-Weymouth Laboratory, England, to improve reliability of detection. The latter method of detection is also used for an extensive phylogenetic analysis to reveal the presence of two potential main viral lineages.

2.5. Carp pox disease

Carp pox disease caused by CyHV-1 is probably the earliest recognized viral disease of fish, dating back to 1563 and had broken out in Europe, North Korea and Japan [1]. It has also been found in Hubei, Henan, Hebei and many other provinces in China. CyHV-1 mainly infects common carp over 1 year old and also has some effect on crucian carp, hybrids of common carp and goldfish, having no harm to black carp, grass carp, silver carp, bream [2].

The proper temperature of carp pox is 9 °C-16 °C. Fish will recover without any administration when over 22 °C. Therefore, temperature control is an efficient method to fight against CyHV-1. Carp pox is an endemic chronic skin disease. In the early days after infection, the fish become thin and transparent. Smooth gray and white patchy growth organisms appear and is covered with a thin layer of white mucus. With the development of the disease, white spots gradually expand and thicken, the number gradually increases and interconnects into pieces. Smooth, creamy to brown appears as pink “paraffin-like or glass-like" pox and sores on blood vessels. These growth organisms are very tightly bound to the surface of infected fish body. The main component of the growth organisms is collagen fibers, which do not transfer but can naturally fall off and reappear in the original affected area. The back, head, fins and caudal peduncle are dense areas of pox and sores. The severely diseased fish is full of pox and sores, and the lesions often have bleeding. When the proliferation of organisms spreads to the bulk of the fish body, it will affect the growth and development of fish. The spine is damaged, the bones are softened, and the appetite diminishes, but generally no death.

The occurrence and disappearance of pox can be controlled by changing certain environmental factors, such as improvement of water quality or rise of temperature leading to the disappearance of symptoms, but affecting the growth and value of the fish. Inoculation of the pox with scratches on healthy common carp can produce the same symptoms as natural conditions [142]. In natural conditions, when water quality deteriorates, toxic substances stimulate the surface of fish and tissues, secreting large amounts of mucus, and finally the mucus falls off.

2.6. Herpesviral haematopoietic necrosis

Herpesviral harmatopoietic necrosis was firstly detected in farming goldfish in Japan [143]. It is also found in Australia [144] and New Zealand [145], spreading to global. The disease caused a large-scale death of goldfish fry in Taiwan in 1995, and CyHV-2 is responsible [146]. CyHV-2 infects crucian carp in many provinces in China [147,148], commonly known as crucian carp gill hemorrhagic disease. The influenced species only include goldfish [149], crucian carp and its common variety [150], and hybrids of goldfish [14].

CyHV-2 is harmful to fish eggs, fry, fingerlings, broodstock, etc., while juveniles are more susceptible to infection than adult fish, and are more likely to cause fulminant deaths of juveniles less than 1 year old [151]. In addition, like other herpesviruses, CyHV-2 can form latent infection and becomes a potential source of transmission [152],while temperature is a key factor for virus replication. At the beginning of the

disease, the fish appear dying, poor appetite and slow swimming, staying at the bottom of the pond or tank. After the skin is pale with mucus, pustules appear on fins. It appears part of the abdomen swells, the eyeballs protrude from both sides and there is freckle bleeding on the sputum [153], blisters on the fins [149], ascites, etc. [3]. The hepatopancreas, spleen and kidney are pale and the spleen and kidney are swollen [154]. Hematopoietic cells in the head kidney and body kidney show obvious nuclear pyknosis and nuclear lysis necrosis, with spleen large areas of necrosis, sometimes accompanied by hemorrhage as well as pancreatic, thymus, intestinal development from multiple lesions to diffuse necrosis, epithelial cell proliferation, degeneration and necrosis of the oropharynx and epidermal cells, focal necrosis of the heart. Other tissues and organs including muscle tissue, brain tissue are found no pathological changes [143,144,146,151].

The partial or complete nucleotide sequences of CyHV-2 such as helicase gene, DNA polymerase gene, terminal enzyme gene, and capsid protein genes have been reported, but the complete genome sequence and gene map have not been determined yet, requiring further researches and improvement. Crucian carp *Carassius auratus* herpesvirus (CaHV) was isolated and its genome was sequenced and analyzed, showing that CaHV is most closely related to CyHV2 and clusters closely with CyHVs of the family *Alloherpesviridae* [155].

At present, PCR is a relatively accurate method for detecting CyHV-2 infection, even a very small amount of viral DNA in tissue [145]. Conventional PCR method based on CyHV-2 helicase gene have high specificity, with no amplification of the genomic DNA of CyHV-1, CyHV-3 and IcHV-1. It can effectively detect CyHV-2 isolates in different regions. A real-time quantitative PCR is established [153], which has high specificity and high sensitivity. It can not only detect CyHV-2 from fish exhibiting clinical symptoms, but also detect carriers. The diagnosis can detect the sick fish in the incubation period in advance so that it can prevent the breakout in time and take appropriate measures to minimize the loss of the disease.

3. Vaccine development

The first successful use of inactivated *Aeromonas salmonicida* for oral immunization in rainbow trout (*Oncorhynchus mykiss*) in 1942 opens up a precedent for the utilization of vaccines in fish [156]. In the following decades, many researchers make extensive explorations of the preparation of fish vaccines to prevent the sudden emergence of fish diseases that are difficult to control with drugs. According to incomplete statistics, the number of commercial vaccines for global commercial production has been more than 140 by the end of 2012 [157]. Currently, there are more than 50 aquaculture vaccines studied in China and nearly 30 species of pathogens are involved, according to incomplete statistics [7]. However, only 5 aquatic vaccines have obtained the national new veterinary drug certificate up to now.

3.1. Vaccine types

According to the preparation method, aquaculture vaccines are classified into live vaccine, inactivated vaccine and gene engineering vaccine (Table 2).

3.1.1. Live vaccine

At present, most of the aquaculture live vaccines are prepared with attenuated or mutated attenuated virus strains whose pathogenicity has been greatly weakened, such as VHSV [158], Canine coronavirus (CCV) [159], IHNV [160], and GCRV [161] vaccines.

3.1.2. Inactivated vaccine

The inactivated vaccine is a managerial method that inactivates pathogenic microorganisms, but it remains immunogenic, providing the ability to induce specific resistance of aquatic animals after inoculation. These vaccines include a variety of tissue fluid inactivated vaccines, vibrio inactivated vaccines, *Aeromonas hydrophila* vaccines, and streptococcal vaccines, for example, VHSV vaccine [162] which is commonly used in trout farming in Europe and the United States.

3.1.3. Gene engineering vaccine

Gene engineering vaccine is further divided into recombinant subunit vaccine, DNA vaccine, gene deletion/mutant vaccine and living-vector vaccine.

3.1.3.1. Recombinant subunit vaccine. The protective antigen genes of pathogens is expressed in different expression systems *in vitro* using recombinant DNA technology to produce pathogenic proteins that can induce protective immune responses in hosts. After separation and purification, recombinant subunit vaccine is completely prepared. Recombinant subunit vaccines do not contain pathogenic virulence factors and are expressed by genetically engineered bacteria. At present, there are many studies on the preparation of subunit aquatic vaccines based on immunogenic components such as bacterial outer membrane proteins, lipopolysaccharides and other protective antigens, but most of them are still in the experimental stage and have not been commercialized. Gene engineering vaccines in aquaculture include IHNV [163], infectious pancreatic necrosis virus (IPNV) [164,165], among which the IPNV VP2 recombinant subunit vaccine is currently the only commercially available fish recombinant vaccine [165].

3.1.3.2. DNA vaccine. A DNA vaccine is a recombinant eukaryotic expression vector that encodes a certain protein antigen and is directly injected into animals. After being taken in by host cells, it expresses an antigen protein, thereby inducing non-specific and specific immune responses, playing an important role in immune protection effect. The research on aquatic DNA vaccine was firstly reported in 1996 [166,167]. Although the DNA vaccine researches start late, encouraging achievements have been made [168]. Till nowadays, DNA vaccines mainly focus on the prevention and control of infectious virus diseases such as IHNV [169], VHSV [170], SVCV [171], and snakehead rhabdovirus (SHRV) [172].

3.1.3.3. Gene deletion/mutant vaccine. Gene deletetion or mutant vaccine is a type of vaccine made by genetic engineering removing a fragment of a virulence gene in a viral or bacterial genome, making it a defective strain. Vaughan reported the first gene mutation vaccine, the attenuated strain of *Aeromonas salmonicida* to prevent salmon furunculosis in 1993 [173]. Gene deleted/mutant vaccine could induce T cell immune responses in rainbow trout [174,175]. This type of vaccine is also researched in channel catfish against channel catfish virus (CCV) [176] and in zebrafish against *Erwinia* [177].

3.1.3.4. Live-vehicle vaccine. Live-vehicle vaccines are genetically engineered vaccines that use a non-pathogenic virus or bacteria to carry and express other highly pathogenic or protective immune-related antigen genes, creating a multivalent vaccine. Under this conditions, attenuated pathogens have become excellent carriers of heterologous antigens in prevention and treatment [178]. The effect of a bacterial live vector vaccine is mainly concentrated on whether it can express a sufficient amount of protein to cause the host immune responses and produce a protective immune response [179]. The initial report of living-vehicle vaccine in aquatic organism was in 1995, preventing *Salmonella* in salmon [180]. Subsequently, the application of living-vehicle vaccine was reported to prevent *Streptococcus* [181], *Edwardsiella tarda*, *A. hydrophila* [182] and CCV [183].

3.2. Delivery methods

Common immunization delivery methods of aquaculture vaccines include injection, immersion and oral administration (Table 3).

Table 2
Frequently-used fish vaccine types.

Classification		Merit	Defect
Live vaccine		Generally attenuated vaccine; Close to natural infection; Effectively stimulate immune system; Pathogen can reproduce *in vivo*; Low dosage; Long protection duration; No adjuvant needed	Poor safety under natural conditions; Short shelf life; Not convenient storage and transportation
Inactivated vaccine		Short development cycle; Safe to use; Easy to preserve	Cannot reproduce *in vivo* after immunization; Large immunize dosage; Short duration; Appropriate adjuvant is needed
Gene engineering vaccine	Recombinant subunit vaccine	Excellent safety; Simple production; Easy control; High stability; High purity	The expression is affected by the expression system; Weak immunogenicity; Short protection duration; Difficult to effectively remove intracellular pathogens
	DNA vaccine	High stability; Induce comprehensive immune response; Easy production in large scale; No risk of return of the virus; Low cost; The most promising vaccine	Uncertain immune response caused by the vaccine; Tissue distribution and expression still unknown; More researches are needed to confirm the stability; Immune tolerance; Risk of integrating into the genome
	Gene deletion/mutant vaccine	High stability; Not easy to reverse under natural conditions; Good immunogenicity; Guaranteed safety; Important direction of vaccine development	Not many successful cases;
	Living-vector vaccine	Can be administered through mucous membrane system; Easy control;	Restricted by its expression level and its persistence; Risk of plasmid loss reducing vaccine function; Environmental safety needs to consider

3.2.1. Injection

Global aquaculture vaccines are mainly immunized with injection. Aquaculture vaccine injection mainly includes intramuscular injection and intraperitoneal injection. Intramuscular injection is mainly used for vaccination of DNA vaccines, while intraperitoneal injection is mainly used for inoculation of traditional inactivated vaccines and attenuated vaccines [184].

3.2.2. Immersion

There are four types of immersion immunization, direct immersion, hypertonic soak, spraying immunization and immersion bath. After the first successfully hypertonic immunized fish in 1976 [185], vibrio vaccine achieves success in soaking immunization in salmon [186] and rainbow trout [187]. However, the mechanism of fish vaccination during immersion immunization is still unclear, such as whether the vaccine enters the body through the skin, tendon, lateral line, or other sites, and whether the vaccine-induced immunity works through the blood circulation system or the mucosal system, etc. In addition, a variety of factors affect the host's uptake of soaked immune antigens, including vaccine concentration, immersion time, aquatic animal size, adjuvants, antigenic forms, water temperature and so on [188].

3.2.3. Oral administration

Traditionally, adjuvant vaccine, biofilm vaccine and antigenic component vaccine can be implemented by oral immunization. Along with the development of new technology, new oral immunization more prefer to slow-releasing micro capsule vaccine, yeast carrier vaccine and transgenic plant vaccine [189]. Oral vaccines are considered to be the most operative immune modes for aquatic vaccines. The main consideration is that the living environment of aquatic animals is inseparable from the water body and the cultured animal scale is large.

3.3. Cyprinid viral disease vaccine

There are only four validation productions for cyprinid viral vaccines in global, which are KHV inactivated vaccine approved in Israel in 2003, carp herpesvirus live vaccine approved in the United States in 2012, SVCV inactivated vaccine approved in Czech Republic in 2012 and GCRV live vaccine in China in 2011 (Table 4). GCRV gene engineering injection vaccine is still under clinical trials and other cyprinid viral disease vaccines are under laboratory researches.

A KHV vaccine patent is granted in the United States in 2018. A attenuated recombinant KHV comprising a genome in which ORF56 and ORF 57 are mutated, wherein the herpesvirus is capable of

Table 3
Comparison of delivery methods of fish vaccines.

	Infection	Immersion	Oral
Target	Systemic	Mucosal system	Digestive system
Administration ease	Laborious	Less convenient	Convenient
Stress	Severe (requires anaesthetization and handling); Moderate for automated vaccination	Moderate for spraying; Severe for bath	Negligible
Potency	Best with adjuvant	Less efficient	Lowest
Immunity duration	6–12 months	Shorter	Shortest
Cost	Cost-effective for high value species	Cost-effective for fish < 10 g	Moderate
Merit	Best immune effect; High serum antibody titer; Low vaccine dosage	Suitable for multiple times immunization; Not restricted by fish body size	Suitable for small size fish species and large-scale immunization; Save time and effort
Defect	Large fish stress response; Suitable for large size fish body; Time-consuming and laborious	Damage vaccine to a certain extent; Large dosage of vaccine; Short immune effect duration	Synergistic measures are needed; Large dosage

Table 4
Cyprinid viral disease vaccine production licensed in global.

Vaccine	Immune object	Preparation method	Country	Approved time
KHV vaccine	Koi	Inactivated	Israel	2003
Carp herpesvirus vaccine	Cyprinid	Live	Unite States	2012
SVCV vaccine	Common carp	Inactivated	Czech Republic	2012
GCRV (strain-892)	Grass carp	Live	China	2011

replication, got the patent granted to protect common carp and koi against KHV indicating excellent immunoprotection capability [190]. KHV liposome oral vaccine can protect common carp from KHV infection [191]. Abundant immune genes are found to participate in the antiviral responses against KHV, indicating that the immune response to KHV is largely controlled by the genetic factors of the host [54].

There are various GCRV vaccines reported in the past decades. GCRV histoplasmic inactivated vaccine was attempted in China since 1960s. Until 1986, Chinese researchers exploited high-efficiency attenuated vaccine with good immune effect and long protection period. GCRV is detoxified by eucalyptus liquid and injected to immunize grass carp demonstrating long-term immunoprotection and up to 90% protection ratio [161]. The immunogenicity of VP5 and VP7, which are the major outer capsid protein of GCRV type I, are researched a lot to evaluate the potential of function as vaccine [192]. Recombinant of VP5 and VP7 vaccine is synergistically orally administrated to grass carp inducing good immunoprotection and less than 10% cumulative mortality [193]. Outer capsid protein VP4 in GCRV type II is also used to prepare GCRV vaccine. The immune protection assay shows that IgM and other antiviral immune responses are significantly triggered and 82% protection ratio is achieved [107]. Other researches on GCRV gene engineering vaccines mainly concentrate on the cell proliferation enhancing activity and immunoprotection of GCRV VP6 and VP7 recombinant plasmid [194–197]. A GCRV VP6 DNA vaccine obtained by baculovirus expression system can achieve a protection ratio of 95% [198]. Using *Bacillus subtilis* as the carrier of GCRV VP4, the recombinant vaccine is orally immunized grass carp. Challenge experiment reveals that the oral recombinant subunit vaccine can protect 50%–60% grass carp from infection and generate immunity against GCRV [199]. GCRV VP5 expressed by *Escherichia coli* is proved to be relative to virus infection and have the potential to produce subunit vaccine [200]. The GCRV inactivated vaccine induces the IFN system of grass carp and strengthens the IgM expression continuously [201]. There are still hundreds of researches unlisted, working on the preparation of GCRV vaccine to improve the utility of vaccines to protect grass carp from GCRV infection. Immune adjuvant is pivotal to enhance the potency of vaccines against GCRV. New type immune adjuvant like CpG [202,203] develops rapidly in the past decades.

Inactivated viral vaccine against SVCV provides limited protection, whereas attenuated vaccine has not been pursued because of a number of limitations, including improper attenuation of the virus, lack of quantitative evaluation for the protection activity provided by the vaccine, and restrictive market and legal regulations [62]. Regardless of the obstacles of technologies and regulatory approvals [204], researches on DNA vaccines against SVCV has been steadily increasing in the last decade [205–207]. The initial SVCV DNA vaccine did not exhibit an expected effectiveness like other fish DNA vaccines against *Novirhabdoviruses*, including IHNV and VHSV [208]. Therefore, multiple trials have been conducted to develop an efficacious SVCV DNA vaccine. The G gene of SVCV encodes a surface glycoprotein which is considered to be a major antigen for inducing the primary host immune responses and is frequently used in DNA vaccine preparation [171,209]. Ten SVCV DNA vaccines carrying SVCV G gene are tested in carp [171]. The majority of treated groups show little protection, with relative survival ranging from 21% to 33%. The strongest protection (48% relative survival) is observed in a group injected with a combination of two constructs expressing the full-length G gene. However, the relative contribution of the two constructs to this protection rate is not determined [171]. The efficacy of another DNA vaccine expressing the G gene of a North American SVCV isolate is investigated in ornamental koi and goldfish [209]. In all trials, immunized fish demonstrate a strong protective response against SVCV, with relative survival ranging from 50% to 88% [209]. Vaccination with an oral or injection subunit vaccine based on the baculovirus recombinant expression of transmembrane SVCV-G protein in insect cells shows positive immune protection [210]. Taken together, these studies put forward the validation for the potential utilization of G gene for DNA vaccine as a prophylactic therapy against SVCV infection.

Although DNA vaccination via the parenteral route has been used as an efficient strategy to elicit antiviral immunity, it is not convenient for large-scale immunization due to the handling stress on fish, high labour and production cost [211]. Furthermore, there is limited scientific literature about the condition of DNA vaccines after injection into fish [212]. Intestine, gills, skin and other mucosal tissues of fish, are important for immune protection against pathogen invasion [213,214]. Activation of mucosal immunity prevents pathogen infections and replications in mucosa, whereas intramuscular vaccines fail to do this [214]. Oral vaccination is an effective way to induce mucosal immunity [215] and this strategy has shown a successful induction of the antiviral responses against viral diseases in different fish species [165]. *Lactobacilli* possess multiple properties to be suitable candidates for vaccine antigen delivery vectors [216]. *Lactobacilli* also play a non-specific immune adjuvant effect [217]. A genetically engineered *Lactobacillus plantarum* co-expressing SVCV G protein and KHV ORF81 protein is investigated for protective immunity in carp and koi through oral vaccination [218]. Immunized carp and koi show effective protection rates of 71% and 53%, respectively [218]. Moreover, immunized carp shows significantly elevated expression of IgM [218]. These results demonstrate that the recombinant *L. plantarum* is able to induce a protective immune response in fish against SVCV and KHV, suggesting a practical approach for large-scale control of cyprinid viral disease [218].

4. Conclusion and prospects

Along with the rapid development of freshwater fish farming industry, increasing number of diseases break out, especially viral diseases. The huge economic losses and food and environment safety risks caused by the diseases lead to the urgent need of vaccine. The variation of pathogens and the diversity of antigens are the main obstacles of efficient vaccine development. At the same time, the immune response mechanisms of vaccines are still uncertain, which not only increases the difficulty of suitable programming, but also leads to the blindness of vaccine researches and applications. The use of fish vaccines lacks effective and convenient ways of administration as well as measures for enhancing vaccine immunity and effective vaccine efficacy assessment systems and methods. More factors restrict the utilization of vaccines, for example, water temperature, water quality, illumination and season. In addition, the organism conditions (age, weight, nutriture, physiological status, group effect) and vaccine quality (immunogenicity, adjuvant, preparation, storage, transportation, administration method, dosage and time) also have a great influence on the potency of vaccine.

In the future, the studies on fish vaccine are needed to improve the theoretical basis. The tendency is to concentrate efforts to solve one or two severe aquatic animal diseases as a breakthrough and promote the development of fish vaccines. Meanwhile, the development of classic and new fish vaccines need to cooperate with each other, with the help of human and veterinary vaccine researches. The research on the administration in relation to the effectiveness of fish vaccines is an important part of vaccine development. It is urgent to establish a simple

and effective fish vaccine evaluation system and accelerate the construction of pilot test bases for fish vaccines. The developments of appropriate immunopotentiators and immune adjuvants also promote the potency of fish vaccines.

As the worldwide rapid development of science and technology as well as the positive attempts in fish and other aquatic animals, aquaculture industry is showing the tendency to flourish. Taking full advantage of the immune responses to diseases, vaccination is a vital strategy to prevent and control the potential diseases, solving the problems of food quality and safety as well as environmental pollution.

Acknowledgements

This work was supported by National Natural Science Foundation of China (31873044) and Huazhong Agricultural University Scientific & Technological Self-Innovation Foundation (2014RC019).

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