Fish and Shellfish Immunology 85 (2019) 132–140

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Correlates of protective immunity for fish vaccines

Hetron Mweemba Munang'andu**, Øystein Evensen*

Norwegian University of Life Sciences, Faculty of Veterinary Medicine, Department of Basic Sciences and Aquatic Medicine, Ullevålsveien 72, P.O. Box 8146, NO-0033, Oslo, Norway

ARTICLE INFO

Keywords:
Antibodies
Correlates
Immunity
Protective
Vaccines

ABSTRACT

Vaccination is one of the most effective disease control strategies that has contributed to the significant reduction of disease outbreaks and antibiotics usage in salmonid aquaculture. To date, licensing of fish vaccines is to a limited extent based on *in vitro* correlates of protection, as done for many mammalian vaccines. This is because the immunological mechanisms of vaccine protection have not been clearly elucidated for most fish vaccines. Herein, we provide an overview of the different steps required to establish correlates of protective immunity required to serve as benchmarks in optimizing vaccine production in aquaculture. We highlight the importance of optimizing challenge models needed to generate consistent results used during vaccine development as a basis for establishing immune correlates of protection. Data generated this far shows that antibodies are potentially the most reliable correlates of protective immunity for fish vaccines. Our findings also show that antigen dose can be optimized to serve as a correlate of protection for fish vaccines. Further, there is need to establish signatures of T-cell protective immunity when antibodies fail to serve as proxies of immune protection, particularly for vaccines against intracellular pathogens. We can anticipate that documentation of efficacy for future vaccines in aquaculture, particularly batch testing will be based on *in vitro* correlates of protective immunity.

1. Introduction

Vaccination is one of the most effective disease control strategies that has contributed to a significant reduction of disease outbreaks and antibiotics use in salmonid aquaculture [1,2]. In Norway vaccination led to the significant reduction of outbreaks of bacterial diseases and reduction in the use of antibiotics in the mid-1990s [3]. Documentation and licensing of these bacterial vaccines were based on lethal *in vivo* challenge [1]. Immunoassays such as the enzyme linked immunosorbent assay (ELISA) are widely used to evaluate antibody responses induced by vaccination, but often antibody levels are not correlated with protective endpoints. From a regulatory and 3Rs standpoint, it is important to establish *in vitro* correlates of protection with a purpose to reduce the use of animals in vaccine testing. It should be that the mechanisms of immune protection for most fish vaccines are not well understood, *i.e.* it is not clear as to whether vaccination prevents establishment of infection, blocks pathogen dissemination, or prevents establishment of pathology in infected fish. That said, to establish *in vitro* correlates of protection, the first point is to identify a quantitative *in vitro* method that correlates with protection obtained from lethal *in vivo* challenge methods using a vaccine that has been shown to meet the acceptance criteria for the vaccine (as defined by the manufacturer or from authorities).

The underlying immunological mechanisms of protection are not necessarily known in detail. The identified immune markers can serve as guides for correct choices of protective antigens, vaccine formulations, antigen dose or vaccination regimes. In this way, the identified immune markers can be used to optimize all factors that are important for protection like vaccine antigens, formulations, antigen dose and immunization regimes and these should be set at optimal conditions to produce immune responses that confer protective endpoints.

Herein we discuss various aspects of correlates of protection for fish vaccines. Given that the bulk of previous studies in fish vaccinology have centered on the use of inactivated whole pathogens or recombinant proteins as antigens, formulations, immunization and relative percent survival (RPS), we start with the importance of the challenge model as a prerequisite to establishing the protective endpoints used as measures of vaccine efficacy. We continue with a discussion of different ways to measure vaccine efficacy like ability to prevent pathogen adherence to mucosal surfaces and at prime sites of entry, ability to protect against systemic pathogen distribution and prevention against pathology in target organs. Then we move over to *in*

* Corresponding author.
** Corresponding author.
E-mail addresses: hetroney.mweemba.munangandu@nmbu.no (H.M. Munang'andu), oystein.evensen@nmbu.no (Ø. Evensen).

https://doi.org/10.1016/j.fsi.2018.03.060
Received 10 November 2017; Received in revised form 25 March 2018; Accepted 31 March 2018
Available online 03 April 2018

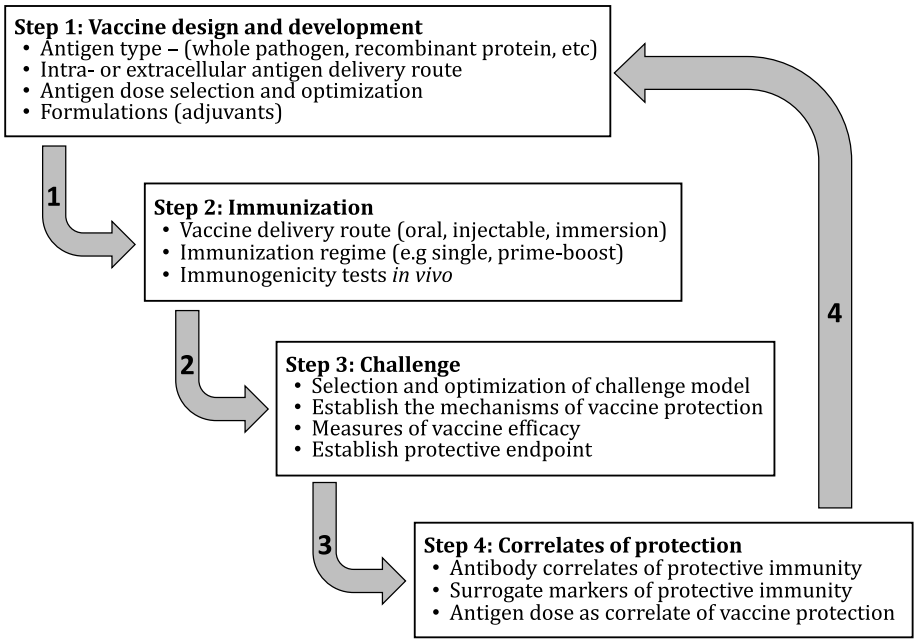

Fig. 1. Shows hypothetical steps involved from vaccine design and development to establishing the correlates of protection. Note that while arrows 1 to 3 show the steps involved from vaccine design (step-1), immunization (step-2), challenge (step-3), and correlates of protection (step-4), arrow 4 serves as a feedback loop in which the established correlates of protection serve as drivers in optimizing all vaccinology inputs (steps 1–3) in order to ensure production of highly protective vaccines, reproducibility and consistence in vaccine production based on outputs of immune markers established in step-4.

vitro immunological methods as measures or proxies of efficacy before we end up discussing if antigen dose of the final product can be used as a correlate of vaccine potency. Overall, the objective of this review is to bridge the gap between fish vaccinology and application of immunological correlates of protection as benchmarks for the documentation and testing of vaccine efficacy and batch potency of fish vaccines in aquaculture.

2. Challenge models

As part of vaccine design and development, *in vivo* vaccine efficacy testing is first used to determine the protective endpoints and then correlate these with *in vitro* quantitative immunological methods (Fig. 1). Fish are typically immunized with test vaccine preparations of decreasing antigen content followed by lethal challenge in order to establish a dose-response curve. For a challenge model to be reliable, it is important that it has a wide discriminatory capacity between the vaccinated and unvaccinated fish to effectively determine vaccine-related protection [4,5]. To ensure consistence and reproducibility it is vital that the challenge model is optimized to generate similar results repeatedly. As pointed out in our previous study [5], it important that all input variables that form the challenge model are individually optimized and their contribution to the overall performance of the model is evaluated. The challenge models commonly used in aquaculture are (i) intraperitoneal, (ii) bath and (iii) cohabitation.

2.1. Intraperitoneal challenge model

Although the intraperitoneal mode of challenge yields high results and is widely used for evaluating the efficacy of different vaccines [6], it has the disadvantage of bypassing the natural routes of infection. Forcibly depositing pathogens in the peritoneum enables the pathogens to gain quick access into the systemic environment thereby reducing the incubation period leading to rapid onset of acute infection but it guarantees identical challenge dose being given to each fish which is the main advantage of this method. Intraperitoneal challenge precludes the ability to evaluate protection at natural portals of pathogen entry on mucosal surfaces and as such precludes the possibility to evaluate the protective role of mucosal immunity in vaccinated fish to the extent this is possible. The infection dose used for intraperitoneal challenge is determined from LD_{50} studies (or similar) and the minimum mortality for testing of vaccine efficacy is typically at or above 60% in the non-vaccinated control fish while at the same time, mortality should not exceed 80–85% (on average), depending on pathogen studied.

2.2. Bath challenge model

The bath challenge method has the advantage of exposing fish to infection by a natural route, *i.e.* through the natural portals of pathogen entry via mucosal surfaces. The major limitation with this method is that the challenge dose per fish will not be equal or uniform and thus will be a source of variation. The experience is also that mortality in non-vaccinated control fish show higher degree of variation than for injection challenge (own experience).

2.3. Cohabitation challenge model

The cohabitation challenge model also has the advantage of mimicking natural disease transmission where infected shedders act as source of infection to cohabitees. The use of shedders mimics the principle of introducing an index case as a primary source of infection to a susceptible population. Therefore, this method simulates natural disease outbreaks in which infection is introduced from a few infected individuals (shedders) or persistent carriers that undergo recrudescence. Hence, cohabitation differs from bath challenge in which the entire susceptible population is introduced into an infected environment while in cohabitation infection starts from a few infected individuals increasing progressively until it reaches an "epidemic proportion" in a susceptible population. Nevertheless, there are similarities between bath and cohabitation challenge in that both models enable pathogens to enter the host through natural portals of entry facilitating the ability to evaluate the mechanisms of protective immunity on mucosal surfaces. In addition, both models allow for sequential progression of infection from the portals of pathogen entry on mucosal surface to establishment of pathology in target organs paving way to identifying the mechanisms of protective immunity both on mucosal surface and the systemic environment. As for bath challenge methods, the challenge dose per fish is unknown and will of course vary with regard to dose and time of exposure, introducing variation into the model.

3. Measures of efficacy and immunological mechanisms of vaccine protection

The central goal for evaluating vaccine efficacy is to determine the protective endpoint induced by vaccination or the antigen dose and vaccine formulation that will confer specified protection, as defined by the vaccine manufacturer or by authorities/European Pharmacopoeia monographs. From a theoretical viewpoint, protective endpoints can be evaluated by different methods like prevention of (i) pathogen adherence on mucosal surfaces, (ii) infection after penetration of mucosal surfaces, (iii) pathogen dissemination into the systemic environment, (iv) pathogen load in target organs, or (v) tissue damage in target organs. For these test points to be of any value for determination of vaccine efficacy they must be quantifiable and they must correlate with protection against mortality as obtained for a batch of vaccine shown to meet the release criteria for a defined vaccine. Below we will discuss some potential approaches to assess protective endpoints related to different stages of infection and corresponding protection.

3.1. Pathogen adherence on mucosal surfaces

An important outcome of vaccination of fish would be to produce immune responses that prevent pathogen adherence on mucosal surfaces. Studies have shown that mucosal antibodies play a role in protection against pathogen adherence on the skin surface of vaccinated fish [7,8]. For example, Dickerson and Clark [8] showed that fish previously exposed to sub-lethal infection of *Ichthyophthirius multifilis* were resistant to subsequent infections corresponding with mucosal antibodies that deterred parasite adherence on the skin surface. Antibodies from resistant fish easily bound to the immobilization antigens (i-antigens) on the parasite cell surface and ciliary membranes. They noted that antibody cross-linking with the i-antigen culminated in expulsion of the parasite from the skin surface clearly demonstrating that antibodies located in the mucosa have the capacity to prevent pathogen adherence on mucosal surfaces. Similar mechanisms of immune protection against *I. multifilis* have been reported in catfish by Wang et al. [7,9] and Zhao et al. [10] who demonstrated the ability of mucosal antibodies to prevent *I. multifilis* adherence on the skin surface of catfish. In their studies Wang et a l [7,9], Zhao et al. [10], and Dickerson and Clark [8] showed a correlation between protection against *I. multifilis* adherence and increase in mucosal antibodies on the skin surface suggesting that a protective antibody titer that prevents *I. multifilis* adherence on the skin surface can be established. More recently gut-specific antibodies named IgT have been described [11] also found in other mucosal organs like skin [12] and gills [13]. The importance of IgT for immune protection of mucosal surfaces remains to be shown although infected fish show IgT responses locally with deposition of IgT onto parasite and bacterial surfaces [11,12]. However, it is not well understood if IgT functions merely as a factor that prevents colonization and proliferation of pathogenic bacteria on mucosal surfaces or plays an effector function (opsonization and complement activation) against mucosal pathogens.

3.2. Prevention of pathogen dissemination and spread

Most pathogens are dispersed to internal organs through the blood stream (viremia) after gaining entry through mucosal surfaces. For example, Finstad et al. [14] showed that piscine reovirus (PRV) was localized in erythrocyte cytoplasmic inclusions while Aamelfot et al. [15–17] showed that infectious salmon anemia virus (ISAV) entered Atlantic salmon red blood cells where it induced hemophagocytosis. ISAV viremia has been associated with replication in leucocytes [18]. Other viruses detected in blood leukocytes include infectious pancreatic necrosis virus (IPNV) [4,19]. Bacterial pathogens disseminated through the blood stream also include a number of pathogenic bacteria [20,21]. To prevent pathogen dispersal, neutralizing antibodies eliminate pathogens entering the blood stream to prevent their deposition in internal organs. Previously we showed that high antibody titers (1.2 OD_{490}, 1:50 dilution) in Atlantic salmon vaccinated against IPNV were linked to low viremia while low circulating antibody levels gave way to high viremia [4]. Consequently, there were few organs infected by the virus in fish that had low viremia compared to fish that had high viremia levels. Hence for viruses such as ISAV, spring viremia of carp virus (SVCV) and IPNV as well as bacteria that are mainly disseminated by the hematogenous route, neutralizing antibodies prevents these pathogens from reaching their target organs. Therefore, the measure of efficacy for fish vaccinated against such pathogens is the absence (elimination) or reduction of the pathogen in blood. However, it is important to point out that not all pathogens are dispersed through the blood stream. For example, nervous necrosis viruses (NNV) use the neuronal system to reach their target organs [22]. The possibility to quantify levels of circulating virus or bacteria by cell culture, plate count or real-time PCR methods makes it possible to create a link between vaccination responses like circulating antibodies, mortality levels post vaccination and challenge and pathogen load. Thus, the basis for establishing correlates of protection against mortality would be possible.

3.3. Prevention of infection establishment in primary replication sites

For pathogens that gain entry into the systemic environment they get deposited in organs that serve as primary replication sites that also serve as amplification sites able to produce high quantities of the pathogen for onward dissemination to target organs. Hence, vaccines that suppress pathogen replication in the primary replication sites have the capacity to prevent infection progression and ultimately block pathogen deposition in the target organs. This was demonstrated in the case of IPNV in which it was shown that high antibody levels corresponded with reduction of viral loads in the headkidney, spleen and liver that served as primary replications sites and consequently prevented the spread of virus to the pancreas, which is one of the target organs [4]. Similarly, Su et al. [23] showed reduced infection rates of *Streptococcus agalactiae* in several organs of vaccinated tilapia that was linked to blocking the bacteria from crossing the brain-barrier to induce infections in the brain, which is one the target organs for this bacterium in tilapia. The key point here is that it is possible to quantify the pathogen load in primary replication sites or target organs and correlate load with level of protection and particularly reduction in pathogen load in surviving, vaccinated fish versus non-vaccinated controls. Similarly, dose-response (i.e. varying antigen dose) studies can also be performed when pathogen load can be quantified and a cut-off can be determined that correlates with protection against mortality.

3.4. Prevention of pathology in target organs

There are several diseases that do not induce high mortality in fish, but they cause pathology in the target organs leading to significant economic losses in aquaculture. For example, salmonid alphavirus (SAV) causes acute necrosis in the exocrine pancreas and skeletal muscles in infected fish. Infection with piscine myocarditis virus (PMCV), the cause of Cardiac myopathy syndrome (CMS) causes myocyte degeneration and necrosis, and inflammation in the ventricle myocardium [24–26]. Infections with piscine orthoreovirus (PRV), the causative agent of heart and skeletal muscle inflammation (HSMI), causes myocarditis, pericarditis and endocarditis as well as red skeletal muscle inflammation [27–29]. These diseases produce tissue damage in the target organs rendering histopathology to be 'the method of choice' for diagnosis [30–33] in which severity is graded according to the level of tissue damage in target organs [26,34,35]. Haugland et al. [36] showed that increase in viral loads in CMS infected target organs correlated with severity of CMS histoscores in which the relationship between histoscores and viral Ct-values showed a correlation of $r^2 = 0.76$.

Scoring for pathology of internal (target) organs represent subjective, ordinal values and these responses (dependent variables) are recorded together with vaccine doses and pathogen load in target organs like virus titer, colony forming units or real-time PCR values etc., all continuous variables. Using logistic regression models make it possible to establish predictive values that indicate level of protection attained for different vaccine preparations (for example). The mechanisms of vaccine protection for these diseases is to prevent or reduce pathology in the target organs of vaccinated fish. Previously, we showed that viral loads > 10^7 $TCID_{50}$/mL were linked to establishment of pathology in the pancreas and liver of Atlantic salmon infected by IPNV. Vaccinated fish that had high antibody levels (> 1.4 OD_{490}, dilution 1:50) showed absence of tissue damage in the pancreas and liver indicating that a cutoff limit of antibody titer could serve as a correlate of protection against pathology in fish vaccinated against IPNV infection [4]. Hence, it is likely that similar cutoff limits that could serve as measures of protection against pathology can be established for various diseases including HSMI, CMS and SAV.

3.5. Relative Percent survival (RPS)

Evaluation of efficacy for most fish vaccines is carried out by determining the relative percent survival (RPS) of vaccinated fish compared to the unvaccinated control fish after lethal challenge with a purpose to determine what vaccine preparation meets the criteria defined by the manufacturer or by regulatory [6,37]. This approach lacks detail on the mechanisms of vaccine protection as it does not show if vaccination prevents pathogen adherence on mucosal surfaces, establishment of infection at the portals of pathogen entry, pathogen dispersal to target organs or suppresses pathogen replication in target organs. Another limitation is that not all pathogens cause high mortality in infected fish, unreliable mortality [38,39] or no mortality at all like infection with (PRV) [35] or PMCV [36]. This precludes the use of RPS as a measure of vaccine efficacy for such diseases. Under such circumstances protection against pathology in target organs can be used to evaluate vaccine efficacy [38] as mentioned above.

4. Correlates of vaccine protection

4.1. Antibody titer as correlate protective immunity

Antibodies are the mostly widely used correlates of protection for mammalian vaccines [40]. Zinkernagel [41] pointed out that the easiest method for demonstrating the protective ability of antibodies is by passive transfer from vaccinated to unvaccinated individuals followed by challenge using an infectious agent homologous to the vaccine strain. In fish, several studies have shown the protective ability of passively transferred antibodies involving different pathogens in various fish species (Table 1). Pasnik et al. [42] found a high correlation between antibody titers and the level of post challenge protection in passively vaccinated Nile tilapia (*Oreochromis niloticus*) against *S. agalactiae* indicating that passively transferred antibodies can be correlated with post challenge protection in fish.

In line with the definition of a correlate of antibody protection by Pulendran et al. [43], an antibody is considered to have reached a 'correlate of protection' when it reaches a cutoff limit above which it confers protective immunity in vaccinated individuals. It follows that an antibody titer that reaches the protective threshold (for a given vaccine) can serve as a signature of protective immunity. Bricknell et al. [44] showed that Atlantic salmon vaccinated with an *Aeromonas salmonicida* vaccine having an antibody titer $\log_2 > 4$ survived lethal challenge while 47% fish having titers $\log_2 < 4$ died indicating that a threshold antibody titer able to produce high protection (RPS > 90%) can be established to serve as a signature of protective immunity against *A. salmonicida* in Atlantic salmon. In three independent studies [4,19,45], we showed that antibody titer > 1.2 OD_{490} (Serum dilution 1:50) corresponded with PCSP > 90% while an antibody titer > 1.4 OD_{490} produced PSCP > 92% indicating that an antibody titer can be established that correlates with PSCP > 90% in Atlantic salmon vaccinated against IPN. Moreover, these findings also show that increasing the antibody titer from 1.2 OD_{490} to 1.4 OD_{490} increased the PCSP from 90% to 92%. Similarly, Dubey et al. [46] showed that an increase in antibody levels from 0.416 OD_{450} to 0.463 OD_{450} corresponded with increased protection, RPS increasing from 37.33% to 79.99% in carp vaccinated against *Aeromonas hydrophila* using an OMP vaccine encapsulated in PLGA nanoparticles. Put together, the increasing trend of antibody titers that correspond with increase in post challenge protection shows that an antibody titer that correlates with protection can be established to serve as a benchmark for which all newly developed vaccines can be measured. All vaccines producing antibody levels below the correlate of protection should be considered as suboptimal while vaccine producing antibody levels at or above the level considered as protective are potent or meets the required potency level.

It is important to reiterate that current advances in fish immunology show a compartmentalization of immunoglobulin (Ig) isotypes distribution in fish [47]. IgT is expected to play a role in protection on mucosal surfaces using mechanisms similar to IgA in humans [47] although the exact mechanisms are not understood and there are no studies that correlate IgT titers with protective immunity [4,6], while IgM provides systemic protection [6,37,48]. As pointed out previously in our study [49], this can be attributed to the lack of immunoassays able to quantify IgT levels in/on mucosal surfaces in response to vaccination so defining protective endpoint as commonly done for IgM in serum is difficult [4,6]. In summary developing appropriate diagnostic tools for measuring IgT titers expressed on mucosal surfaces in response to vaccination is needed and further to correlate these responses with prevention of microbial infections on mucosal surfaces. Therefore, IgM is still by far the best-known correlate of protective immunity in vaccinated fish [48,50–53].

4.2. Gene expression as surrogate markers of protective immunity

It is noteworthy that while several studies show that cellular mediated immune (CMI) responses are activated by replicative viral vaccines [37,54–56], they are also activated by bacterial DNA and attenuated live vaccines in fish. For example, Yamasaki et al. [57] showed significant increase in $CD4^+$ and $CD8^+$ T-cell responses together with upregulation of the Th1 cytokines such as IFNγ and T-bet linked to high RPS in ginbuna crucian carp (*Carassius auratus langsdorfii*) vaccinated against *Edwardsiella tarda* using a live attenuated vaccine. On the contrary, fish vaccinated using a formalin inactivated whole cell (IWC) vaccine had high post challenge mortality despite having high antibody and IL-4/13 responses when Th1 responses were suppressed. In another study, Yamasaki et al. [58] observed *E. tarda* clearance in the head-kidney and spleen following high cytotoxic T-lymphocyte (CTL) activity induced by increased CD8α+ cell responses. They also observed that *E. tarda* specific antibodies did not increase until after bacterial clearance indicating that humoral responses appear too late to provide protective immunity against intracellular *E. tarda* infection. To consolidate the notion that CMI responses are crucial for induction of protective immunity against intracellular bacterial infections, Yamasaki et al. [59] adoptively transferred T-cells sensitized by *E. tarda* to isogenic naïve ginbuna crucian carp in order to identify T-cell subsets involved in protecting fish against intracellular bacterial infection. They observed that recipients of $CD4^+$ and $CD8^+$ cells acquired high resistance against *E. tarda* infection. In addition, transfer of sensitized CD8α+ cells upregulated the expression of Th1 cytokines such as IFNγ and perforin involved in cytotoxic mediated elimination of infected cells. Put together, these studies show that CMI responses play a crucial role in protection against intracellular bacterial infections and that they can be activated by vaccination in a similar manner with viral induced CMI response in fish [37,54–56]. However, what has not been established is

Table 1
Passive immunization studies in fish.

Pathogen	Fish species	Ref
Aeromonas hydrophila	Rainbow trout (*Oncorhynchus mykiss*)	[76]
Aeromonas hydrophila	Indian major carp (*Labeo rohita*)	[77]
Aeromonas salmonicida	Coho salmon (*Oncorhynchus kisutch*)	[78]
Aeromonas salmonicida	Brook trout (*Salvelinus fontinalis*)	[79]
Flavobacterium columnare	Channel catfish (*Ictalurus punctatus*)	[80]
Flavobacterium psychrophilum	Rainbow trout (*Oncorhynchus mykiss*)	[81]
Lactococcus garvieae	Yellowtail (*Seriola quinqueradiata*)	[82]
Streptococcus agalactiae	Nile tilapia (*Oreochromis niloticus*)	[42]
Streptococcus ictaluri	Channel catfish (*Ictalurus punctatus*)	[83]
Streptococcus iniae	Nile tilapia (*Oreochromis niloticus*)	[84]
Streptococcus iniae	Rainbow trout (*Oncorhynchus mykiss*)	[85]
Streptococcosis	Rainbow trout (*Oncorhynchus mykiss*)	[86]
Ichthyophthirius multifiliis	Channel catfish (*Ictalurus punctatus*)	[87]
Ichthyophthiriasis	Tilapias (*Oreochromis aureus*)	[88]
Infectious hematopoietic necrosis virus	Rainbow trout (*Oncorhynchus mykiss*)	[89,90]
Yersinia ruckeri	Rainbow trout (*Oncorhynchus mykiss*)	[91]
Viral hemorrhagic septicemia virus	rainbow trout (*Oncorhynchus mykiss*)	[92]
Viral hemorrhagic septicemia	Pacific herring (*Clupea pallasii*)	[93]
Vibrio alginolyticus	IgY-encapsulated	[94]
Vibrio anguillarum	Rainbow trout (*Oncorhynchus mykiss*)	[95]

the duration for which activated $CD8^+$ T-cells remain protective after vaccination. Moreover, the quantity of activated $CD8^+$ cells that correspond with protection against intracellular infections has not been established in fish. And as such, the protective endpoint of activated $CD8^+$ cells against infection remains unknown for fish vaccines. Dissimilar to antibodies that serve as correlates of vaccine protection for antigens that stimulate B-cell responses in which an antibody titer can directly be correlated with RPS, reduction in the quantity of infectious agents and prevention of pathology in target organs after challenge [60–62], there are no studies shown to directly correlate the quantity of $CD8^+$ T-cells activated by vaccination with RPS, reduction in the quantity of infectious agents and prevention of pathology in target organs after challenge.

Based on the aforementioned above, for vaccine preparations that evoke immune responses biased towards CMI responses, such as DNA or attenuated live vaccines, measuring antibody responses as correlates of protection might not give a full picture of immune responses involved (like T-lymphocyte responses). Most DNA or live attenuated vaccines will however raise an antibody response and antibodies can thus serve as proxies of protective immunity, a surrogate marker of immune protection where cellular immune responses do the actual job. Plotkin [40,63,64] defined a surrogate as an immune marker that can be used to substitute for the clinical endpoint, which can reliably be used to predict vaccine efficacy [65]. Several studies have shown a high correlation between the expression of Mx and protection in fish vaccinated against viral hemorrhagic septicemia (VHSV) and infectious hematopoietic virus (IHNV) using DNA vaccines at early time post vaccination [65,66]. For example, McLauchlan et al. [65] showed early correlation within three days between Mx and protection in rainbow trout vaccinated against VHSV at early time post vaccination. Antibodies were expressed 8 weeks later indicating that Mx profiles at early time could serve as an early surrogate marker of protection or predictor of protection against VHSV infection. Similarly, Byon et al. [67] showed significant upregulation of cellular mediated immune genes such as Mx, CD8α, CD40 and BLAM within three days after vaccination against VHS with a DNA vaccine encoding the VHSV G-protein linked to high protection (RPS = 93%) while antibodies were expressed earliest by 8 weeks post vaccination in Japanese flounder (*Paralichthys olivaceus*). Similarly, Kim et al. [66] showed a high correlation between protection and Mx expressed within a few days and antibodies were expressed several weeks after vaccination in fish vaccinated against IHNV infection.

Surrogate markers of protective immunity can potentially be established for non-replicative vaccines as shown in our previous studies in which we found high correlation ($r^2 = 0.896$, $P < 0.0000$) between GATA-3 and antibody responses which in turn corresponded with post challenge survival proportions (PCSP) in Atlantic salmon vaccinated against IPN [68]. Hence, GATA-3 can serve as a surrogate marker predictive of the outcome of post challenge protection. Similarly, Bridle et al. [69] found a biosignature of multiple genes inclusive of immunoglobulin mu heavy chain, hepcidin, Lim and acting binding protein 1 as predictors of vaccine induced protection in Atlantic salmon vaccinated against *Yersinia ruckeri*. Put together, these studies show that surrogate markers can potentially be established for fish but more studies are needed to fully validate these findings.

4.3. Antigen dose as a correlate of vaccine protection

Yamashita et al. [70] immunized red-spotted grouper against nervous necrosis virus (RGNNV) using different antigen doses ranging from $10^{6.5}$ to $10^{8.5}$ $TCID_{50}$/fish. After challenge, they observed that fish immunized with an antigen dose > $10^{7.5}$ were highly protected and yet there was no significance difference in fish vaccinated with an antigen dose of $10^{6.5}$ and control fish indicating that reducing the antigen dose by one $\log_{10}$ accounted for a significant difference between highly protected fish and less (or none) protected fish. In their findings, they showed that an antigen dose of $10^{7.0}$ $TCID_{50}$/fish served as the cutoff limit for protection against NNV in grouper. Similarly, we showed that an antigen dose of 2×10^{10} $TCID_{50}$/mL corresponded with PCSP > 90% in Atlantic salmon vaccinated against IPN while reducing the antigen dose by a $\log_{10}$ significantly lowered the PCSP to < 42% [4]. Hence, an antigen dose of 2×10^{10} $TCID_{50}$/mL could serve as correlate of protection for PCSP > 90% in Atlantic salmon vaccinated against IPN. Li et al. [71] showed an antigen dose dependent increase in protection levels in tilapia vaccinated against *S. agalactiae* in which increasing the antigen doses from 10^5 to 10^9 CFU/fish showed a corresponding increase of RPS from 10.2% to 67.7% resulting in a significant linear correlation ($r^2 = 0.965$, $P < 0.008$) (Fig. 2). Huang et al. [72] also found a dose-dependent increase in protection in Nile tilapia vaccinated against *S. agalactiae* in which increasing the antigen dose from 10^7 to 10^9 CFU/fish increased the RPS from 20% to 33%. Table 2 shows different studies in which increasing the antigen dose was shown to correspond with increased protection for different vaccines administered in different fish species against various diseases. Overall, these studies show that increasing the antigen dose induces a corresponding increase in protection in vaccinated fish and that a cutoff limit of

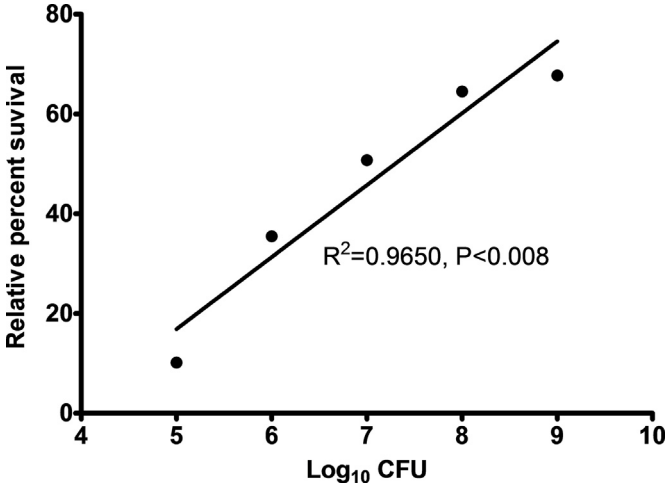

Fig. 2. Shows a significantly high linear correlation between increase in antigen dose and relative percent survival (RPS). Data was extrapolated from Li et al. [71] in vaccination of tilapia using *Streptococcus agalactiae* vaccine.

antigen dose can be established that correlates with protection to serve as benchmark against which all future vaccine batches can be assessed. Vaccines of an antigen dose at or above the protective antigen dose can be considered as protective while vaccines below the protective antigen dose would be considered as suboptimal, given that the antigens are identical.

However, it is interesting to note that LaPatra et al. [73] showed that increase in protection levels induced by DNA vaccination against IHNV infection in rainbow trout was not dependent on increase of plasmid dose, administered over a range of 0.1–2.5 mg/fish. This is a very high dose for a 2 g rainbow trout which likely explains the lack of a dose-response to vaccination and challenge. To consolidate these findings, Corbeil et al. [74] showed that a single dose as low as 1–10 ng of DNA for the IHN vaccine protected rainbow trout fry against lethal challenge. An optimal dose of 100 ng/fish was chosen to ensure a strong protection. These studies are all performed under laboratory conditions and the best documentation of vaccine effect under field conditions have been obtained with the Apex-IHN® vaccine used to protect Atlantic salmon against IHNV in British Columbia where more than 100 million fish have been vaccinated between 2005 and 2015 [75] and clinical cases of IHN have not been recorded over these years.

5. General discussion and conclusions

In this overview, we have shown that it takes several steps to establish correlates of vaccine protection. These steps include optimization of challenge models used for vaccine efficacy testing to document that the vaccine meets the specified efficacy criteria and these results must be consistent for different vaccine batches. It is also possible to establish methods that can quantify for example pathogen adherence on mucosal surfaces, penetration into mucosal organs and/or replication in primary replication sites. Prevention of tissue damage in target organs can also be assessed (quantified) and correlated with for example antigen dose but appropriate statistical methods would have to be used to obtain solid correlates with protection against pathology since these are subjective measurements (scoring of histopathological findings). It is also possible to establish 'signatures of protective immunity' from host immune response like antibody responses or cellular responses and these should be established for vaccine batches that have shown to meet efficacy criteria, i.e. protection for example against mortality or clinical disease. The defined 'signature of protective immunity' serves as a benchmark upon which all new vaccine batches (of the same vaccine) should be evaluated. Thus, all batches that elicit immune responses at or above the established protective cutoff limit are potent while those below are considered sub-potent. This is the main point with any batch release method, to separate potent from sub-potent batches.

Based on studies carried out this far [73], it is evident that antibodies are the most reliable correlate of protective immunity in vaccinated fish. However, indications are that antigen dose can also be used as a correlate of protection but here good dose-response studies have to be carried out. For vaccines that induce biased T-cell responses, it is conceivable that cellular mediated immune protection or genomic markers related to T-cell responses can serve as surrogate markers of protective immunity. However, in fish immunology we have few studies that demonstrate the protective ability of cellular mediated immune responses in vaccinated fish. Moreover, most replicative vaccines that induce cellular mediated immune responses also produce humoral responses rendering antibodies to be the most reliable correlate of vaccine protection for both replicative and non-replicative vaccines. Yet another factor that we have not discussed in any detail is the ability of the vaccine to elicit immune responses in the vaccinated animal that prevent against replication of the specific pathogen, i.e. a form of sterilizing immunity. Recent studies have shown that vaccination of Atlantic salmon with Apex-IHN® completed abolish transfer of the virus to cohabiting naïve fish [75]. This was an interesting observation since vaccinated fish were not completed protected against infection and

Table 2
Examples of vaccination showing increase in antigen dose that correspond with increase in protection.

Pathogen	Fish species	Vaccine type	Increase in vaccine dose	Increase in RPS	Ref
A: Bacterial vaccine					
Aeromonas salmonicida	Atlantic salmon	Inactivated whole cell	0%–200%	10.5–89.5%	[96]
Aeromonas hydrophila	Rohu	PLGA nanoparticles	4μg/g-8μ/g	48.3–73.3%	[46]
Aeromonas hydrophila	Major carp	Inactivated whole cell	10^5 to 10^{10} CFU/ml	40-80%	[97]
Edwardsiella ictaluri	Channel Catfish	Live attenuated strain	2.5×10^5-2.4^{10} CFU/fish	31–94.8%	[98]
Mycobacterium marinum	striped bass	DNA vaccine	5 - 50μg/fish	0.0–90.0%	[99]
Piscirickettsia	Atlantic salmon	Subunit	1:5 dilution to neat	17.5–35%	[100]
Vibrio anguillarum	Japanese flounder	DNA vaccines	5 - 50μg/fish	57.5–85.7%	[101]
Yersinia ruckeri	Rainbow trout	Inactivated whole cell	10 CFU/fish-10^8 CFU/fish	60-100%	[102]
Streptococcus agalactiae	Nile tilapia	Live attenuated strain	10^3-10^9 CFU/fish	10-100%	[71]
B: Viral vaccines					
Betanodavirus	Seven band grouper	Inactivated whole virus	$10^{6.5}$-$10^{8.5}$$TCID_{50}$/fish	9.2–82.6%	[70]
Betanodavirus	European Sea Bass	Virus like particles SB2	0.1–20μg/fish	46.5–88.9%	[103]
Betanodavirus	European Sea Bass	Virus like particles MGNNV	20-100 μg/fish	71.7–89.4%	[103]
Lymphocystis disease virus	Japanese flounder	Genetically engineered	0.1–15 μg/fish	19.6–2.6%[a]	[104]
Nodavirus	Atlantic halibut	Recombinant protein	10-50μg/fish	17-67%	[105]
Infectious pancreatic necrosis virus	Atlantic salmon	Inactivated whole virus	10^9-$10^{10}$$TCID_{50}$/mL	45-90%	[4]
Viral nervous necrosis	Seven band grouper	Recombinant protein	0.8 μg - 20μg/fish	50-90%	[106]

[a] Percentage of tumor growth induce by lymphocystic disease virus.

mortality, 2.6% mortality in vaccinated versus 97% in controls [75].

In summary, what we put forth herein underscores the use of established correlates of protection as important benchmarks in optimizing vaccine production. From a 3Rs perspective we advocate that the licensure of future vaccines in aquaculture should move towards *in vitro* methods for batch release in line with the current policy of the European Union and the European Pharmacopoieia.

Conflicts of interest

Authors declare no competing interests.

Author contribution

Both authors have made direct and intellectual contribution and approved publication of the manuscript.

Acknowledgements

This study was funded by the Targeted disease prophylaxis in European Fish farming project, Grant agreement number 311993.

References

[1] R. Gudding, W.B. Van Muiswinkel, A history of fish vaccination: science-based disease prevention in aquaculture, Fish Shellfish Immunol. 35 (6) (2013) 1683–1688.
[2] R. Gudding, T. Goodrich, The history of fish vaccination, Fish Vaccination (2014) 1–11.
[3] R. Gudding, Vaccination as a preventive measure, Fish Vaccination (2014) 12–21.
[4] H.M. Munang'andu, B.N. Fredriksen, S. Mutoloki, R.A. Dalmo, O. Evensen, Antigen dose and humoral immune response correspond with protection for inactivated infectious pancreatic necrosis virus vaccines in Atlantic salmon (Salmo salar L), Vet. Res. 44 (2013) 7.
[5] H.M. Munang'andu, N. Santi, B.N. Fredriksen, K.-E. Løkling, Ø. Evensen, A systematic approach towards optimizing a cohabitation challenge model for infectious pancreatic necrosis virus in Atlantic salmon (Salmo salar L.), PLoS One 11 (2) (2016 Feb 19), http://dx.doi.org/10.1371/journal.pone.0148467 eCollection 2016.
[6] H.M. Munang'andu, J. Paul, O. Evensen, An overview of vaccination strategies and antigen delivery systems for Streptococcus agalactiae vaccines in nile Tilapia (Oreochromis niloticus), Vaccines (Basel) 4 (4) (2016).
[7] X. Wang, T.G. Clark, J. Noe, H.W. Dickerson, Immunisation of channel catfish, Ictalurus punctatus, with Ichthyophthirius multifiliis immobilisation antigens elicits serotype-specific protection, Fish Shellfish Immunol. 13 (5) (2002) 337–350.
[8] H. Dickerson, T. Clark, Ichthyophthirius multifiliis: a model of cutaneous infection and immunity in fishes, Immunol. Rev. 166 (1) (1998) 377–384.
[9] X. Wang, H.W. Dickerson, Surface immobilization antigen of the parasitic ciliate Ichthyophthirius multifiliis elicits protective immunity in channel catfish (Ictalurus punctatus), Clin. Diagn. Lab. Immunol. 9 (1) (2002) 176–181.
[10] X. Zhao, R.C. Findly, H.W. Dickerson, Cutaneous antibody-secreting cells and B cells in a teleost fish, Dev. Comp. Immunol. 32 (5) (2008) 500–508.
[11] Y.A. Zhang, I. Salinas, J. Li, D. Parra, S. Bjork, Z. Xu, S.E. LaPatra, J. Bartholomew, J.O. Sunyer, IgT, a primitive immunoglobulin class specialized in mucosal immunity, Nat. Immunol. 11 (9) (2010) 827–835.
[12] Z. Xu, D. Parra, D. Gomez, I. Salinas, Y.A. Zhang, L. von Gersdorff Jorgensen, R.D. Heinecke, K. Buchmann, S. LaPatra, J.O. Sunyer, Teleost skin, an ancient mucosal surface that elicits gut-like immune responses, Proc. Natl. Acad. Sci. U. S. A. 110 (32) (2013) 13097–13102.
[13] Z. Xu, F. Takizawa, D. Parra, D. Gomez, L. von Gersdorff Jorgensen, S.E. LaPatra, J.O. Sunyer, Mucosal immunoglobulins at respiratory surfaces mark an ancient association that predates the emergence of tetrapods, Nat. Commun. 7 (2016) 10728.
[14] Ø.W. Finstad, M.K. Dahle, T.H. Lindholm, I.B. Nyman, M. Løvoll, C. Wallace, C.M. Olsen, A.K. Storset, E. Rimstad, Piscine orthoreovirus (PRV) infects Atlantic salmon erythrocytes, Vet. Res. 45 (1) (2014) 35.
[15] M. Aamelfot, O.B. Dale, S.C. Weli, E.O. Koppang, K. Falk, Expression of the infectious salmon anemia virus receptor on atlantic salmon endothelial cells correlates with the cell tropism of the virus, J. Virol. 86 (19) (2012) 10571–10578.
[16] M. Aamelfot, Tropism of Infectious Salmon Anaemia Virus and Distribution of the 4-o-acetylated Sialic Acid Receptor, 978-82-7725-243-8. Thesis for the degree of Philosophiae Doctor at the Norwegian School of Veterinary Science Oslo, Norway (2013).
[17] M. Aamelfot, O. Dale, K. Falk, Infectious salmon anaemia–pathogenesis and tropism, J. Fish. Dis. 37 (4) (2014) 291–307.
[18] E. Moneke, B.O. Ikede, F.S. Kibenge, Viremia during infectious salmon anemia virus infection of Atlantic salmon is associated with replicating virus in leucocytes, Dis. Aquat. Org. 66 (2) (2005) 153–157.
[19] H.M. Munang'andu, B.N. Fredriksen, S. Mutoloki, B. Brudeseth, T.Y. Kuo, I.S. Marjara, R.A. Dalmo, O. Evensen, Comparison of vaccine efficacy for different antigen delivery systems for infectious pancreatic necrosis virus vaccines in Atlantic salmon (Salmo salar L.) in a cohabitation challenge model, Vaccine 30 (27) (2012) 4007–4016.
[20] M. Sakai, K. Taniguchi, K. Mamoto, H. Ogawa, M. Tabata, Immunostimulant effects of nucleotide isolated from yeast RNA on carp, Cyprinus carpio L, J. Fish. Dis. 24 (8) (2001) 433–438.
[21] M. Zamri-Saad, M. Amal, A. Siti-Zahrah, Pathological changes in red tilapias (Oreochromis spp.) naturally infected by Streptococcus agalactiae, J. Comp. Pathol. 143 (2–3) (2010) 227–229.
[22] J.Z. Costa, K.D. Thompson, Understanding the interaction between Betanodavirus and its host for the development of prophylactic measures for viral encephalopathy and retinopathy, Fish Shellfish Immunol. 53 (2016) 35–49.
[23] Y.L. Su, J. Feng, Y.W. Li, J.S. Bai, A.X. Li, Development of a quantitative PCR assay for monitoring Streptococcus agalactiae colonization and tissue tropism in experimentally infected tilapia, J. Fish. Dis. 39 (2) (2016) 229–238.
[24] G. Timmerhaus, A. Krasnov, P. Nilsen, M. Alarcon, S. Afanasyev, M. Rode, H. Takle, S.M. Jørgensen, Transcriptome profiling of immune responses to cardiomyopathy syndrome (CMS) in Atlantic salmon, BMC Genom. 12 (1) (2011) 459.
[25] G. Timmerhaus, A. Krasnov, H. Takle, S. Afanasyev, P. Nilsen, M. Rode, S.M. Jørgensen, Comparison of Atlantic salmon individuals with different outcomes of cardiomyopathy syndrome (CMS), BMC Genom. 13 (1) (2012) 205.
[26] C. Fritsvold, R.T. Kongtorp, T. Taksdal, I. Ørpetveit, M. Heum, T. Poppe, Experimental transmission of cardiomyopathy syndrome (CMS) in Atlantic salmon Salmo salar, Dis. Aquat. Org. 87 (3) (2009) 225–234.
[27] R. Kongtorp, M. Halse, T. Taksdal, K. Falk, Longitudinal study of a natural outbreak of heart and skeletal muscle inflammation in Atlantic salmon, Salmo salar L, J. Fish. Dis. 29 (4) (2006) 233–244.
[28] R. Kongtorp, T. Taksdal, A. Lyngøy, Pathology of heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon Salmo salar, Dis. Aquat. Org. 59 (3) (2004) 217–224.
[29] M.N. Yousaf, M.D. Powell, The effects of heart and skeletal muscle inflammation and cardiomyopathy syndrome on creatine kinase and lactate dehydrogenase levels in Atlantic salmon (Salmo salar L.), Sci. World J. 2012 (2012).
[30] H. Hauge, M. Dahle, T. Moldal, E. Thoen, A.-G. Gjevre, S. Weli, M. Alarcón, S. Grove, Piscine orthoreovirus can infect and shed through the intestine in experimentally challenged Atlantic salmon (Salmo salar L.), Vet. Res. 47 (1) (2016) 57.
[31] T. Taksdal, J. Wiik-Nielsen, S. Birkeland, P. Dalgaard, T. Mørkøre, Quality of raw and smoked fillets from clinically healthy Atlantic salmon, Salmo salar L., following an outbreak of pancreas disease (PD), J. Fish. Dis. 35 (12) (2012) 897–906.
[32] L.-H. Johansen, M.K. Dahle, Ø. Wessel, G. Timmerhaus, M. Løvoll, M. Røsæg, S.M. Jørgensen, E. Rimstad, A. Krasnov, Differences in gene expression in Atlantic salmon parr and smolt after challenge with Piscine orthoreovirus (PRV), Mol. Immunol. 73 (2016) 138–150.
[33] E. Di Cicco, H.W. Ferguson, A.D. Schulze, K.H. Kaukinen, S. Li, R. Vanderstichel, Ø. Wessel, E. Rimstad, I.A. Gardner, K.L. Hammell, Heart and skeletal muscle inflammation (HSMI) disease diagnosed on a British Columbia salmon farm through a longitudinal farm study, PLoS One 12 (2) (2017) e0171471.
[34] Ø.W. Finstad, K. Falk, M. Løvoll, Ø. Evensen, E. Rimstad, Immunohistochemical detection of piscine reovirus (PRV) in hearts of Atlantic salmon coincide with the course of heart and skeletal muscle inflammation (HSMI), Vet. Res. 43 (1) (2012) 27.
[35] A.B. Mikalsen, O. Haugland, M. Rode, I.T. Solbakk, O. Evensen, Atlantic salmon reovirus infection causes a CD8 T cell myocarditis in Atlantic salmon (Salmo salar L.), PLoS One 7 (6) (2012) e37269.
[36] Ø. Haugland, A.B. Mikalsen, P. Nilsen, K. Lindmo, B.J. Thu, T.M. Eliassen, N. Roos, M. Rode, Ø. Evensen, Cardiomyopathy syndrome of Atlantic salmon (Salmo salar L.) is caused by a double-stranded RNA virus of the Totiviridae family, J. Virol. 85 (11) (2011) 5275–5286.
[37] H.M. Munang'andu, O. Evensen, A review of intra- and extracellular antigen delivery systems for virus vaccines of finfish, J Immunol Res 2015 (2015) 960859.
[38] C. Xu, S. Mutoloki, O. Evensen, Superior protection conferred by inactivated whole virus vaccine over subunit and DNA vaccines against salmonid alphavirus infection in Atlantic salmon (Salmo salar L.), Vaccine 30 (26) (2012) 3918–3928.
[39] C. Xu, T.C. Guo, S. Mutoloki, O. Haugland, O. Evensen, Gene expression studies of host response to Salmonid alphavirus subtype 3 experimental infections in Atlantic salmon, Vet. Res. 43 (2012) 78.
[40] S.A. Plotkin, Correlates of protection induced by vaccination, Clin. Vaccine Immunol. 17 (7) (2010) 1055–1065.
[41] R.M. Zinkernagel, On immunity against infections and vaccines: credo 2004, Scand. J. Immunol. 60 (1–2) (2004) 9–13.
[42] D.J. Pasnik, J.J. Evans, P.H. Klesius, Passive immunization of Nile tilapia (Oreochromis niloticus) provides significant protection against Streptococcus agalactiae, Fish Shellfish Immunol. 21 (4) (2006) 365–371.
[43] B. Pulendran, R. Ahmed, Immunological mechanisms of vaccination, Nat. Immunol. 12 (6) (2011) 509–517.
[44] I. Bricknell, J. King, T. Bowden, A. Ellis, Duration of protective antibodies, and the correlation with protection in Atlantic salmon (Salmo salarL.), following vaccination with anAeromonas salmonicidavaccine containing iron-regulated outer membrane proteins and secretory polysaccharide, Fish Shellfish Immunol. 9 (2) (1999) 139–151.
[45] H.M. Munang'andu, A. Sandtro, S. Mutoloki, B.E. Brudeseth, N. Santi, O. Evensen, Immunogenicity and cross protective ability of the central VP2 amino acids of

infectious pancreatic necrosis virus in Atlantic salmon (Salmo salar L.), PLoS One 8 (1) (2013) e54263.
[46] S. Dubey, K. Avadhani, S. Mutalik, S.M. Sivadasan, B. Maiti, J. Paul, S.K. Girisha, M.N. Venugopal, S. Mutoloki, O. Evensen, I. Karunasagar, H.M. Munang'andu, Aeromonas hydrophila OmpW PLGA nanoparticle oral vaccine shows a dose-dependent protective immunity in rohu (Labeo rohita), Vaccines (Basel) 4 (2) (2016).
[47] D. Parra, F. Takizawa, J.O. Sunyer, Evolution of B cell immunity, Annu. Rev. Anim. Biosci 1 (1) (2013) 65–97.
[48] H.M. Munang'andu, S. Mutoloki, O. Evensen, Acquired immunity and vaccination against infectious pancreatic necrosis virus of salmon, Dev. Comp. Immunol. 43 (2) (2014) 184–196.
[49] H.M. Munang'andu, S. Mutoloki, O. Evensen, An overview of challenges limiting the design of protective mucosal vaccines for finfish, Front. Immunol. 6 (2015) 542.
[50] H.M. Munang'andu, S. Mutoloki, Ø. Evensen, A review of the immunological mechanisms following mucosal vaccination of finfish, Front. Immunol. 6 (2015) 427.
[51] H.M. Munang'andu, J. Paul, Ø. Evensen, An overview of vaccination strategies and antigen delivery systems for Streptococcus agalactiae vaccines in nile Tilapia (Oreochromis niloticus), Vaccines 4 (4) (2016) 48.
[52] H.M. Munang'andu, S. Mutoloki, Ø. Evensen, Non-replicating vaccines, Fish Vaccination 22–32.
[53] S. Mutoloki, H.M. Munang'andu, Ø. Evensen, Oral vaccination of fish–antigen preparations, uptake, and immune induction, Front. Immunol. 6 (2015) 519.
[54] S. Ahmadivand, M. Soltani, M. Behdani, Ø. Evensen, E. Alirahimi, E. Soltani, R. Hassanzadeh, J. Ashrafi-Helan, VP2 (PTA motif) encoding DNA vaccine confers protection against lethal challenge with infectious pancreatic necrosis virus (IPNV) in trout, Mol. Immunol. 94 (2018) 61–67.
[55] J.-Z. Zhao, L.-M. Xu, M. Liu, Y.-S. Cao, S.E. LaPatra, J.-S. Yin, H.-B. Liu, T.-Y. Lu, Preliminary study of an oral vaccine against infectious hematopoietic necrosis virus using improved yeast surface display technology, Mol. Immunol. 85 (2017) 196–204.
[56] K. Utke, H. Kock, H. Schuetze, S.M. Bergmann, N. Lorenzen, K. Einer-Jensen, B. Köllner, R.A. Dalmo, T. Vesely, M. Ototake, Cell-mediated immune responses in rainbow trout after DNA immunization against the viral hemorrhagic septicemia virus, Dev. Comp. Immunol. 32 (3) (2008) 239–252.
[57] M. Yamasaki, K. Araki, K. Maruyoshi, M. Matsumoto, C. Nakayasu, T. Moritomo, T. Nakanishi, A. Yamamoto, Comparative analysis of adaptive immune response after vaccine trials using live attenuated and formalin-killed cells of Edwardsiella tarda in ginbuna crucian carp (Carassius auratus langsdorfii), Fish Shellfish Immunol. 45 (2) (2015) 437–442.
[58] M. Yamasaki, K. Araki, T. Nakanishi, C. Nakayasu, Y. Yoshiura, T. Iida, A. Yamamoto, Adaptive immune response to Edwardsiella tarda infection in ginbuna crucian carp, Carassius auratus langsdorfii, Vet. Immunol. Immunopathol. 153 (1–2) (2013) 83–90.
[59] M. Yamasaki, K. Araki, T. Nakanishi, C. Nakayasu, A. Yamamoto, Role of CD4+ and CD8α+ T cells in protective immunity against Edwardsiella tarda infection of ginbuna crucian carp, Carassius auratus langsdorfii, Fish Shellfish Immunol. 36 (1) (2014) 299–304.
[60] A.B. Romstad, L.J. Reitan, P. Midtlyng, K. Gravningen, O. Evensen, Development of an antibody ELISA for potency testing of furunculosis (Aeromonas salmonicida subsp salmonicida) vaccines in Atlantic salmon (Salmo salar L), Biologicals 40 (1) (2012) 67–71.
[61] A.B. Romstad, L.J. Reitan, P. Midtlyng, K. Gravningen, O. Evensen, Antibody responses correlate with antigen dose and in vivo protection for oil-adjuvanted, experimental furunculosis (Aeromonas salmonicida subsp. salmonicida) vaccines in Atlantic salmon (Salmo salar L.) and can be used for batch potency testing of vaccines, Vaccine 31 (5) (2013) 791–796.
[62] A.B. Romstad, L.J. Reitan, P. Midtlyng, K. Gravningen, V. Emilsen, O. Evensen, Comparision of a serological potency assay for furunculosis vaccines (Aeromonas salmonicida subsp. salmonicida) to intraperitoneal challenge in Atlantic salmon (Salmo salar L.), Biologicals 42 (2) (2014) 86–90.
[63] S.A. Plotkin, Immunologic correlates of protection induced by vaccination, Pediatr. Infect. Dis. J. 20 (1) (2001) 63–75.
[64] S.A. Plotkin, Correlates of vaccine-induced immunity, Clin. Infect. Dis. 47 (3) (2008) 401–409.
[65] P. McLauchlan, B. Collet, E. Ingerslev, C. Secombes, N. Lorenzen, A. Ellis, DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injection and duration of protection—early protection correlates with Mx expression, Fish Shellfish Immunol. 15 (1) (2003) 39–50.
[66] C.H. Kim, M.C. Johnson, J.D. Drennan, B.E. Simon, E. Thomann, J.-A.C. Leong, DNA vaccines encoding viral glycoproteins induce nonspecific immunity and Mx protein synthesis in fish, J. Virol. 74 (15) (2000) 7048–7054.
[67] J.Y. Byon, T. Ohira, I. Hirono, T. Aoki, Use of a cDNA microarray to study immunity against viral hemorrhagic septicemia (VHS) in Japanese flounder (Paralichthys olivaceus) following DNA vaccination, Fish Shellfish Immunol. 18 (2) (2005) 135–147.
[68] H.M. Munang'andu, B.N. Fredriksen, S. Mutoloki, R.A. Dalmo, O. Evensen, The kinetics of CD4+ and CD8+ T-cell gene expression correlate with protection in Atlantic salmon (Salmo salar L) vaccinated against infectious pancreatic necrosis, Vaccine 31 (15) (2013) 1956–1963.
[69] A.R. Bridle, B.F. Koop, B.F. Nowak, Identification of surrogates of protection against yersiniosis in immersion vaccinated Atlantic salmon, PLoS One 7 (7) (2012) e40841.
[70] H. Yamashita, K. Mori, A. Kuroda, T. Nakai, Neutralizing antibody levels for protection against betanodavirus infection in sevenband grouper, Epinephelus septemfasciatus (Thunberg), immunized with an inactivated virus vaccine, J. Fish. Dis. 32 (9) (2009) 767–775.
[71] L. Li, R. Wang, W. Liang, T. Huang, Y. Huang, F. Luo, A. Lei, M. Chen, X. Gan, Development of live attenuated Streptococcus agalactiae vaccine for tilapia via continuous passage in vitro, Fish Shellfish Immunol. 45 (2) (2015) 955–963.
[72] H.-Y. Huang, Y.-C. Chen, P.-C. Wang, M.-A. Tsai, S.-C. Yeh, H.-J. Liang, S.-C. Chen, Efficacy of a formalin-inactivated vaccine against Streptococcus iniae infection in the farmed grouper Epinephelus coioides by intraperitoneal immunization, Vaccine 32 (51) (2014) 7014–7020.
[73] S.E. LaPatra, S. Corbeil, G.R. Jones, W.D. Shewmaker, N. Lorenzen, E.D. Anderson, G. Kurath, Protection of rainbow trout against infectious hematopoietic necrosis virus four days after specific or semi-specific DNA vaccination, Vaccine 19 (28) (2001) 4011–4019.
[74] S. Corbeil, S. LaPatra, E. Anderson, G. Kurath, Nanogram quantities of a DNA vaccine protect rainbow trout fry against heterologous strains of infectious hematopoietic necrosis virus, Vaccine 18 (25) (2000) 2817–2824.
[75] A. Long, J. Richard, L. Hawley, S.E. LaPatra, K.A. Garver, Transmission potential of infectious hematopoietic necrosis virus in APEX-IHN(R)-vaccinated Atlantic salmon, Dis. Aquat. Org. 122 (3) (2017) 213–221.
[76] S. LaPatra, K. Plant, S. Alcorn, V. Ostland, J. Winton, An experimental vaccine against Aeromonas hydrophila can induce protection in rainbow trout, Oncorhynchus mykiss (Walbaum), J. Fish. Dis. 33 (2) (2010) 143–151.
[77] P. Swain, S. Dash, J. Bal, P. Routray, P. Sahoo, S. Sahoo, S. Saurabh, S. Gupta, P. Meher, Passive transfer of maternal antibodies and their existence in eggs, larvae and fry of Indian major carp, Labeo rohita (Ham.), Fish Shellfish Immunol. 20 (4) (2006) 519–527.
[78] A. Ellis, A. Burrows, T. Hastings, K. Stapleton, Identification of Aeromonas salmonicida extracellular protease as a protective antigen against furunculosis by passive immunization, Aquaculture 70 (3) (1988) 207–218.
[79] H. Marquis, R. Lallier, Efficacy studies of passive immunization against Aeromonas salmonicida infection in brook trout, Salvelinus fontinalis (Mitchill), J. Fish. Dis. 12 (3) (1989) 233–240.
[80] R. Shelby, C. Shoemaker, P. Klesius, Passive immunization of channel catfish Ictalurus punctatus with anti-Flavobacterium columnare sera, Dis. Aquat. Org. 77 (2) (2007) 143–147.
[81] B. LaFrentz, S. LaPatra, G. Jones, K. Cain, Passive immunization of rainbow trout, Oncorhynchus mykiss (Walbaum), against Flavobacterium psychrophilum, the causative agent of bacterial coldwater disease and rainbow trout fry syndrome, J. Fish. Dis. 26 (7) (2003) 377–384.
[82] T. Ooyama, A. Kera, T. Okada, V. Inglis, T. Yoshida, The protective immune response of yellowtail Seriola quinqueradiata to the bacterial fish pathogen Lactococcus garvieae, Dis. Aquat. Org. 37 (2) (1999) 121–126.
[83] D.J. Pasnik, J.J. Evans, P.H. Klesius, Specific serum antibody responses in channel catfish (Ictalurus punctatus) provide limited protection against Streptococcus ictaluri challenge, Vet. Immunol. Immunopathol. 144 (1) (2011) 144–146.
[84] R. Shelby, P.H. Klesius, C. Shoemaker, J. Evans, Passive immunization of tilapia, Oreochromis niloticus (L.), with anti-Streptococcus iniae whole sera, J. Fish. Dis. 25 (1) (2002) 1–6.
[85] A. Eldar, A. Horovitcz, H. Bercovier, Development and efficacy of a vaccine against Streptococcus iniae infection in farmed rainbow trout, Vet. Immunol. Immunopathol. 56 (1–2) (1997) 175–183.
[86] M. Akhlaghi, B. Munday, R. Whittington, Comparison of passive and active immunization of fish against streptococcosis (enterococcosis), J. Fish. Dis. 19 (3) (1996) 251–258.
[87] T.L. Lin, T.G. Clark, H. Dickerson, Passive immunization of channel catfish (Ictalurus punctatus) against the ciliated protozoan parasite Ichthyophthirius multifiliis by use of murine monoclonal antibodies, Infect. Immun. 64 (10) (1996) 4085–4090.
[88] Y. Sin, K. Ling, T. Lam, Passive transfer of protective immunity against ichthyophthiriasis from vaccinated mother to fry in tilapias, Oreochromis aureus, Aquaculture 120 (3–4) (1994) 229–237.
[89] S. LaPatra, K. Lauda, G. Jones, Antigenic variants of infectious hematopoietic necrosis virus and implications for vaccine development, Dis. Aquat. Org. 20 (1994) 119–119.
[90] S. LaPatra, K. Lauda, G. Jones, S. Walker, W. Shewmaker, Development of passive immunotherapy for control of infectious hematopoietic necrosis, Dis. Aquat. Org. 20 (1994) 1–1.
[91] N.J. Olesen, Detection of the antibody response in rainbow trout following immersion vaccination with Yersinia ruckeri bacterins by ELISA and passive immunization, J. Appl. Ichthyol. 7 (1) (1991) 36–43.
[92] N. Lorenzen, E. Lorenzen, K. Einer-Jensen, J. Heppell, T. Wu, H. Davis, Protective immunity to VHS in rainbow trout (Oncorhynchus mykiss, Walbaum) following DNA vaccination, Fish Shellfish Immunol. 8 (4) (1998) 261–270.
[93] P. Hershberger, J. Gregg, C. Grady, S. LaPatra, J. Winton, Passive immunization of Pacific herring against viral hemorrhagic septicemia, J. Aquat. Anim. Health 23 (3) (2011) 140–147.
[94] C.-J. Wu, H. Wang, Y.-L. Chan, T.-L. Li, Passive immune-protection of small abalone against Vibrio alginolyticus infection by anti-Vibrio IgY-encapsulated feed, Fish Shellfish Immunol. 30 (4) (2011) 1042–1048.
[95] M. Akhlaghi, Passive immunisation of fish against vibriosis, comparison of intraperitoneal, oral and immersion routes, Aquaculture 180 (3) (1999) 191–205.
[96] A.B. Romstad, L.J. Reitan, P. Midtlyng, K. Gravningen, Ø. Evensen, Antibody responses correlate with antigen dose and in vivo protection for oil-adjuvanted, experimental furunculosis (Aeromonas salmonicida subsp. salmonicida) vaccines in Atlantic salmon (Salmo salar L.) and can be used for batch potency testing of

vaccines, Vaccine 31 (5) (2013) 791–796.
[97] S. Dash, S.K. Das, J. Samal, P.K. Ojha, J.K. Patra, H. Thatoi, Dose dependence specific and non-specific immune responses of Indian major carp (L. rohita Ham) to intraperitoneal injection of formalin killed Aeromonas hydrophila whole cell vaccine, Vet. Res. Commun. 35 (8) (2011) 541–552.
[98] D.J. Wise, J.S. Terhune, The relationship between vaccine dose and efficacy in channel catfish Ictalurus punctatus vaccinated as fry with a live attenuated strain of Edwardsiella ictaluri (RE-33), J. World Aquacult. Soc. 32 (2) (2001) 177–183.
[99] D.J. Pasnik, S.A. Smith, Immunogenic and protective effects of a DNA vaccine for Mycobacterium marinum in fish, Vet. Immunol. Immunopathol. 103 (3) (2005) 195–206.
[100] M.A. Kuzyk, J. Burian, D. Machander, D. Dolhaine, S. Cameron, J.C. Thornton, W.W. Kay, An efficacious recombinant subunit vaccine against the salmonid rickettsial pathogen Piscirickettsia salmonis, Vaccine 19 (17) (2001) 2337–2344.
[101] H. Yang, J. Chen, G. Yang, X.-H. Zhang, R. Liu, X. Xue, Protection of Japanese flounder (Paralichthys olivaceus) against Vibrio anguillarum with a DNA vaccine containing the mutated zinc-metalloprotease gene, Vaccine 27 (15) (2009) 2150–2155.
[102] T.J. Welch, S. LaPatra, Yersinia ruckeri lipopolysaccharide is necessary and sufficient for eliciting a protective immune response in rainbow trout (Oncorhynchus mykiss, Walbaum), Fish Shellfish Immunol. 49 (2016) 420–426.
[103] R. Thiéry, J. Cozien, J. Cabon, F. Lamour, M. Baud, A. Schneemann, Induction of a protective immune response against viral nervous necrosis in the European sea bass Dicentrarchus labrax by using betanodavirus virus-like particles, J. Virol. 80 (20) (2006) 10201–10207.
[104] F. Zheng, X. Sun, X.a. Wu, H. Liu, J. Li, S. Wu, J. Zhang, Immune efficacy of a genetically engineered vaccine against lymphocystis disease virus: analysis of different immunization strategies, Evid. base Compl. Alternative Med. 2011 (2011).
[105] I. Sommerset, R. Skern, E. Biering, H. Bleie, I.U. Fiksdal, S. Grove, A.H. Nerland, Protection against Atlantic halibut nodavirus in turbot is induced by recombinant capsid protein vaccination but not following DNA vaccination, Fish Shellfish Immunol. 18 (1) (2005) 13–29.
[106] J.-O. Kim, J.-O. Kim, W.-S. Kim, M.-J. Oh, Development of a recombinant protein vaccine based on cell-free protein synthesis for sevenband grouper Epinephelus septemfasciatus against viral nervous necrosis, J. Microbiol. Biotechnol. 25 (10) (2015) 1761–1767.