Developmental and Comparative Immunology xxx (2016) 1–20

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Oral vaccination of fish: Lessons from humans and veterinary species

Carmen W.E. Embregts, Maria Forlenza*

Cell Biology and Immunology Group, Department of Animal Sciences, Wageningen University, Wageningen, The Netherlands

ARTICLE INFO

Article history:
Received 2 January 2016
Accepted 17 March 2016
Available online xxx

Keywords:
Live vaccines
Adenoviruses
Encapsulation
M-like cells
Adjuvants
Zebrafish

ABSTRACT

The limited number of oral vaccines currently approved for use in humans and veterinary species clearly illustrates that development of efficacious and safe oral vaccines has been a challenge not only for fish immunologists. The insufficient efficacy of oral vaccines is partly due to antigen breakdown in the harsh gastric environment, but also to the high tolerogenic gut environment and to inadequate vaccine design. In this review we discuss current approaches used to develop oral vaccines for mass vaccination of farmed fish species. Furthermore, using various examples from the human and veterinary vaccine development, we propose additional approaches to fish vaccine design also considering recent advances in fish mucosal immunology and novel molecular tools. Finally, we discuss the pros and cons of using the zebrafish as a pre-screening animal model to potentially speed up vaccine design and testing for aquaculture fish species.

1. Introduction

Aquaculture has been the fastest growing food-producing sector for years and the yield of aquaculture has overgrown the yield of wild capture fisheries (FAO, 2014). As a downside of this sector-wide intensification, increasing stocking densities give rise to high stress levels which in turn make fish more vulnerable to infections. Prevention of disease outbreaks is therefore essential to prevent serious economic losses and thus the development or refinement of targeted vaccines for aquaculture species is imperative.

To date vaccines are available for most aquaculture fish species; most are targeting bacterial pathogens and only a few are raised against viruses. Depending on the age and size of the fish, commercial vaccines are administered either orally (by mixing with the feed), by immersion (dip or bath) or by injection through the intraperitoneal (i.p.) or intramuscular (i.m.) route. Intraperitoneal injection is conventionally used to deliver water-in-oil (w/o)-based injectable vaccines whereas intramuscular injection is most often used to deliver DNA plasmids (reviewed in Brudeseth et al. (2013)). While protection is generally highest with injection-vaccination, it is also associated with intensive handling and stress for the fish. Furthermore, depending on the type of adjuvant used, w/o-based injectable vaccines have been associated with local side effects including tissue inflammation, adhesion and necrosis. Fish that are too small to be injected are usually vaccinated orally or by immersion, but these routes usually result in low efficacy and short protection. To ensure protection throughout the entire production cycle, vaccination regimes have been developed for various species in which a combination of immersion, oral and injection vaccination is used. In most cases, it is only after injection with w/o-based vaccines that strong and long lasting protection is achieved.

There is no doubt that with respect to animal welfare and handling costs, the mucosal route of vaccination, and in particular the oral route, would be the ideal method of vaccine delivery. Nevertheless, owing to the high costs of vaccine production required for immersion vaccination or the limited efficacy of the current oral formulations, mass vaccination of fish, exclusively *via* the mucosal routes, is not common practice.

While nowadays improvement of current oral vaccination strategies is a major topic in fish vaccine development, the first report on a successful oral vaccine was already reported in 1942. The study showed protection of trout against a challenge with *Bacterium salmonicida* after prolonged feeding (64–70 consecutive days) with chloroform-inactivated bacteria (Duff, 1942). In the 80s and 90s considerable attention was drawn to the development of oral vaccines for fish. During this period, morphological and functional differences within the intestine were investigated and the second segment was identified as the main place of antigen uptake

Abbreviations: TLR, Toll-like receptor; NLR, NOD-like receptor; RLR, RIG-like receptor.

* Corresponding author.
E-mail address: maria.forlenza@wur.nl (M. Forlenza).

http://dx.doi.org/10.1016/j.dci.2016.03.024
0145-305X/

(Fujino et al., 1987; Georgopoulou et al., 1986; Iida and Yamamoto, 1985; Noaillac-Depeyre and Gas, 1973; Rombout and van den Berg, 1989; Rombout et al., 1989; Stroband and Kroon, 1981). This knowledge highlighted the necessity of protecting antigens from being broken down in the stomach and foregut of the fish. The need for antigen protection was confirmed by comparing the uptake of antigens, induced immune responses and vaccine efficacy after oral or anal administration. Higher vaccine efficacy of anal vaccination in comparison to oral vaccination using the same vaccine was reported for many studies including vaccination against *Vibrio anguillarum* (*V. anguillarum*) and *Yersinia ruckeri* (*Y. ruckeri*) for salmon (Johnson and Amend, 1983) or against *V. anguillarum* in carp (Rombout et al., 1986).

The overall limited efficacy of oral vaccines in fish, as in other veterinary species or in humans, is not only due to problems linked to antigen breakdown in the harsh gastric environment, but also to the highly tolerogenic gut environment. The phenomenon of (oral) tolerance is well known in fish as well (Joosten et al., 1997; Maurice et al., 2004; Rombout and Kiron, 2014; Rombout et al., 1989), but the mechanisms associated to its development have not been systematically addressed. The potential risk of tolerance induction upon oral vaccination, especially in immunologically immature young fish, is therefore an additional factor that fish immunologists have to take into account while developing mucosal vaccines.

From studies in mammals it is well established that mucosal tolerance is dependent on antigen dose and route of administration (Faria and Weiner, 2005). Mucosal tolerance is driven by the expression of high local levels of anti-inflammatory cytokines (IL-10 and TGFβ) that sustain the generation and maintenance of tolerogenic regulatory T cells and dendritic cells (DCs) (Weiner and Wu, 2011). Therefore potent mucosal adjuvants as well as targeted delivery strategies are being used for oral vaccine development in humans and veterinary species. For example, the use of live attenuated vaccines targeting mucosal dendritic cells or microfold epithelial cells (M cells), administered along with strong adjuvants (i.e. bacterial toxins) has received significant attention and holds great promise to facilitate the induction of effective mucosal responses (Fujkuyama et al., 2012; Neutra and Kozlowski, 2006).

This review will summarize current knowledge on experimental oral vaccines in human and veterinary species, with a particular focus on fish, focusing on antigen type, dose, encapsulation as well as delivery methods. First, based on all summarized work we will try to extrapolate communalities among all (successful) approaches with a special focus on the nature of the pathogen, nature of the fish and vaccination strategy. Next, using several examples from the human and veterinary field, we will dissect the conditions that might lead to tolerance to orally delivered antigens in fish and later, the protective immune mechanisms that need to be rationally targeted to overcome tolerance and achieve successful oral vaccination. Among others, we will discuss the possibility to target local antigen presenting cells (APCs) or M-like antigen-sampling cells (Fuglem et al., 2010) as well as the choice of mucosal adjuvants that can either promote antigen adhesion to M-like cells, or strongly activate local innate and adaptive immune responses. Finally, we will discuss the pros and cons of using zebrafish as a pre-screening platform for novel oral vaccination approaches for cultured fish species.

2. Is development of fish oral vaccines really lagging behind? Current status on human and veterinary oral vaccines

In mammals it is well known that parenteral antigen administration triggers weak mucosal responses, whereas antigen administration at mucosal surfaces efficiently triggers local as well as systemic humoral and cell-mediated responses (Neutra and Kozlowski, 2006). The importance of triggering specific immune responses at mucosal surfaces for protection against mucosal pathogens is well recognized. Despite this, in humans, as in many veterinary species, the development of effective oral vaccines has gone at a slow paste. In fact, after more than 100 years of research, only five mucosal vaccines have been approved for human use. These include four oral vaccines against polio virus, rotavirus, *Salmonella typhi* (*S. thypi*), and *Vibrio cholera* (*V. cholera*) and one nasal vaccine against influenza virus (Böhles et al., 2014; Lamichhane et al., 2014; Neutra and Kozlowski, 2006). Perhaps not surprising, but also these vaccines do not trigger a long-lasting protection and they all require boosting after 2 years from first administration in adults, and 6 months in children aged 2–5 years. Many oral vaccines for human application are currently in various developmental phases and clinical testing, but often safety issues or adverse side effects slow their implementation. Altogether this underlines how, also in humans, optimal conditions to achieve effective oral vaccination have not been defined yet and how challenging the field of mucosal vaccine development can be.

The situation for veterinary oral vaccines (for non-fish species) is perhaps a little better, with a very successful oral vaccine against rabies virus, which helped eradicate rabies disease from wildlife reservoirs in Europe and most of the United States, and one oral vaccine for pigs against the intracellular bacterium *Lawsonia intracellularis* (Meeusen et al., 2007). Several mucosal (oral) vaccines are available from various vaccine companies for pigs and cows against rotavirus and for poultry against several pathogens: turkey adenovirus; infectious bronchitis virus; Newcastle virus; infectious bursitis virus; chicken herpesvirus; turkey herpesvirus; reovirus; *Bordetella avium*; *Pasteurella multocida* (Gerdts et al., 2006).

Considering the total number of commercially available vaccines against fish pathogens, only a small but significant proportion is administered orally (reviewed in (Brudeseth et al. (2013); Dhar et al. (2014); Sommerset et al. (2014)). For salmonid species, commercial oral vaccines are available for Atlantic salmon and coho salmon against *Piscirickettsia salmonis* (*P. salmonis*), infectious pancreatic virus (IPNV), and infectious salmon anaemia virus (ISAV); for rainbow trout against IPNV, *Y. ruckeri*, *P. salmonis* and for rainbow trout and sea bass against *V. anguillarum*. For Great amberjack one oral vaccine has been licensed in Japan against *Lactococcus garviae* (*L. garviae*). Despite their commercialisation however, oral vaccines for fish are in use in a few countries only (mainly Chile, Norway and Scotland) (Brudeseth et al., 2013). Similar to the human oral vaccines, oral vaccines for fish provide only a weak or short protection and are therefore mostly used as prime and/or booster vaccination. Depending on the fish species and production cycle, farmers will optimally choose for a vaccination regime starting with dip vaccination, followed by booster vaccination (dip or oral) and finally an injection vaccination (Brudeseth et al., 2013). Such vaccination regime generally confers strong and long-lasting protection during the entire production cycle. However, it causes more stress to the fish and is more costly and labour intensive for the farmer. Therefore considerable effort is still dedicated to the improvement of currently available vaccines and to design new oral vaccination strategies that can provide stronger and longer lasting protection in fish.

3. Successes and concerns of current mucosal human and veterinary vaccines

When considering the nature of the pathogen, all oral vaccines licensed for use in humans and veterinary species are against mucosal pathogens that either infect the mucosal surface itself or use the mucosa as portal of entry to then establish a systemic

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

infection. Furthermore, with only few exceptions, successful oral vaccines are based on live attenuated viruses or bacteria that closely mimic the route of infection of the pathogen and trigger strong local immune responses without the need for any additional adjuvant. For example, the live attenuated poliovirus and *S. typhi* vaccines are derived from pathogens that preferentially adhere to M cells and exploit M-cell transport to invade organized mucosal lymphoid tissues in the intestine (Jones et al., 1994; Sicinski et al., 1990).

As a downside, live attenuated vaccines are less safe as it cannot be guaranteed that the pathogen would not revert to the virulent form or cause pathogenesis in few individuals. This has been already observed for the oral polio vaccine in humans for which 1 in a million doses caused a so-called 'vaccine-associated paralytic polio' (WHO, 2014). This reflects the importance and risks associated to the use of live attenuated vaccines and the need for development of even safer and rationally designed vaccines.

The challenges however are not only in the development of safer vaccines for humans, but also for veterinary species including poultry. It is now been ascertained that imperfect vaccination against Marek's disease virus (MDV) can drive the generation and enhance the transmission of highly virulent pathogens (Read et al., 2015). Spray-vaccination of chickens with live attenuated MDV gives rise to the considerable risk that not all chickens are exposed to the same dose of the vaccine, if at all. The vaccine effectively protects vaccinated individuals but fails to induce sterile immunity, as vaccinated chickens are still able to shed the vaccine virus. In this situation, in order to be able to survive in a population of largely vaccinated animals, the virus is driven towards increased virulence. This example clearly highlights how not only the design but also the administration route of a successful vaccine plays a crucial role in tilting the balance between immunity and pathogenesis.

When considering the currently licensed oral vaccines for fish we realise that all are based on heat- or formalin-killed pathogens. Most of these pathogens are however either enteric pathogens, such as *Y. ruckeri* and *V. anguillarum*, or enter and affect mucosal organs, such as *P. salmonis* or IPNV. Furthermore, although no specific adjuvants are mentioned to be administered along with the vaccine antigen, most vaccines are either incorporated in an Antigen Protecting Vehicle (*Y. ruckeri, V. anguillarum* and IPNV vaccines, MSD-Animal Health) or in patented MicroMatrix™ delivery system (*P. salmonis*, ISAV and IPNV, Centrovet). Such encapsulations or delivery methods are aimed at protecting the antigens from gastric degradation, but might also promote antigen uptake by enhancing adhesion to mucosal surfaces. Moreover, they might possibly act as adjuvants by providing local inflammatory signals (Plant and Lapatra, 2011). The use of inactivated pathogens certainly poses an advantage with respect to (environmental) safety but, together with the lack of strong mucosal adjuvants, it might still be one of the causes of the weak and short protection provided by these vaccines.

Interestingly, prolonged protection against *P. salmonis* and ISA virus can be achieved in farmed salmonids, but only upon administration of MicroMatrix™-encapsulated oral vaccines at repeated intervals of approximately 1200 degree-days (approximately 3 month for salmon kept at 11–12°) (Tobar et al., 2015). Protection was found to correlate to high serum IgM levels whereas the window of disease susceptibility was shown to coincide with the decline in IgM serum levels. Therefore, repeated oral administrations assured that serum IgM remained high throughout the entire production cycle and this confirmed once again that oral antigen administration can elicit systemic responses (Rombout et al., 1989). Furthermore, while it is clear that on the one hand fish do not develop a strong memory response to the vaccine, they on the other hand also do not develop tolerance. The possible mechanisms behind such phenomenon are not fully understood. It is possible that the vaccine dose combined with the MicroMatrix™ provides sufficient signals (inflammatory and co-stimulatory) to activate B cells directly and drive them towards few rounds of division and antibody production. Nevertheless, as it will be further discussed in section 6.2, the lack of immune memory and tolerance upon antigen re-exposure might be explained by the failure of the vaccine to activate cell-mediated T cell responses required for effective B cell activation and memory formation. Given the promising but insufficient progress on fish oral vaccine development, substantial research effort is still being dedicated to the study of mucosal immune responses in fish and the generation of novel oral vaccines.

4. Current strategies and efficacy of experimental non-encapsulated oral fish vaccines

Given the relative success of the currently licensed fish vaccines, as well as the convenience and safety of inactivated pathogens, a large number of studies focused on the use of non-encapsulated (inactivated) bacteria, yeast or plants expressing bacterial or viral antigens. This approach is largely based on the hypothesis that antigens expressed in whole cells might not need further encapsulation since the cell wall itself might act as natural protection barrier against the intestinal environment. A selected overview of the current experimental approaches for the development of oral vaccines against bacterial and viral pathogens using non-encapsulated antigens is presented in Table 1 and will be discussed in this section. A more extensive and detailed summary is presented in Supplementary Table 1. Although many reports could be found describing vaccine uptake and local responses, we selectively focused on those studies that also tested vaccine efficacy upon pathogen challenge (see references in (supplementary) Table 1).

Independently of the dose, regime, nature of the pathogen or vaccine type, it is apparent that when protection could be observed, this was either assessed after only a very short time following last vaccine administration or was found to decrease within 2–3 months after vaccination. In most cases single or consecutive administrations, or consecutive administration with varying time intervals, did not make a significant difference in survival. This suggests that the dose achieved by a single administration or by consecutive administration is sufficient to induce a response and confer protection, at least within the time tested.

Interestingly, two studies showed that the addition of common injectable adjuvants (alum and Freund's incomplete) to the oral vaccine had a positive effect on vaccine efficacy. Addition of 25% v/v of 10% potassium aluminium sulphate (alum) increased survival of rainbow trout against *V. anguillarum* using different types of whole cell vaccines, when compared to the same vaccines without adjuvant (Agius et al., 1983), Supplementary Table 1. The addition of incomplete Freund's adjuvant (20% v/v) to a formalin-killed *Streptococcus agalactiae* (*S. agalactiae*) vaccine increased survival of red tilapia upon i.p. challenge with *S. agalactiae,* when compared to the non-adjuvanted vaccine (Firdaus-Nawi et al., 2013), Table 1. Nevertheless, long-term protection and potential side effects were not investigated.

Of interest, few approaches showed the potential of using enteric pathogens not affecting fish or commensal microbes as vaccine vehicles (Table 1). Formalin-inactivated *Escherichia coli* (*E. coli*) expressing *S. agalactiae* or grass carp reovirus (GCRV) antigens, and live attenuated *Salmonella typhimurium* (*S. typhimurium*) expressing surface immunogenic protein (Sip) of *S. agalactiae* generally conferred good protection at various vaccination doses. Similarly, live *Lactococcus lactis* expressing *Aeromonas hydrophila* antigens, *Lactobacillus plantarum* (*L. plantarum*) expressing Spring Viremia of Carp Virus (SVCV) or Cypinid

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

Table 1
Experimental approaches for the development of oral vaccines using non-encapsulated antigens.

Pathogen	Fish species	Regime and formulation	Antigen	Dose	Protection (survival)	Time of challenge	Challenge route	Reference
Bacteria								
Aeromonas hydrophila (A.h.)	Catla (3.8 g), rohu (3.4 g), common carp (4 g)	15d; Mixed with feed	h.i. *A.h.* biofilm grown on chitin flakes	10^7 CFU/fish/day	0–25% RPS 25–35% RPS 30–60% RPS	20 dpv 40 dpv 60 dpv	i.m.	(Azad et al., 1999)
				10^{10} or 10^{13} CFU/fish/day	20–45% RPS 35–50% RPS 75–80% RPS	20 dpv 40 dpv 60 dpv		
		10d, 15d or 20d; Mixed with feed	h.i. *A.h.* biofilm on chitin flakes	10^{10} CFU/fish/day; for 10d	15–55% RPS 45–44% RPS 35–75% RPS 25–40% RPS	20 dpv 40 dpv 60 dpv 150 dpv		
				10^{10} CFU/fish/day; for 15d	35–60% RPS 65–80% RPS 45–100% RPS 35–60% RPS	20 dpv 40 dpv 60 dpv 150 dpv		
				10^{10} CFU/fish/day; for 20d	20–55% RPS 75–90% RPS 35–100% RPS 30–55% RPS	20 dpv 40 dpv 60 dpv 150 dpv		
Aeromonas hydrophila	Gibel carp (50 g)	2 times 5d, 14d interval; Mixed with feed	F.i. whole cells or cell ghosts	4×10^8 cell ghosts/g fish/day	78%	30 dpv	i.p.	(Tu et al., 2010)
				4×10^8 cells/g fish/day	61%			
				Control	5%			
Aeromonas hydrophila	Tilapia (17–18 g)	4 weeks; Mixed with feed	*L. lactis* expressing aerolysin genes D1 and D4 from A.h.	10^6 CFU/g of feed	D1: 85% D4: 70	1 dpv	i.p.	(Anuradha et al., 2010)
				10^8 CFU/g of feed	D1: 100% D4: 85%			
				Controls	25–40%			
Edwardsiella tarda (E.t.)	Olive flounder (4–5 g)	One time only, or with boost at 14d; Gavage	Autotrophic mutant ΔalrΔasd *E. tarda*	10^8 CFU/fish	90–100%	3 wpv	i.p., Bath	(Choi et al., 2011)
				10^9 CFU/fish	100%			
				10^8 CFU/fish, 10^8 CFU/fish for boost	60–65%			
				10^9 CFU/fish, 10^8 CFU/fish for boost	100%			
				Controls	0–18%			
Edwardsiella tarda	Olive flounder (4–5 g)	One time only, or with boost at 14d; Gavage	Autotrophic mutant ΔalrΔasd *E. tarda*	10^8 CFU fish	90–100%	3 wpv	i.p., Bath	(Choi et al., 2011)
				10^9 CFU/fish	100%			
				10^8 CFU/fish, 10^8 CFU for boost	60–65%			
				10^9 CFU/fish, 10^8 CFU for boost	100%			
				Controls	0–18%			
Edwardsiella ictaluri (E.i.)	Channel catfish (7–9 cm)	One time; Mixed with feed	Live attenuated *E. ictaluri*	4.6–6 x 10^6 CFU/g feed	92–100%	29 dpv	Bath, cohab	(Wise et al., 2015)
				4.6–6 x 10^7 CFU/g feed	97–100%			
				Field trial; 1.9×10^6 CFU/g feed	V: 54% C: 28%			
Photobacterium damsela (P.d.)	Sea bass (20 g)	One time; Gavage	F.i. whole *P.d.* and extracellular components	500 μl	V: 64% C: 31%	6 wpv	Bath	(Bakopoulos et al., 2003)
					V: 55% C: 19%	12 wpv		
Flavobacterium psychrophilum (F.p.)	Ayu (0.5 g)	3d or 15d with 5d interval; repeated 5 or 15 times; Mixed with feed	F.i. *F. psychrophilum*	0.1–0.2 g bacteria/kg;	5 times: 53–94% 15 times: 92–97% C: 35–69%	3 wpv	Bath	(Kondo et al., 2003)
					5 times: 76–87% 15 times: 79–88% C: 42–76%	7 wpv		
Flavobacterium psychrophilum	Rainbow trout (1.6 g)	10 times on alternating days; Mixed with feed	Membrane vesicles (supernatatant) and f.i. *F.p.* of stationary (SP) and logarithmic phase (LP)	1250 μl supernatant (SN) and 0.2 g cells/kg of fish	SP cells: 66–68% LP cells: 94% SP + SN: 47–53% LP + SN: 51% LP + SN + SP: 59% SP + SN + SP: 97–100% C: 56.3–44.1%	3 wpv	Bath	(Aoki et al., 2007)
Streptococcus agalactiae (S.a.)	Nile tilapia (100 g)	1,2 or 3 times with 1 week interval Gavage	Live attenuated *S. typhimurium* expressing surface	1×10^7 CFU/fish; 1–3 times	1: 20% RPS 2: 30% RPS 3: 47% RPS	7 dpv	i.p.	(Huang et al., 2014)

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

Table 1 (*continued*)

Pathogen	Fish species	Regime and formulation	Antigen	Dose	Protection (survival)	Time of challenge	Challenge route	Reference
			immunogenic protein of *S.a.*	1×10^8 CFU/fish; 1–3 times	1: 27% RPS 2: 40% RPS 3: 53% RPS			
				1×10^9 CFU/fish; 1–3 times	1: 33% RPS 2: 47% RPS 3: 57% RPS			
				Controls	0%			
Streptococcus agalactiae	Nile tilapia (30 g)	1, 2 (6d interval) or 3 times (3d interval); Mixed with feed	Live attenuated *S. agalactiae*	10^7 CFU/fish; 1–3 times	1: 50% 2: 64% 3: 67%	15 and 30 dpv	i.p.	(Li et al., 2015)
				10^8 CFU/fish; 1–3 times	1: 69% 2: 81% 3: 79%			
				10^9 CFU/fish; 1–3 times	1: 72% 2: 85% 3: 72%			
				Control	33%			
Streptococcus agalactiae	Red hybrid tilapia (100 g)	2 times with 14d interval; Mixed with feed	F.i. *E. coli* expressing *S.a.* cell wall surface anchor family protein	1×10^6 CFU/g of feed	V: 70% C: 0%	4 wpv	Bath	(Nur-Nazifah et al., 2014)
Streptococcus agalactiae	Red tilapia (100 g)	2 times with 14d interval; Mixed with feed	F.i. *S. agalactiae*	6.7×10^7 CFU/ml	50%	14 dpv	i.p.	(Firdaus-Nawi et al., 2013)
				6.7×10^7 CFU/ml with 1:6 v/v FIA	100%			
				Control	12%			
Streptococcus iniae (S.i.)	Nile tilapia (12.7 g)	1d or 5d; Mixed with feed	Oralject technique-1 (O-1); lyophilized *S. iniae*	O-1, 2×10^9 bacteria/fish/day; 1d	82%	23 dpv	i.p.	(Shoemaker et al., 2006)
			Oralject technique-2 (O-2); lyophilized *S. iniae*	O-2, 2×10^9 bacteria/fish/day; 1d	77%			
				O-1, 2×10^8 bacteria/fish/day; 5d	78%			
				O-2, 2×10^8 bacteria/fish/day; 5d	68%			
				Control	53%			
Vibrio anguillarum (V.a.)	Atlantic halibut (40 g)	One time; Gavage	Fy-i. *V.a.*	100 µl bacteria/fish	V: 50% C: 0%	12 wpv	i.p.	(Bowden et al., 2002)
Yersinia ruckeri (Y.r.)	Rainbow trout (13.4 g)	One time or boosted after 4 m; Mixed with feed	F.i. *Y. ruckeri* or AquaVac ERM Oral (AV)	10^8 CFU/fish	Y.r: 72% AV: 48%	2 m after booster	Bath	(Villumsen et al., 2014)
				10^8 CFU/fish plus 5×10^7 CFU booster	Y.r: 72% AV: 80%			
				5×10^9 CFU plus booster	Y.r: 100%			
				Control	44%			
Viruses								
Viral heamorrhagic septicemia virus (VHSV)	Rainbow trout (40–80 g)	3d; Mixed with feed	Attenuated lyophilized VHSV, PEG pellets	4.8–5.7 $TCID_{50}$/fish total	V: 77–91% C: 30–41%	6 wpv	bath	(Adelmann et al., 2008)
Viral heamorrhagic septicemia virus (VHSV)	Olive flounder (4–5 g)	1 time or boost after 2w; Gavage	Live recombinant VHSV eGFP-NV (ΔNV)	10^4 PFU/fish	25–60%	4 wpv	i.m.	(Kim et al., 2011)
				10^5 PFU/fish	50–75%			
				2×10^5 PFU/fish	70–90%			
				Control	0%			
Red-spotted grouper nervous necrosis virus (RGNNV)	Convict grouper (71.5 g)	1 time; Gavage	RGNNV VLPs produced in *S. cerevisiae*	50 µg of purifies VLPs/fish	V: 100% C: 37%	56 dpv	i.p.	(Wi et al., 2015)
Grass carp reovirus (GCRV)	Grass carp (150 g)	21d; Mixed with feed	f.i. *E. coli* expressing capsid proteins VP5-VP7	10^8 bacteria/feed pellet	V: 90% C: 10%	7 dpv	i.p.	(Lu et al., 2011)
Cyprinid Herpes Virus-3 (CyHV3), Spring Viremia of Carp Virus (SVCV)	Common carp (500 g)	3d; followed by 2 boosts for 3d after 8d and 25d from first administration; Mixed with feed	SVCV-G and CyHV3-ORF81 expressed in *L. plantarum*	10^9 CFU/g feed	V: 71% (SVCV) C: 11% (SVCV)	5 dpv	Oral	(Cui et al., 2015)
	Koi carp (350 g)		SVCV-G and CyHV3-ORF81 expressed *in L. plantarum*	10^9 CFU/g feed	V: 53% (CyHV3) C: 8% (CyHV3)			
Rock bream iridovirus (RBIV)	Rock bream (7–8 g)	4 times, weekly interval; Mixed with feed	Major capsid protein expressed in *Pichia pastoris*	1×10^6 yeast cells/ 2.5 g feed/fish	V: 92% C: 0%	10 dpv	Bath	(Seo et al., 2013)

(*continued on next page*)

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

Table 1 (*continued*)

Pathogen	Fish species	Regime and formulation	Antigen	Dose	Protection (survival)	Time of challenge	Challenge route	Reference
				(250 μg recombinant/fish)				
Rock bream iridovirus	Rock bream (10 g)	3 times, 10d interval; Mixed with feed	Major capsid protein rMCP in rice callus	10 μg lyophilized calli powder/fish	80%	10 dpv	Bath	(Shin et al., 2013)
				30 μg lyophilized calli powder/fish	90%			
				Control	0%			

Abbreviations: d: day(s); w: week(s); dpv: days post vaccination; wpv: weeks post vaccination, mpv: months post vaccination; h.i.: heat-inactivated; f.i.: Formalin-inactivated; fy-i. formaldehyde-inactivated, UV-:. UV-inactivated; cohab: cohabitation; FIA: Freund's incomplete adjuvant; BW: body-weight; i.p.: intraperitoneal; i.m: intramuscular.

Herpesvirus-3 (CyHV-3) antigens, and *Lactobacillus casei* expressing IPNV non-structural proteins, all showed promising levels of protection. These results together suggest that enteric microbes might be suitable vaccine vehicles. Live commensals might retain their ability to colonize the gut environment and persist long enough to deliver the antigen at mucosal surfaces. However, as a result of the intrinsic ability to be tolerated by the host, on their own they might not be sufficient to trigger strong local responses. In contrast, inactivated enteric pathogens might still be able to diffuse through the mucus layer and adhere to enterocytes. By doing so, they might possibly provide inflammatory signals for local immune reactions owing to the presence of Pathogen Associated Molecular Patterns (PAMPs) triggering danger signals.

Production of vaccine antigens in plants including algae, potatoes, tobacco or rice has been proposed as a viable approach for the development of mucosal vaccines especially in veterinary species, owing among others, to their relatively low production cost and scalability (Liew and Hair-Bejo, 2015). Studies in mice demonstrated the ability of rice-expressed proteins to effectively deliver antigens to M cells and trigger local as well as systemic response (Nochi et al., 2007). In fish, the first report on an effective oral vaccine produced in plants was reported only recently (Shin et al., 2013). In the latter study, lyophilized rice calli of *Oriza sativa* expressing the major capsid protein of Rock Bream Iridovirus (RBIV) were mixed with the fish feed and were shown to confer very good protection against a RBIV challenge.

Another expression system exploited for the generation of vaccine antigens is yeast. Antigens expressed and produced in yeast benefit from a similar protection from its host as antigens expressed in bacteria. The wall of yeast makes the antigens less vulnerable to degradation and decreases the need for further encapsulation. Furthermore, yeast can easily be mixed with fish feed and might even act as an adjuvant by its richness in β-glucans. When administered orally with the fish feed, the yeast *Pichia pastoris* expressing the major capsid protein of RBIV conferred significant protection against RBIV challenge. However, protection was only evaluated shortly after vaccination. The yeast expression system has also been exploited for its ability to produce large quantities of Virus-Like Particles (VLPs), which are multimeric protein complexes whose shape mimic natural occurring virions. VLPs composed of the capsid protein of the Red-spotted Grouper Nervous Necrosis Virus (RGNNV) were produced in *Saccharomyces cerevisiae* (*S. cerevisiae*) and conferred low but significant protection against viral challenge when administered orally (Wi et al., 2015).

Despite the many and promising approaches summarized in Table 1 (and Supplementary Table 1), it is difficult to draw general conclusions on dose, vaccine type, and vaccine efficacy since long-term protection (>3 months) was not systematically addressed. Most studies performed challenges 10–30 days after last vaccination, and only a few also assessed protection at later time points (Azad et al., 1999; Bakopoulos et al., 2003; Bowden et al., 2002; Nayak et al., 2004; Siriyappagouder et al., 2014). Furthermore, in most of the summarized studies, pathogen challenge was not performed using the natural route of infection. It has already been mentioned that oral vaccination can lead to increased serum IgM levels, and that in some infections serum IgM levels correlated to diseases resistance or susceptibility (Rombout et al., 1989; Tobar et al., 2015). Nonetheless, it cannot be excluded that bypassing the mucosa by directly injecting the pathogen into the peritoneal cavity or muscle might also bypass crucial mucosal immune mechanisms. As a consequence, the subsequent lack of activation of local as well as systemic responses might lead to an underestimation of vaccine efficacy.

5. Current strategies and efficacy of experimental encapsulated oral fish vaccines

In contrast to the abovementioned whole bacteria or yeast- and plant-derived vaccines, more vulnerable vaccines such as DNA plasmids, purified recombinant proteins and sub-unit vaccines certainly need a form of protection to prevent breakdown and ensure antigen uptake in the second gut segment of fish. In many animal species, soluble, non-adherent antigens are generally found to be taken up at low levels, if at all, and in the intestine, such antigens commonly induce immune tolerance (Companjen et al., 2006; Mayer and Shao, 2004; Neutra and Kozlowski, 2006). On the contrary, enhanced mucosal immune responses were observed when using antigens that could be retained at mucosal surfaces by delivery in adherent polymers or were coupled to proteins that themselves are adherent to epithelial surfaces (Companjen et al., 2006; Gerdts et al., 2006; Neutra and Kozlowski, 2006).

It was clearly shown in mice and several other veterinary species that micro-encapsulated vaccines have several advantages for mucosal delivery. Microfold (M) cells are particularly accessible to microparticles and actively transport them into Peyer's patches, which consist of large clusters of lymphoid follicles localized in the mucosa of the distal small intestine. Microparticles that are both small (up to 1 μm diameter) and adherent to M cells are taken up most efficiently (Frey et al., 1996; Mantis et al., 2000; Neutra et al., 2001). Furthermore, specific glycolipids and protein-linked oligosaccharides on the apical surfaces of villus enterocytes, follicle-associated enterocytes above Peyer's patches, and M cells play a crucial role in antigen adherence (Frey et al., 1996; Lamichhane et al., 2014; Mantis et al., 2000) Cells that are functionally equivalent, but phenotypically distinct, from mammalian M cells have been identified in the gut of Atlantic salmon (Fuglem et al., 2010). The role of M-like cells in gut immunity together with gut barrier function and anatomical construction are reviewed elsewhere in this issue (Løkka and Koppang, 2016).

Given the presence of specialized antigen-sampling (M-like) cells in the fish intestine, together with the already described antigen-sampling ability of enterocytes and gut-associated

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

macrophages (Companjen et al., 2006; Rombout Jan et al., 2011), we can safely state that micro-encapsulation can be exploited to further enhance uptake of the abovementioned inactivated bacteria or yeast- and plant-derived vaccines (section 4). In this way vaccines that show a sub-optimal protection when delivered 'naked', possibly due to high intestinal antigen breakdown, can be improved upon encapsulation. An overview of the current experimental approaches for the development of oral vaccines against bacterial and viral pathogens using (bio)encapsulated antigens is presented in Table 2 and will be discussed in this section.

Bio-encapsulation in live vehicles such as artemia, rotifer and water flea was investigated for its suitability to vaccinate fish larvae that do not yet feed on pelleted food (Table 2). Although some reports describe the induction of protection after oral delivery of bio-encapsulated vaccines for fry and larvae, protection was generally assessed shortly after antigen administration pointing more towards an immunostimulatory effect of the vaccine. Furthermore, maximum dosage of vaccination is restricted by the daily feed intake and encapsulation efficiency of the vaccine since the bio-encapsulated vaccines are incorporated in the natural starter diet of the fish fry or larvae. This efficiency strongly depends on the chosen vehicle in combination with the state of the bacteria to be encapsulated and their stability after bioencapsulation (Kawai et al., 1989). Factors such as vaccine dose and time of exposure, especially in young animals, are extremely crucial for vaccine efficacy. As it will be further discussed in section 7.2, these factors can certainly make the difference between induction of immune responses or tolerance.

Micro-encapsulation of vaccines in polymers such as chitosan, Poly D,L-lactic-co-glycolic acid (PLGA), alginates, liposome, and MicroMatrix™ are more practical and efficient for juvenile and older fish as they can be incorporated in the feed. The properties and formulation of the aforementioned micro-encapsulation methods have been extensively reviewed elsewhere (Plant and Lapatra, 2011; Tafalla et al., 2013). Given this, we will concentrate on a few studies that specifically focused on vaccine efficacy upon pathogen challenge (Table 2). Most evident is the success of alginate-encapsulated live attenuated or avirulent enteric pathogens (e.g. *Edwardsiella tarda, Staphylococcus iniae, Vibrio* ssp) and DNA vaccines against viruses. As further discussed later in section 7.3, such success might reside in the ability of the alginates to protect the microbe and the plasmid while passing through the digestive tract, and to diffuse through the gut mucus layer, thereby reaching the enterocyte surface. Once in contact with the epithelium, alginates might be actively taken up by antigen-sampling cells and deliver the microbe or DNA plasmid. Enteric pathogens will most likely have the intrinsic ability to activate local mucosal responses, whereas DNA plasmids can then enter the nucleus and trigger antigen expression in the host cell in a manner similar to that triggered during a viral infection. The latter mechanism has been previously described as the base of the great success of i.m. injected DNA vaccines in fish (Lorenzen and LaPatra, 2005). The efficacy of DNA vaccines also confirms that delivering nucleic acids fulfils the requirements of closely mimicking antigen expression and presentation during an intracellular pathogen infection. This in turn will most likely trigger cellular as well as humoral immune responses appropriate to the pathogen.

6. Rational immunological approach to oral vaccine design for fish

There is no doubt that, independently of the administration routes, vaccines that best mimic the natural pathogen infection and trigger appropriate immune pathways against the pathogen, are most effective; but how is this appropriate immune response achieved?

6.1. Triggering humoral responses

For pathogens against which an antibody-dependent systemic humoral response is appropriate to confer protection, killed or inactivated microbes, protein-adjuvant vaccines, virus-like particles (VLPs), or other subunit vaccines are sufficient to trigger adequate systemic immune responses. When inactivating the pathogen or producing the subunits in heterologous expression systems, care should be taken to assure that the antigenic epitope against which the antibody response is directed remains intact. Parenteral administration of such vaccines in the presence of adjuvants will in fact elicit antigen-specific neutralizing antibodies that will protect the host upon natural infection. Briefly, B cells can directly recognize protein antigens and can be stimulated to proliferate and secrete antibodies. In parallel, the adjuvant present in the vaccine formulation will trigger the activation of local innate immune cells, including Antigen Presenting Cells (APCs), through recognition of viral PAMPs *via* Pattern Recognition Receptors (e.g. TLRs, NLRs, RLRs). APCs will then upregulate the expression of pro-inflammatory cytokines (i.e. IL-12, IFNα/β and TNFα) and co-stimulatory molecules (i.e. CD80/86). These molecules are crucial for the activation of cell-mediated immunity through antigen presentation to $CD4^+$ helper T cells *via* the MHC-II pathway. In turn, $CD4^+$ helper T cells, will differentiate to the Th1 subset under the influence of the pro-inflammatory cytokines. Subsequent production of IFNγ by Th1 T cells will sustain antibody production, affinity maturation and memory formation of antigen-specific B cells. In most mammals and in birds these antibodies, mostly of the IgG isotype, will be able to fix complement and will be present in the circulation and peripheral immune organs.

Vaccines that exclusively elicit systemic humoral immunity, however, might not be efficacious against mucosal (enteric) pathogens, as parenteral immunization does not effectively elicit mucosal responses (Neutra and Kozlowski, 2006). Pathogens that undergo rapid antigenic variation will also not be stopped by vaccines exclusively inducing humoral immunity, as cross-protection will not be achieved. Most importantly, pathogens against which not only humoral but also cell-mediated cytotoxic T cell responses are required (e.g intracellular microbes) will also not be affected by vaccines that mainly elicit humoral responses. This last category, which also includes most mucosal pathogens, comprises the largest group of microbes against which effective vaccines are lacking in most animal species. Perhaps it is not surprising that most target pathogens are viruses and intracellular bacteria, with the exception of few protozoan subspecies (e.g. plasmodium, trypanosomes) (Koff et al., 2013).

6.2. Triggering cell-mediated T cell responses

To address the need to elicit systemic cell-mediated T cell responses in humans and several veterinary species, live attenuated pathogens (bacteria or viruses) and non-replicating DNA plasmids have been developed that are administered *via* the parenteral route in the presence of strong adjuvants. One of the advantages of for example live attenuated viruses is their ability to replicate in the host but at much lower rate than the wild type virulent pathogen. Such feature allows for the use of relatively low vaccine doses. Next, and most importantly, vaccine replication in the host and the presence of the adjuvant will trigger the activation of local innate immune cells and APCs. This latter aspect is extremely crucial for the activation of cell-mediated immunity through antigen presentation to $CD8^+$ T cells *via* the MHC-I pathway, as well as to $CD4^+$ helper T cells *via* the MHC-II pathway through cross-presentation. The presence of viral particles as well as the expression of viral antigens on the APC surface will also activate antigen-specific B

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

Table 2
Experimental approaches for the development of oral vaccines using (bio)encapsulated antigens.

Pathogen	Encapsulation method	Fish species	Regime	Antigen	Dose	Protection (%)	Time of challenge	Challenge route	Reference
Bacteria									
Vibrio anguillarum (V.a.)	Bioencapsulation	Ayu (63 mg)	22d	F.i. *V. a.* in water flea and rotifer	0.64 μg LPS/fish detected	V: 92.4% C: 64.2%	1 dpv	Bath	(Kawai et al., 1989)
Vibrio harveyi	Alginates	Rainbow trout (15 g)	6 times, 1w interval	Outer membrane proteins (OMPs)	50 μg OMPs/fish/day	V: 90% C: 10%	12 wpv	i.p.	(Arijo et al., 2008)
Edwardsiella tarda (E.t.), Streptococcus iniae (S.i.)	Alginates	Japanese flounder (12.4 g)	3d; followed by oral and immersion boost at 2 m	Attenuated *E. t.* expressing *S. i.* antigen Sia10	10^8 CFU/fish/day	V: 82% *(E.t.)* C: 26% *(E.t.)* V: 84% *(S.i.)* C: 14% *(S.i.)*	1 mpv	Bath (E.t.); i.p. (S. i)	(Sun et al., 2012)
					10^8 CFU/fish/day, 8 h immersion boost 10^7 CFU/ml	V: 76% *(E.t.)* C: 22% *(E.t.)* V: 86% *(S.i.)* C: 26% *(S.i.)*			
Edwardsiella tarda	Alginates	Japanese flounder (10 g)	5d	Avirulent *E. tarda* strain ATCC 15947	10^9 CFU/fish/day	V: 64–71% C: 14–18%	5 wpv	i.p. and bath	(Cheng et al., 2010)
Edwardsiella tarda (E.t.)	Alginates	Japanese flounder (9.6 g)	5d	Pseudomonas strain FP3 expressing *E.t.* antigen Esa1	10^9 CFU/fish/day	V: 56% C: 8%	1 mpv	i.p.	(Sun et al., 2010)
Edwardsiella tarda, Vibrio harveji (V.h.)	Alginates	Turbot (14 g)	3d	Live attenuated *E.t.* expressing *V.h.* DegQ soluble antigen	10^8 CFU/fish/day	V: 91% *(E.t.)* C: 20% *(E.t.)* V: 94% *(V.h.)* C: 28% *(V.h.)*	1 mpv	Bath	(Hu et al., 2011)
Flavobacterium columnare	Alginates	Nile tilapia (15.7 g)	7d	F.i. *F. columnare*	2.65×10^{10} CFU/fish/day	No protection	21 dpv	Bath	(Leal et al., 2010)
Flavobacterium psychrophilum	Alginates	Rainbow trout (1 g)	3 times for 7d, followed by 11d standard food	Live attenuated *F. psychrophilum*	7.7×10^5 CFU/fish/	V: 8% C: 0%	3 dpv	Sub-cutaneous	(Ghosh et al., 2015)
Aeromonas hydrophila	Chitosan (600 nm); alginate-chitosan (1100 nm)	Indian major carp (100 g)	7d	F.i. *A. hydrophila*	1×10^{10} CFU/fish/day	V: 20% C: 10%	7 wpv	i.p.	(Behera and Swain, 2014)
Vibrio anguillarum	Chitosan	Asian sea bass (10 g)	21d	Porin gene, outer membrane protein OMP38 DNA plasmid	50 μg plasmid/fish total	V: 55% C: 10–15%	1 dpv	i.m.	(Rajesh Kumar et al., 2008)
Vibrio parahaemolyticus	Chitosan	Black seabream (80 g)	3d	Outer membrane protein K in DNA plasmid	50 μg plasmid/fish total	V: 80% C: 17%	21 dpv	i.m.	(Li et al., 2013)
Aeromonas hydrophila	Liposome (5–10 μm)	Common carp (30 g)	3d	F.i. *A. hydrophila*	10 μl of liposomes/day (33 μg/ml protein)	V:60–80% C: 55–100%	22 dpv	Intra-subcutaneous	(Yasumoto et al., 2006b)
Aeromonas salmonicida	Liposome	Common carp (350 g)	3 times, 2w interval	Ultrasound-inactivated *A. salmonicida*	100μg/vaccination	V: 84% C: 63%	2 wpv	Bath	(Irie et al., 2005)
Piscirickettsia salmonis	MicroMatrix	Atlantic salmon (30 g)	10 times, 2d interval	*P. salmonis*	1×10^{10} cells/g feed (2% BW fed every 3d)	V: 80% C: 10–20%	300 or 600 degree-dpv	i.p.	(Tobar et al., 2011)
Stenotrophomonas maltophilia	PLGA (2.2 μm)	Channel catfish (100 g)	3 times, 14d interval	Ultrasone-inactivated *S. maltophilia*	50 μg/fish 100 μg/fish 200 μg/fish Control	43.3% 65% 75% 0%	35 dpv	i.p.	(Wang et al., 2011)
Lactococcus garviae	PLGA, alginates	Rainbow trout (20 g)	7d; booster at 61d	Formalin-inactivated *L. garviae* in PLGA or alginate (Alg) microspheres	1×10^{10} bacteria/fish/day in PLGA or alginate microspheres (alg); boost at 61d	PLGA: 68% Alg: 60% C: 14% PLGA: 50% Alg: 44% C: 10% PLGA: 76% Alg: 72% C: 14% PLGA: 70% Alg: 68% C: 14%	30 dpv 60 dpv 90 dpv (with boost) 120 dpv (with boost)	i.p.	(Altun et al., 2009)
Viruses									
Nervous necrosis virus (NNV)	Bioencapsulation	Orange spotted grouper (18 dph)	2d	Fy.-i. *E. coli* expressing NNV capsid protein in Artemia	10^5 bacteria/Artemia	V: 80–86% C: 44–54%	35 dph	i.p.	(Lin et al., 2007)
Nervous necrosis virus (NNV)	Bioencapsulation	Orange spotted	2d	h.i. *V. a.* or *E. coli* (*E.c*) expressing	8.3×10^4 CFU *E. c*/Artemia (200/fish/day)	25–45%	7 dpv	i.p.	(Chen et al., 2011)

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

Table 2 (*continued*)

Pathogen	Encapsulation method	Fish species	Regime	Antigen	Dose	Protection (%)	Time of challenge	Challenge route	Reference
		grouper (30 dph)		NNV coat protein in Artemia	4.1 × 10^6 CFU *V. a*/ Artemia (200/fish/ day)	52.5–87.5%			
					Control	2.5–42.5%			
Infectious haematopoietic necrosis virus (IHNV)	Alginates	Rainbow trout (3 –4 g)	1 time or boost 15d later	IHNV Glycoprotein gene and promotor region upstream of interferon regulatory factor 1A gene	10 μg DNA (1 time)	22%	30 dpv	Bath	(Ballesteros et al., 2015a)
					25 μg DNA (1 time)	30%			
					20 μg DNA (plus boost)	46%			
					50 μg DNA (plus boost)	56%			
					Control	0%			
Infectious pancreatic necrosis virus (IPNV)	Alginates (<10 μm)	Rainbow trout (1 g)	1 time	IPNV capsid protein VP2 gene in DNA plasmid	10 μg DNA/fish	V: 80–85% C: 10–15%	15 dpv	Bath	(de las Heras et al., 2010)
						V: 70–85% C: 10–20%	30 dpv		
		Brown trout (1.5 g)	1 time	IPNV capsid protein VP2 gene in DNA plasmid	10 μg DNA/fish	V: 85% C: 10–15%	15 dpv		
						V: 85% C:5–10%	30 dpv		
Infectious pancreatic necrosis virus (IPNV)	Alginates	Rainbow trout (1.5 g)	1 time	IPNV capsid protein VP2 gene in DNA plasmid	10 μg DNA/fish	V: 80% C: 20–25%	15 dpv	Bath	(Ballesteros et al., 2015b)
Infectious pancreatic necrosis virus (IPNV)	Alginates	Rainbow trout (1.5 g)	1 time (gavage) or 3d (mixed with feed)	IPNV capsid protein VP2 gene in DNA plasmid	10 μg/fish/day	1 time: 80% 3d: 88% C: 17%	15 dpv	Bath	(Ballesteros et al., 2014)
						1 time: 78% 3d: 82% C: 17%	30 dpv		
Infectious Salmon Anaemia Virus (ISAV)	Chitosan 333 nm (NV) and 41.7 nm (NV + Adjuvant)	Atlantic salmon (70 g)	7d	UV-i. ISAV (with alphavirus replicase DNA vaccine as adjuvant)	1 × 10^5 $TCID_{50}$/fish	71%	450 UTA pv	i.p.	(Rivas-Aravena et al., 2015)
					7 μg DNA/fish and 1 × 10^5 $TCID_{50}$/fish	88%			
					Control	52%			
Nodavirus (NV)	Chitosan	Asian sea bass (10 –15 g)	1 day	NV RNA2 capsid protein gene in DNA plasmid	100 μg plasmid/fish	V: 85–90% C: 50%	3 wpv	i.m.	(Vimal et al., 2014)
Cyprinid Herpes Virus-3 (CyHV3)	Liposome	Common carp (25 –30 g)	3d	F.i. CyHV3	20 μl liposomes/ day/fish (20 μg tot. protein/ ml)	V: 77% C: 10%	22 dpv	Gill inoculation	(Miyazaki et al., 2008; Yasumoto et al., 2006a)
Infecious heamatopoietic necrosis virus (IHNV)	PLGA (500 nm average, 200 –1000 nm)	Rainbow trout (5 g)	4d or 8d	IHNV Glycoprotein gene in DNA plasmid	22 μg plasmid DNA	17%	6 wpv	i.p.	(Adomako et al., 2012)
					43 μg plasmid DNA	27%			
					Control	7%			
					22 μg plasmid DNA	17%	10 wpv		
					43 μg plasmid DNA	33%			
					Control	17%			

Abbreviations: d: day(s); w: week(s); dpv: days post vaccination; dph: days post hatch; wpv: weeks post vaccination, mpv: months post vaccination; h.i.: heat-inactivated; f.i.: Formalin-inactivated; fy-i. formaldehyde-inactivated, UV-:. UV-inactivated; cohab: cohabitation; FIA: Freund's incomplete adjuvant; BW: body-weight; i.p.: intraperitoneal; i.m: intramuscular.

cells. IFNγ-secreting $CD4^+$ Th1 cells will further sustain B cells activation, antibody production and isotype switch in a manner similar to the one described above (section 6.1). Altogether, such response contributes to a full activation of effector humoral as well as cell-mediated responses against viruses or other intracellular pathogens. Interestingly, DNA plasmids, although not able to replicate in the host cells, have been shown to effectively trigger both arms of the immune system. Especially in fish, they have been proven very successful and can be used at doses 100 times lower than in larger mammals (Lorenzen and LaPatra, 2005; Meeusen et al., 2007). Unfortunately, mainly due to legislative concerns, only the DNA vaccine for vaccination of salmon again IHNV in Canada is commercially available.

In fish, most of the aforementioned immune mechanisms are believed to be generally conserved, although they have not been investigated in details in all farmed fish species, and are reviewed elsewhere (Gudding et al., 2014; Mucosal health in aquaculture, 2015). Humoral as well as cell-mediated immune responses have been associated with protection upon systemic as well as mucosal vaccination in fish (Mutoloki and Jørgensen, 2014; Salinas, 2015). Serum IgM levels have been associated with protection in several vaccination strategies, especially against extracellular pathogens. The relatively recent development of monoclonal antibodies in ginbuna crucian carp and trout against the T cell markers CD8α and CD4 (Toda et al., 2011, 2009) has allowed for a better characterization of T cell responses to infection and vaccination (Fischer et al., 2013). Most of the signature cytokines associated to Th1, Th2 and Th17 responses have been identified in fish, although their full functional characterization is still underway in most relevant fish species. Finally, the discovery of a novel immunoglobulin type in 2005, named IgT (for Teleost (Hansen et al., 2005)), and the generation of monoclonal antibodies against trout IgT, has provided fish vaccinologists with a new tool to better characterize systemic and mucosal responses (Salinas et al., 2011; Xu et al., 2016). A

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

detailed description of innate as well as adaptive immune responses identified and characterized thus far in fish has been recently reviewed (Jørgensen, 2014; Mutoloki and Jørgensen, 2014). A recent review focusing especially on the immunological mechanisms following mucosal vaccination of finfish is also available (Munang'andu et al., 2015).

7. Triggering protective humoral and cell-mediated responses upon oral vaccination

7.1. Targeting M-(like) cells and antigen presenting cells in the fish gut

When antigens or vaccines are administered *via* the oral route they are faced with the same host defence mechanisms as do commensals and enteric microbes. Oral vaccines will have to survive the gastric environment, attacks by proteases or nucleases, and stick to and penetrate the thick mucus to finally reach the epithelial barrier. For these reasons relatively large amounts of antigens need to be delivered orally, as it is impossible to exactly determine the amount of antigen that is delivered to the epithelial layer. When and if the vaccine will be in contact with the apical epithelium, it will have to be actively taken up and delivered to the basal side. At this side, the lamina propria, innate immune cells and specifically APCs would process it. In mammals specialized antigen sampling cells, M cells, are present in the follicle associated epithelium surmounting Payer's patches but also on the apical part of the villus epithelium (Mantis et al., 2000; Wang et al., 2014). Owing to their location, antigen sampling ability, and structure, M cells play a key role in exposing leukocytes in the lamina propria to the variety of antigens and microbes present in the lumen of the gut. Furthermore, M cells efficiently sample luminal content (bacteria, viruses, soluble microbial products or particulate antigens) and deliver it to leukocytes on their basolateral membrane by using vesicles that are transported through the cytosol (Neutra et al., 2001). M cells form a pocket within which leukocytes can aggregate. This pocket reduces the distance between the apical and basal membrane thereby increasing the efficiency at which antigens are delivered to underlining leukocytes (Jang et al., 2004). Besides M cells, specialized dendritic cells (DCs) extend protrusion through the epithelial layer and directly sample antigens in the lumen (Owen et al., 2013; Scott et al., 2011). As a result of their efficient sampling ability, strategic positions and specialized roles in activating local immune responses, M cells and intraepithelial DCs have become specific targets for vaccine delivery (Kim and Jang, 2014). For example, a monoclonal antibody carrying a vaccine antigen has been used to specifically deliver antigens to murine M cells (Nochi et al., 2007), and several immunostimulatory molecules are exploited to target DCs (Fujkuyama et al., 2012). In this way local mucosal responses can be rationally triggered.

In salmon, a distinct population of M-like cells has been identified. These cells were found to be intermingled within the epithelial layer, extend cytoplasmic protrusion to the luminal side and be able to sample gold-BSA microparticles (Fuglem et al., 2010). Although M-like cells specific markers have not yet been identified in fish, salmon M-like cells show a staining similar to mammalian M cells as were found positive for *Ulex europaeus* agglutinin (UEA-1 from gorse) and negative for wheat germ agglutinin (WGA). UEA-1 single-positive cells were only found deep in the mucosal folds of the villi and not in the apical region. This suggests that they might have a strategic position that allows them to interact with microbes that gain access to the deeper area of the intestinal folds preventing uptake and possible reaction to luminal (commensal) microbes. Although leukocytes were not present in the basal pocket underneath the antigen-sampling cells, macrophage-like cells have been seen associated to the basal side of M-like cells. In fish, both enterocytes and intraepithelial macrophages have been shown to play a role in antigen uptake in the second gut segment. Intraepithelial macrophages were shown to efficiently take up and transport antigens especially when antigens where coupled to enteric adhesion molecules (Companjen et al., 2006). Besides this, the presence of motile non-resident macrophages that can potentially transport antigens at peripheral sites has also been observed (Rombout and van den Berg, 1989). Markers for fish macrophages are available only in a few fish species (Ellett et al., 2010; Mulero et al., 2008; Weyts et al., 1997). Nevertheless, they could be used as a proof of concept to specifically target this putative APC in a model fish species to demonstrate the suitability of cell-specific antigen delivery approaches.

7.2. Induction of anergy or tolerance and antigen dose

Perhaps expected, but in mammals the majority of antigens or microorganisms sampled from the lumen through M cells or DCs do not trigger a local immune response. The lack of responsiveness to ingested or orally administered antigens is referred to as oral tolerance, which is speculated to be the prevention of harmful responses to otherwise beneficial food components and commensal bacteria. This intrinsic homeostatic mechanism is the major obstacle to overcome when designing oral vaccines. The mechanisms leading to oral tolerance are still not fully understood, not even in mammals. Nevertheless there is a general consensus that T regulatory cells (Tregs) and tolerogenic DCs (tol-DCs) play an important role in gut immunity and induction of oral tolerance (Weiner and Wu, 2011). In mammals, Tregs express high levels of the IL-2 receptors and may deprive other cell populations of this growth factor by adsorbing IL-2. Tregs also express high levels of the inhibitory receptor CTLA4 and through this compete with normal T cells for co-stimulatory molecules on APCs. Finally, both Tregs and tol-DC secrete high amounts of the anti-inflammatory cytokines IL-10 and TGFβ that greatly increase the threshold required for leukocyte activation (Weiner and Wu, 2011). In fact, under these conditions, an orally administered antigen that is taken up and presented by DC to antigen-specific T cells will never trigger an appropriate stimulatory signal, unless the antigen itself has the ability to trigger strong pro-inflammatory responses. The latter signals should then translate into the upregulation of MHC-II molecules and co-stimulatory molecules (CD80/86) on the APC surface as well as secretion of pro-inflammatory cytokines. In the absence of pro-inflammatory signals and in the presence of IL-10 and TGFβ, antigen presentation by non-activated APCs will trigger the development of Tregs and render antigen-specific T cells anergic or drive them to apoptosis (deletion) (Weiner and Wu, 2011). This in turn will contribute to depletion or unresponsiveness of the pool of antigen-specific T cells leading to tolerance upon re-exposure to the same antigens.

From a vaccination point of view it is important to note that induction of Tregs and anergy has been associated to antigen dose. In mammals, high doses of orally administered antigens will induce anergy and deletion of antigen-specific T cell whereas low antigen dose will rather trigger Tregs development. In fish, although the mechanisms of tolerance induction have not been systematically addressed, antigen dose and route of administration have also been associated to tolerance (Joosten et al., 1997; Maurice et al., 2004). Given the difficulty to precisely quantify the amount of orally administered antigen that is taken up at mucosal surfaces, it might prove extremely difficult to standardize the conditions at which the vaccine will not induce tolerance. From an oral vaccine development perspective, the vaccine antigen should therefore not only be actively taken up and delivered to APCs in the lamina propria, but

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

should also trigger strong stimulatory signals of a magnitude sufficient to break intestinal tolerance. In this respect, the use of strong mucosal adjuvants or replicating vaccines will prove instrumental.

7.3. Mimicking pathogen entry and activation of mucosal responses

7.3.1. Live attenuated enteric pathogens as vaccine vectors

Live attenuated enteric pathogens, even in the absence of adjuvants, will have the ability to enter and replicate at the mucosal surface and trigger appropriate mucosal responses, owing to the presence of PAMPs that can trigger stimulatory signals. One of the major hurdles is in the development of safe attenuated vaccines that are least likely to revert to virulence. Attenuation by repeated passages of viruses or bacteria *in vitro* does not always allow controlling the number and sites of the mutations, making it more difficult to predict how likely the vaccine is to revert to the virulent form. Furthermore, based on the nature of the pathogen, live attenuated vaccines might not confer sterile immunity implying that the vaccinated host will be carrier and shedder of the vaccine pathogen. As discussed above, lack of sterile immunity might come at great costs for the entire vaccinated and non-vaccinated population.

The development of safe vaccines is of utmost importance when considering animals, including fish, that are reared at high stocking densities, under relatively stressful conditions and are transported all over the world. In this situation vaccines should preferably induce sterile immunity and not be harmful to any kind of animal, the environment as well as the end consumer.

Due to the safety concerns, to date, one live bacterial vaccine has been licensed for sale in aquaculture in Canada and Chile (Renogen® only) and three in the US (Shoemaker et al., 2009). These vaccines include Renogen® against bacterial kidney disease (BKD), AQUAVAC-ESC® against enteric septicemia of catfish, and AQUAVAC-COL® against columnaris disease of catfish. A live viral hemorrhagic septicemia virus (VHSV) vaccine is available in Germany (Gomez-Casado et al., 2011). Of interest the Renogen® vaccine is based on cross-reactive immunity generated upon vaccination with an environmental avirulent bacterium *Arthrobacter (Ar.) davidanieli,* phylogenetically and antigenically related to *Renibacterium salmoninarum,* which is the causative agent of BKD (Griffiths et al., 1998). Unfortunately all aforementioned live attenuated vaccines are delivered by immersion in catfish fry one week post-hatch and are not sufficiently protective when administered orally at this stage (Lillehaug, 2014; Shoemaker and Klesius, 2014). A similar approach was used to orally vaccinate Japanese flounder against *Edwardsiella tarda* (*E. tarda*). Very good protection upon i.p. and bath challenge was found after oral administration of avirulent strains encapsulated in alginate microparticles (Cheng et al., 2010). Moreover, alginate encapsulated avirulent strains of *E. tarda* were used to generate a cross-protective divalent oral vaccine against *E. tarda* and *Vibrio harveji* or *E. tarda* and *Streptococcus iniae* and were found to induce very good protection (Table 2).

Of note is the fact that *E. tarda* is an enteric pathogen with a very wide host and temperature range. For what discussed above, it might retain its intrinsic ability to adhere to mucosal surfaces and trigger strong mucosal responses even in the absence of adjuvants. Using avirulent *E. tarda* as vehicle to express heterologous antigens might prove a viable option against microbes for which attenuated strains still show safety concerns or for which environmental avirulent strains are not available. The safety level of avirulent or attenuated *E. tarda* strains, together with serotype variability, certainly needs to be systematically evaluated before it can find its commercial application. Regardless of the aforementioned challenges when developing live enteric pathogens as vaccine vehicles, the large body of experimental work performed so far and the availability of a few commercial vaccines that can be administered by immersion, shows their great potential (Brudeseth et al., 2013; Park et al., 2012). Similar approaches have been pursued using other enteric pathogens such as *E. coli* and *V. anguillarum* that were engineered to express the nervous necrosis virus (NVV) capsid protein and were delivered as inactivated vehicles through artemia (Table 2), (Chen et al., 2011; Lin et al., 2007). Although the length of the vaccine administration as well as the duration of protection need further optimization, the principle of using enteric pathogens as oral vehicle seems very promising in fish as well.

7.3.2. Encapsulated DNA plasmid and activation of effective mucosal responses

As summarized in Table 2, it appears that alginate-encapsulated DNA plasmid might also fulfil most requirements for effective antigen administration at mucosal surfaces followed by activation of local immune responses. Considering their biochemical properties, and possibly those of fish gut mucus, alginates might assure plasmid protection, migration through the mucus layer, and efficient uptake by the epithelial layer. Once the plasmid enters the cytoplasm, CpG motives present in the plasmid backbone might deliver stimulatory signals acting as adjuvants. Once expressed by host cells, the protein antigen encoded by the plasmid can be recognized as a PAMP, activating local innate immune cells but also antigen-specific B cells or NK cells through antibody-dependent cell-mediated cytotoxicity (ADCC).

Of interest, alginate microspheres were used to successfully vaccinate brown trout and rainbow trout against IPNV using VP2-encoding DNA plasmid or against IHNV using glycoprotein (G)-encoding plasmid (Ballesteros et al., 2015a, b; 2012; de las Heras et al., 2010; Davison et al., 2003). Microspheres were generated using a $CaCl_2$ method that generally leads to relatively large particles (10–100 μm or larger). This size is much bigger than the one considered to be most effectively taken up by M cells in mammals (Frey et al., 1996). This suggests that cells other than antigen-sampling M-like cells might be involved in the uptake of alginate microparticles in fish. In our laboratory, an heterogeneous suspension of alginate microparticles was generated using an alternative approach. Microspheres ranging in size between 1.6 and 9 μm containing a total of 20 μg of DNA plasmid encoding for the SVCV-G protein (unpublished data) were delivered by oral gavage to carp of 20 g. Immuno-histochemical analysis revealed strong G protein expression throughout the epithelial layer of the second gut segment at 14 days post-vaccine administration. Furthermore, discrete macrophage-like cells and other leukocytes in the lamina propria were also found to be strongly positive for G protein expression. Our results, combined with the results in trout, suggest that alginates, independently of the size, might be particularly suitable to deliver antigens at mucosal surfaces in fish. They seem to be effectively taken up or perhaps can directly fuse with the gut epithelial membrane. Such efficient antigen uptake by enterocytes was observed previously (Rombout and van den Berg, 1989) but can however also pose a limitation to the efficacy of oral vaccines: on the one hand, persistence of the antigen in the enterocytes might guarantee sufficient antigen exposure and activation of local immune responses, on the other hand however it might create conditions leading to tolerance due to high local antigen dose if the antigen persists for too long. Additionally, in fish kept at 20 °C, the intestinal epithelium in the second gut segment is completely renewed within 10–15 days (Rombout et al., 1985; Stroband and Debets, 1978). This time is of course temperature and fish species dependent. In zebrafish kept at 26 °C, renewal was faster (7–10 days) in the second gut segment and took 5–7 days in the first gut segment (Wallace et al., 2005). Although the renewal period is significantly longer than observed in mammals (3–4 days), the

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

antigen might be lost unless it is efficiently transferred to leukocytes at the basal side of the epithelium, or taken up by intra-epithelial macrophages. This might partly help explain why relatively high plasmid doses need to be used upon oral administration when compared to i.m. injection. Antigens might then have to be delivered repeatedly, increasing the costs of oral DNA vaccination. Furthermore, considering the importance of the antigen dose in preventing the tilting of the balance towards induction of oral tolerance, optimizing the time and dose of DNA plasmid delivery will be of utmost importance for successful oral DNA vaccination.

7.3.3. Recombinant attenuated live vaccine vectors

Rather than relying on live attenuated pathogens, avirulent strains, or DNA plasmids, a safer and rational attenuation of candidate vaccine pathogens would be preferred. This could circumvent risks linked to the potential reversion to virulence or legislative concerns linked to the use of DNA vaccinated animals. Attenuation can be achieved by engineering the pathogen by for example selectively deleting metabolic pathway(s) or virulence gene(s). Such approach also allows the generation of DIVA vaccines facilitating the Discrimination of Infected from Vaccinated Animals, by the addition of tags or other markers. DIVA vaccines are particularly relevant in the veterinary vaccine industry to allow transport and trading of vaccinated animals. As a trade-off, a great deal of knowledge needs to be gathered about the pathogen infectious cycle and genes function in order to be able to rationally modify the pathogen. For large, complex viruses such as herpesviruses, or bacteria, this can greatly delay and increase the costs of vaccine development. For pathogens containing a segmented genome, such as influenza virus, engineering of genes in one or few particular segments might not prevent resorting of genomic segments among influenza serotypes within the populations, thereby quickly diluting out and losing the vaccine strain.

Rational pathogen attenuation has been attempted in fish as well (Table 1). For example, live attenuated *E. tarda* mutants were generated in which the alanine racemase gene and aspartase semialdehyde dehydrogenase gene were knocked out, rendering the mutants dependent on exogenous alanine and aspartate (Choi et al., 2011). The mutants conferred good protection upon challenge with virulent strains when administered to Olive flounder by oral gavage. Live attenuated recombinant VHSV was generated by removing the non-structural protein NV (ΔNV) and inserting eGFP (Kim et al., 2011). Such attenuated strain was able to confer good protection, in a dose-dependent manner, when administered orally. Despite the promising results, only few studies investigated the efficacy of recombinant attenuated vaccines for oral vaccine delivery. Still, long-term protection needs to be validated for the abovementioned studies as challenge was performed relatively shortly after last vaccination. Nonetheless, the use of enteric pathogens, like *E. tarda*, proves again to be a viable strategy for oral vaccine delivery.

8. Rational vaccine design and novel approaches for the vaccines of the future

8.1. Live viral vectors as vaccine vehicles

Is it therefore at all possible to generate safe live vaccines? In the last 30 years a great effort has been directed towards the identification of live viral or bacterial vectors that are not necessarily derived from the pathogen against which the vaccine is developed. For this purpose baculoviruses, lentiviruses, retroviruses, alphaviruses and adenoviruses have received great attention. All these vectors have several features in common: i) they can be modified or are naturally non-pathogenic to the species in which they will be used in; ii) they are able to deliver nucleic acids in eukaryotic cells, allowing for vaccine antigen expression by the host cell itself; iii) through viral pseudotyping, they can be designed to specifically target the cells to be infected; iv) they have very straightforward cloning and modification strategy.

Due to their ability to insert their genome in the host chromosome, baculoviruses, lentiviruses and retroviruses have been removed from the list of suitable vaccine vectors as they are considered unsafe. Incorporation of viral genes in a random location in the host genome can cause the onset of cancer (oncogenic insertion), or in the case of species used for human or animal consumption, the animals might be considered a Genetically Modified Organism (GMO). The latter is a problem especially linked to consumers' perception of safety; in fact, even though the foreign gene might not necessarily incorporate in the gonads of the vaccinated animals and therefore cannot be transmitted to the progeny, consumers and legislative bodies are not willing to take the risk. A very good case about how extremely successful vaccines are currently not widely used on the market, is the one about DNA vaccines in fish. As extensively discussed above, i.m. injection of DNA plasmid or oral administration of alginate-encapsulated DNA can be very effective in protecting fish against a variety of viral pathogens. Unfortunately, with the only exception of the DNA vaccine against IHN virus licensed in Canada for vaccination of salmon, no other DNA vaccine is available on the marked for edible species.

8.1.1. Alphaviruses

Alphaviruses are positive-sense, single-stranded RNA viruses with a genome of approximately 11.5 kb in length that do not insert their genome in the host chromosome. For this and for the relative simplicity and ease of manipulation of their RNA genome, they also represent interesting targets for vaccine vector development. Two types of Alphavirus-based vectors have been designed (Vander Veen et al., 2012) but none of them have so far been approved by any regulatory agency for use in animals or humans. The first vector is based on the generation of a self-replicating RNA (replicon) originating from a DNA plasmid also containing the sequence of the gene of interest. The second vector is based on Alphavirus replicon particles (RPs) that are single-cycle, propagation-defective particles that carry RNA encoding for the gene of interest but are not able to spread beyond the initial infected cells. As discussed above, the second generation of Alphavirus RPs carrying RNA certainly have an increased safety profile with respect to DNA-based replicon RNA as it does not require delivery of DNA sequences to the host. Nonetheless, DNA-based salmonid alphavirus (SAV)-based replicon vaccines encoding the infectious salmon anaemia virus (ISAV) hemagglutinin-esterase (HE) have been shown to be extremely effective when delivered i.m, whereas i.p. administration did not provide the same protection (Wolf et al., 2014). Intramuscular delivery triggered strong local as well as systemic innate, humoral and possibly cell-mediated responses, further supporting the suitability of alphavirus-based vectors to trigger strong immune responses. Another study also showed the broad temperature range (4°C–37 °C) and tropism of the SAV-based replicon as it was functional in fish, mammalian and insect cells *in vitro* as well as in shrimps *in vivo* (Olsen et al., 2013). The level of heterologous protein expression by the SAV-based replicon is however lower than observed in mammalian replicon-based systems. To date, such vectors have not been tested for oral delivery. For this reason it will be interesting to raise their safety profile by developing Alphavirus replicon particles that could deliver RNA rather than DNA. Furthermore, based on their proven ability to trigger the immune system of fish, immunity to the vaccine vector will also have to be

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

evaluated if Alphaviruses are to be used as vaccine vehicles.

8.1.2. Adenoviruses

Taking into account the abovementioned safety considerations, adenoviruses are currently at the vanguard of live viral vectors for use in humans and veterinary species (Appaiahgari and Vrati, 2015). Adenoviruses are medium-sized, non-enveloped double-stranded DNA viruses. Adenoviruses have been isolated from all vertebrate species, including fish (Davison et al., 2003), and are known to cause mild to severe respiratory disease in warm-blooded animals and enteric/renal diseases in aquatic animals. The best characterized adenoviruses are those of mammalian and avian origin (Bangari and Mittal, 2006). In fish, only one representative has been isolated from white sturgeon and has been temporarily assigned to a separate new genus (Kovács et al., 2003). Advantages of adenoviral vectors include their large packaging capacity (>8 kb), high titres and high levels of transgene expression when placed under a strong promotor. Moreover, they are able to target a broad range of dividing and non-dividing cell types with almost 100% efficiency. Unlike lentiviruses or retroviruses, adenoviruses do not integrate into the host genome which greatly increases their safety profile.

Good examples of the great success of adenoviruses as vaccine vectors are the recently developed experimental vaccines against Ebola virus (Gilbert, 2015), malaria (Ogwang et al., 2015; Schuldt and Amalfitano, 2012), Respiratory Syncytia Virus (RSV) (Green et al., 2015) Foot-and-Mouth Disease (Porta et al., 2013). They are generally based on a combined vaccination regime starting with prime vaccination with a modified non-human adenovirus, followed by booster vaccination with a modified poxvirus (mostly Modified Vaccinia Ankara (MVA)). The adenovirus vector, very effectively triggers strong humoral and cell-mediated responses to the vaccine antigen but also to the adenovirus vector itself. Therefore booster vaccination with an heterologous vector is required to prevent elimination of the adenovirus-based vaccine in primed vaccinated individuals.

The discovery and pioneering work performed on mammalian and avian adenoviruses, combined with the presence of cold-blooded adenoviruses, represents a novel, unexploited strategy that could be undertaken by fish vaccinologists for future vaccine development. For this, the further characterization of the currently identified adenovirus genome, the search for additional strains infecting fish species other than white sturgeon, and the generation of tools for their genetic manipulation, will be essential to ascertain the potential of adenoviruses as effective vaccine vehicles also in fish.

9. Improvement of currently suboptimal oral vaccines: mucosal adjuvants

Non-replicating antigens such as soluble proteins, plasmid DNA or killed pathogens are poorly immunogenic when delivered orally. Moreover, they generally induce tolerance because of their inability to trigger appropriate inflammatory stimuli and costimulatory signals. Therefore, the use of potent mucosal adjuvants can greatly enhance the efficacy of such suboptimal vaccines. The use of novel adjuvant formulations for improvement of fish vaccine efficacy has been extensively reviewed elsewhere (Tafalla et al., 2013), therefore in this section we will focus on mucosal adjuvants that can specifically enhance oral vaccine efficacy.

The relative success of the few commercial oral vaccines for fish can perhaps be partly attributed to their protective encapsulation method: antigen protective vehicle (APV) by MSD or MicroMatrix™ by Centrovet (Brudeseth et al., 2013). Even though these vehicles might provide stimulatory signals, they are obviously not sufficient to trigger a strong memory response (Tobar et al., 2015) as the oral vaccines alone are not sufficient to provide long-lasting protection. These vaccines however, can be used as a base to start testing novel combinations of mucosal adjuvant-vaccine antigens. Suboptimal vaccines could be administered in combination with conventional adjuvants or molecules that have been shown to exert strong immunostimulatory activities also in fish and are therefore able to trigger danger signals. Examples of the latter type include enterotoxins or PAMPs such as: the non-toxic part of the *Escherichia coli* heat-labile enterotoxin (LTB), cholera toxin β-subunit (CTB), polyI:C, beta-glucans, bacterial flagellin or CpG motifs.

9.1. Conventional adjuvants

Some of the adjuvants generally used for injection vaccination in humans or veterinary species (i.e. alum and Freund's incomplete) have also been tested in fish in combination with oral vaccines (section 4 and Table 1). Although the reports showed the applicability of using standard injection vaccine adjuvants in an oral vaccination strategy, possible side effects need to be assessed.

Other promising adjuvants components considered in experimental human and veterinary vaccines are liposomes, saponins (e.g Quil-A, QS-21) or highly immunogenic immune stimulating complex (ISCOMS). At this moment, Pharmaq is studying the introduction of the latter adjuvants in commercialised fish vaccines (Tafalla et al., 2013). Two studies already investigated the efficacy or liposomes for oral vaccination of fish (Table 2). The first study showed successful oral vaccination of carp against CyHV-3 using liposome-encapsulated formalin-inactivated (f.i) CyHV-3 (Miyazaki et al., 2008; Yasumoto et al., 2006a). The second study, showed more variable degrees of protection against *A. hydrophila* using liposome-encapsulated f.i. *A. hydrophila* (Yasumoto et al., 2006b). In both cases, long term protection and the underlining protective mechanisms have not been investigated in details. The saponing Quil-A was found to increase systemic antibody levels against the model antigen Human Gamma Globulin (HGG) after oral delivery to Mozambique tilapia but protection after challenge was not assessed (Jenkins et al., 1991).

Additional adjuvants that are included in licensed human and veterinary vaccines and can be used for both, parenteral or mucosal administration include: MF59® (oil-in-water emulsion), a squalene-based adjuvant system 03 (AS03®) and AS04® (monophosphoryl lipid A (MPL-A) + alum) (Savelkoul et al., 2015). They all have been shown to effectively induce humoral as well as cell-mediated responses to various degrees, at least when administered *via* the parenteral route. Squalene is a cholesterol precursor that is added to adjuvant emulsions (i.e. MF59 and AS03) and was shown to enhanced antigen uptake by DCs. Local reactions and its persistence in oil residues however, drive the demand for alternatives. Non-toxic MPL-A, derived from LPS, is a TLR4-targeting adjuvant that was shown to greatly enhance APCs activity as well as T and B cell memory responses. Muramyl dipeptide (MDP) present on bacterial cell walls is currently included in various experimental formulation and, similarly to MPL-A, is a strong activator of APCs and of cellular responses. To date, none of the abovementioned licensed adjuvants or their components have been tested for oral vaccine delivery in fish. Considering their full characterization, safety profile, and most of all, their ability to induce humoral and cell-mediated responses, the aforementioned adjuvants or their components, represent promising novel tools to further improve currently insufficient oral vaccines.

9.2. Enterotoxins as strong mucosal adjuvants

To improve delivery and uptake of antigens to the hindgut,

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

antigens can be fused to enteric carrier molecules that also retain the ability to induce strong inflammatory signals. For example, fusion of Green Fluorescent Protein (GFP) to LTB (LTB-GFP) led to increased GFP uptake in the carp mucosa and enhanced GFP-specific serum antibody levels. While almost no GFP was detected after non-LTB-fused GFP administration, high levels of GFP were detected in enterocytes and macrophage-like cells after delivery of LTB-GFP (Companjen et al., 2006). In earlier studies, the enteric protein CTB was found to increase systemic antibody levels against the model antigen Human Gamma Globulin (HGG) after oral delivery to Mozambique tilapia (Jenkins et al., 1994). Enterotoxins hold great promise as mucosal adjuvants as very little amounts have been shown to trigger strong mucosal responses in humans and in veterinary species, including fish. Modified subunits have been developed that retain their immunostimulatory capacity while limiting the exacerbated inflammatory response (Elson and Dertzbaugh, 2005; Lycke, 2005). These toxins are effective as mucosal adjuvants, presumably because they retain the ability to penetrate the mucus layer, are taken up by M cells or mucosal DCs, and ultimately activate key innate signalling pathways. Activated DCs in turn orchestrate adaptive immune responses that are appropriate for defence against live pathogens. Given the already ascertained ability of these molecules to enhance uptake by intra-epithelial macrophages, it will be interesting to evaluate their possible uptake by the recently described fish M-like antigen-sampling cells and follow their faith within the vaccinated animal. This information might certainly contribute to a better understanding of the protective mechanisms that need to be triggered to elicit protective mucosal responses.

Perhaps acting in a similar manner as enterotoxins, a new microparticle-based oral adjuvant containing LPS was described. In this study, LPS from meningococcus bacteria was found to significantly induce IgM production and protection against *A. hydrophila* in African catfish after oral administration (Pérez et al., 2013). LPS molecules, similarly to enterotoxins, might in fact retain the ability to trigger strong local responses and enhance the efficacy of suboptimal inactivated vaccines.

As discussed previously, not only enterotoxins but also enteric pathogens themselves (e.g. *E. tarda, V. anguillarum, E. coli, Y. ruckeri*), either as live vehicles or as inactivated pathogens, can provide adequate stimulatory signals and act as adjuvants for currently suboptimal oral vaccine formulations.

9.3. Molecular adjuvants

Although studies show the potential of using molecular adjuvants such as DNA plasmid-encoded molecules or RNA-based vectors, their use in oral vaccination for fish is very limited. For example, Alphavirus replicon vaccines have been widely used in mammalian vaccines and are known to activate the innate and adaptive immune system at various levels. This approach was also used in an oral DNA vaccine for Atlantic salmon against Infectious Salmon Anaemia Virus (ISAV). It was observed that addition of the alphavirus replicon DNA sequence significantly increased protection upon ISAV challenge when compared to the oral DNA vaccine without the molecular adjuvant (Rivas-Aravena et al., 2015). The large number of dsRNA sequences accumulated upon delivery of the Alphavirus replicon might contribute to the observed adjuvant effect.

A chitosan-encapsulated DNA vector encoding heat shock protein 70 (hsp70) of the protozoan *Cryptocaryon irritans* (*C. irritans*) was found to significantly increase survival of orange spotted grouper when administered orally along with a DNA plasmid encoding the immobilization antigen (iAg) of *C. irritans*. This study not only showed that oral DNA vaccination is also effective against extracellular parasites, but also that heat shock proteins can act as molecular adjuvants when administered orally (Josepriya et al., 2015).

Several cytokines and chemokines have been tested as molecular adjuvants in injected DNA vaccines (Tafalla et al., 2013) For example, strong adjuvant effects have been described for *ifna, ifnb* and *ifnc* in Atlantic salmon that were vaccinated with a suboptimal DNA vaccine against ISAV. Besides increased protection, significantly higher antibody levels and expression of several B- and T-cell markers was found in the muscle, indicating an interferon-induced influx of leukocytes to the site of injection (Chang et al., 2015). Other cytokines such as Il8 and Il1b (peptides) have also been used but never in combination with oral vaccine delivery. Given the necessity to trigger strong inflammatory signals that are able to overcome the high tolerogenic threshold of the gut environment, cytokines such as Il12, Ifnγ or Tnfa could also be used alone or in combination. Most of these cytokine induce activation of APCs by triggering the upregulation of pro-inflammatory cytokines, MHC-II and CD80/86 co-stimulatory molecules. For this reason, they could be used in combination with inactivated/killed pathogens that alone trigger little activation of APCs.

9.4. PAMPs as mucosal adjuvants

Great advances have been made in fish research in trying to identify and characterize several Pattern Recognition Receptors (PRRs), scavenger receptors, C-type lectins etc, because these in fact, represent the targets for rational adjuvant design.

In fish, PAMPs such as bacterial flagellin (targeting TLR5) or CpG motives (potentially targeting TLR9), have been shown to exert strong immunostimulatory activities and to have adjuvant activities when administered with suboptimal parenteral vaccines, as reviewed in (Tafalla et al. (2013)). Flagellin, especially from enteric pathogens (i.e. *Vibrio* ssp, *Salmonella* ssp), holds great promise. In fact, similar to enterotoxins, it has the ability to withstand the gastric environment and the potential to target receptors on leukocytes at mucosal surfaces, hereby triggering the necessary costimulatory signals on APCs. CpG motives, either incorporated in the plasmid backbone of DNA vaccines or administered along with antigens, have been shown to have significant immunostimulatory activities. Their effects are, however, sequence- and species-dependent (Pietretti and Wiegertjes, 2014) suggesting that the choice of CpG motives has to be tailored to the fish species. This implicates that, when administered to different fish, the same vaccine antigen might have to be combined to different CpGs in order to exert its adjuvant effect. Despite their extensive characterization *in vitro*, and *in vivo* upon parenteral administration of antigens, the use of both flagellin and CpGs as mucosal adjuvants awaits further characterization.

Among the most promising immunostimulants that could be used as mucosal adjuvant in oral vaccine formulations, beta-glucans are perhaps the best known and characterized (Bonaldo et al., 2007; De Smet et al., 2013; Ogier de Baulny et al., 1996; Selvaraj et al., 2006, 2005; Skov et al., 2012; Petit & Wiegertjes, 2016). Beta-glucans are easily incorporated into the fish standard feed, making them suitable candidate adjuvants for oral vaccination. Adjuvant activities of beta-glucans have been demonstrated in fish most often in combination with i.p. vaccination against bacterial diseases (Fredriksen et al., 2011; Suanyuk and Itsaro, 2011). Their potential in oral vaccination, however, has not yet been investigated.

In addition to the use as an immunostimulant, beta-glucans can also function as a vehicle to encapsulate and protect antigens. Although this has not yet been exploited for fish vaccines, subcutaneous injection of mice with OVA-loaded beta-glucan

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

particles (GPs) induced an increased $CD4^+$ T-cell proliferation when compared to mice injected with OVA absorbed to the adjuvant alum (Huang et al., 2010). The potential use of GPs in oral vaccination was investigated using human intestinal cell lines (Caco-2 and HT-29) and mice (De Smet et al., 2013). It was found that *in vitro* GP-OVA complexes were internalized by Caco-2 and HT-29 cells and that the complexes did not affect cell viability. Internalization induced an increased expression of *il23p19*, *il8,* and a downregulation of *tgfβ*. The above mentioned results show the adjuvant potential of GPs as vaccine vehicle but future research is necessary to test their applicability for fish vaccines.

Another well-known and widely used adjuvant is polyinosinic:polycytidylic acid (poly I:C), which has been used in multiple fish vaccination studies by injection vaccination but not yet as an mucosal adjuvant. In mammals, poly I:C is most often delivered in nano- or microspheres because of its vulnerability to serum nucleases and because high doses of systemic poly I:C are toxic and can induce autoimmunity (de Clercq, 1979). In fish, co-delivery of chitosan-encapsulated poly I:C and inactivated whole VHSV by i.p. injection was found to induce significant protection in zebrafish against a challenge with VHSV. However, there was no significant difference in survival between groups vaccinated with or without poly I:C as adjuvant (Kavaliauskis et al., 2015). Similar protective effects were found when zebrafish were co-vaccinated with the VHSV-G protein and poly I;C (Kavaliauskis, manuscript in preparation). Poly I:C can also be combined with other adjuvants to further enhance its immunostimulating properties. Combinations of poly I:C and CpG were found to increase protection in Atlantic salmon against SAV when combined with i.p. injection of an inactivated SAV vaccine (Thim et al., 2014, 2012). Altogether, the above studies show the potency of poly I:C as an adjuvant for fish vaccines. However, its use as an adjuvant for oral vaccination is yet to be evaluated.

9.5. Plant-based proteins as immunostimulants to break mucosal tolerance

Plant-based proteins, including soy bean meal and concentrates thereof, are becoming increasingly important as protein source to reduce fishmeal content. However, it is known that the substitution of fishmeal with soy bean meal may have adverse effects such as induction of intestinal lesions and subacute enteritis (Chikwati et al., 2013; Couto et al., 2014). Enteritis leads to a widening of the lamina propria and a subsequent influx of immune cells. The influx of immune cells interferes with the barrier function of the intestine and increases the ease of pathogens or antigens to pass the intestinal barrier (Knudsen et al., 2008). While intestinal lesions and severe enteritis needs to be prevented, soy bean meal might be used as a feed additive to increase vaccine antigen uptake by increasing permeability of the intestinal wall. This possibility was evaluated in rainbow trout orally vaccinated with PLGA nanospheres containing a DNA vaccine against IHNV. Fish were fed diets either containing 35% soy bean meal or 35% soy bean concentrate but no significant differences were found in nanoparticle uptake or survival when being challenged ten weeks after vaccination (Adomako et al., 2012). Since severe enteritis induced by high soy bean meal concentration leads to endocytosis block rather than an increase in uptake in a species-specific manner, care should be taken to find an optimal low dose for every fish species and size (Urán et al., 2008).

Altogether, a rational selection of enteric (live) vectors or encapsulation methods might aid the development of new effective, and the improvement of current suboptimal, oral vaccines for fish. Moreover, it will certainly provide a matrix of tools when combined with a more targeted selection of mucosal adjuvants based on their immunostimulatory ability and affinity for the gut environment.

10. Consideration on the nature of the fish and its environment

Oral vaccine development in fish might present an additional degree of complexity when considering the vast number of cultured fish species and the diversity within. Therefore, different fish species may require a different strategy for the development of oral vaccines, even against the same pathogen. Major differences in gut morphology as well as intestinal environment can be found between stomachless fish and fish with a stomach, and between carnivorous, herbivorous and omnivorous fish species.

Oral vaccination of fish that do not have a stomach may require less protection of the antigen since they are not exposed to the harsh environment and low pH of the stomach. However, factors others than stomach pH are involved and even within the group of fish that possess a stomach, large differences exist. For example, fish with a thin stomach wall, like rainbow trout, have high stomach acidity because they are more dependent on pH than on muscle strength for food kneading and breakdown. This is in contrast to fish with a thick muscular stomach wall, like African catfish, that are better able to knead food and are therefore less dependent on stomach pH for food breakdown (Weber, 2014).

Carnivorous fish species have higher protease activity compared to herbivorous and omnivorous species. Non-carnivorous fish might have high α-amylase activity, since plant materials are difficult to digest. It is important to gain knowledge on the fish intestinal tract and its environment to make an estimate of the level of stress that the vaccine will encounter. Depending on the estimated degree and source of breakdown, an antigen protection or encapsulation technique can be chosen or designed. Besides feeding preferences, differences in digestion enzymes and intestinal environment are caused by several other factors including age of the fish, temperature and season (Chakrabarti et al., 1995; Kolkovski, 2001).

In humans, success or failure of mucosal vaccines in Latin American, European, American individuals versus African patients has been largely ascribed to differences in microbial compositions (Valdez et al., 2014). In mice it has been shown that gut microbiota largely influences the development of an healthy mucosal immune system and most importantly that oral tolerance could not be induced in the absence of signals derived from the gut flora (Bauer et al., 1963; Hapfelmeier and Macpherson, 2010; Hill and Artis, 2010; Kiyono et al., 1982; Sudo et al., 1997). Studies have demonstrated that also in zebrafish the composition the gut microbiota plays a crucial role of in the onset of enterocolitis (Brugman et al., 2009) and recent reviews have summarized how also in teleost fish a delicate arm race between the host, commensals and pathogens is taking place at mucosal surfaces (Gomez et al., 2013). Therefore, considering the immense heterogeneity of fish species, their environment and eating habits, a careful consideration of the enterotypes (gut microbiota community profiles) of aquaculture species should also be taken into account when developing oral vaccines.

While antigen coating or encapsulation can be the key to ensure sufficient antigen delivery to the hindgut, the biochemical properties of the encapsulation vehicle can strongly influence the degree of uptake and antigen release. Consequently, choosing a suboptimal antigen coating or encapsulation system can have detrimental effects on the efficacy of the delivered vaccine. As an example, oral vaccination of rainbow trout against IHNV using PLGA-microencapsulated DNA vaccine resulted in low protection, while uptake of the microspheres was significant. This apparent discrepancy was found to be caused by the property of PLGA to not

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

dissolve efficiently at 14°, the water temperature at which vaccination was performed (Adomako et al., 2012). This study shows the importance of choosing an encapsulation method whose characteristics match with the environment of the fish.

Last but not least, in experimental vaccination and challenge experiments of fish, temperature might be a key element determining the level of success. It is commonly known that the fish immune system is rather slow compared to mammals, especially that of fish living in cold environments. Besides temperature, changes in water type and culturing conditions will also play a role as for example salmon having to move from fresh to salt water conditions and from tanks to sea cages. Only few studies systematically addressed the effect of temperature on vaccine efficacy. For example, vaccination of coho salmon against *V. anguillarum* at temperatures ranging from 3.9 to 20.6° did not lead to significant differences in vaccine efficacy as all groups showed very good protection (Fryer et al., 1978). On the contrary, a temperature-dependent effect was observed on the protective innate and adaptive mechanisms induced upon i.m. DNA vaccination of rainbow trout against VHSV. While the vaccine protected the fish well at temperatures of 5, 10 and 15°, no neutralizing antibodies and a delayed *mx3* expression were observed only in fish vaccinated at 5° (Lorenzen et al., 2009).

Altogether, given the vast diversity of fish species, heterogeneity of their environment and culturing conditions, testing the vaccine in the target species and under field conditions will prove to be crucial to finally validate vaccine efficacy.

11. The zebrafish as animal model for aquaculture animals

Vaccine validation should certainly be performed in the species of interest and under conditions that best resemble the natural rearing conditions and environment. Still, the use of fish models might help speed up part of the process linked to the characterization of the vaccine and of the immune response of the host. In the past 20 years, the small zebrafish has managed to climb the pyramid of animal models commonly used for biomedical research in humans. Despite its established reputation in the biomedical field, zebrafish has been largely underestimated and poorly used as a model in the aquaculture field. The availability of numerous transgenic fish lines, including those specifically marking IgM + B cells, T cells, macrophages, or neutrophils, creates an unique opportunity to investigate the real-time kinetics of cell recruitment, proliferation and migration in response to specific vaccine antigens. Double transgenic and reporter fish lines for specific cytokines or chemokines are also available. These lines might help elucidate which cell types express which molecules, when and where, in response to antigens or adjuvants. This type of analysis is in general rarely possible in most fish species due to the scarcity of antibodies.

The use of transgenic fish lines in combination with labelled antigens might help predict the faith of for example orally delivered antigens and the relative contribution of specific cell types in the uptake, presentation and activation of the immune response. A detailed review of zebrafish gut physiology and its potential use as a model to study intestinal responses is reviewed elsewhere in this issue (Brugman, 2016). Fluorescently labelled inactivated or live vectors could be easily traceable in zebrafish. Delivery and uptake of (encapsulated) antigens could be monitored in all gut segments and suitability of various encapsulation methods, based on size or stability, could be performed in real-time. Chemical ablation of specific cell types is also possible in zebrafish (Gray et al., 2011), allowing the determination of the role of specific leukocytes in the response to the vaccine. For example, the ultimate proof that M-like sampling cells transport and deliver antigens to leukocytes in the lamina propria could come from the generation of novel transgenic zebrafish lines, as soon as M-like cell markers are identified in fish. Besides the availability of transgenic lines, several mutants are also available. For example, Il10$^{-/-}$ knockout zebrafish are available and could be used to investigate the role of Il10 in the onset or maintenance of gut tolerance. Furthermore, live imaging is not only limited to the transparent larval stages, but with the combined use of for example two-photon microscopy and casper mutant fish that lack pigmentation, it can be extended to the juvenile and adult fish as well.

Despite the many advantages of the zebrafish model, including the ones mentioned above, the use of zebrafish as a model for aquaculture species poses some limitations. Zebrafish is a cyprinid fish living in fresh waters at an optimal temperature of 27° and is a stomachless, omnivorous fish. Due to these characteristics, the zebrafish is certainly a suitable pre-screening model for cyprinids and some fresh water fish. In contrary, it might be less suitable for other commercially relevant species, including salmon, trout, turbot, seabream, or sea bass. As discussed above, the microbiota plays an extremely crucial role in influencing gut development, homeostasis as well as induction of tolerance. As a consequence the microbiota and the gut environment will be extremely different between omnivores and carnivores. Nevertheless, zebrafish can provide the proof of principle of the validity of novel approaches and may help accelerate the selection of antigens, adjuvant, vaccine vehicles or encapsulation methods, which without doubt, will ultimately have to be validated in the species of interest.

12. Concluding remarks

Based on the status of oral vaccines in humans and veterinary species, there is no doubt that the generation of safe and efficacious oral vaccines is among one of the most difficult tasks of immunologists. This is illustrated by the very limited number or oral vaccines approved for use in humans and the slightly larger number approved for use in poultry, pigs and cattle. In this respect fish are not lagging much behind, with 5 oral vaccines available on the market. These vaccines however, are against only a very limited number of pathogens and are available for an even smaller number of fish species. When considering the vast diversity of cultured fish species and their pathogens, the current oral vaccines are by far insufficient to fulfil the market requirements. Such species diversity is in fact larger than the diversity in species and pathogens faced by for example the poultry or cattle vaccine industry.

Nonetheless, fish oral vaccine development can greatly profit from the progress made on human and veterinary oral vaccines. For example, the use of live vectors, e.g adenoviruses, or a more rational attenuation of enteric pathogens, e.g *E. tarda* or *V. anguillarum*, as well as the combination of weak oral antigens with strong mucosal adjuvants, e.g enterotoxins, leaves a vast number of combinations that have not been fully exploited in fish vaccine development. Fish mucosal immunology, despite the large body of work performed in the last 30 years, is still in its infancy, mostly due to the great heterogeneity in teleost species. Nevertheless, the discovery of new players in fish mucosal immunity within the last 5–10 years, including IgT or M-like sampling cells, keeps the field of mucosal immunology and vaccinology a dynamic and developing area. The possibility to specifically target M-like cells or putative APCs in the fish gut is becoming a viable option in fish vaccine delivery as well. This is being realized through the great advances in gene discovery and the several genome sequencing initiatives for several fish species and their pathogens. The bottleneck will of course be the functional characterization of most of the novel genes and the translation of this fundamental knowledge into practical applications linked to vaccine development. Molecular traceable and genetically modifiable models such as transgenic or mutant

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

zebrafish can support and accelerate fish vaccine development as much as other animal models have helped the human and veterinary field. Information on host mucosal responses, together with insights in how fish gut microbiota might influence the response to oral vaccination is increasing at a rapid paste. This information will be essential to design strategies aimed at breaking mucosal tolerance while preventing inflammation for a greater variety of fish species. Finally, collaborations between academia, industrial partners and farmers will be instrumental to produce safe and efficacious vaccines for most commercially relevant fish species.

Acknowledgments

This research was partly funded by the European Commission under the 7th Framework Programme for Research and Technological Development (FP7) of the European Union (Grant Agreement 311993 TARGETFISH) and by the Netherlands Organisation for Scientific Research under Veni project number 11200.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2016.03.024.

References

Adelmann, M., Köllner, B., Bergmann, S.M., Fischer, U., Lange, B., Weitschies, W., Enzmann, P.J., Fichtner, D., 2008. Development of an oral vaccine for immunisation of rainbow trout (Oncorhynchus mykiss) against viral haemorrhagic septicaemia. Vaccine 26, 837–844. http://dx.doi.org/10.1016/j.vaccine.2007.11.065.

Adomako, M., St-Hilaire, S., Zheng, Y., Eley, J., Marcum, R.D., Sealey, W., Donahower, B.C., LaPatra, S., Sheridan, P.P., 2012. Oral DNA vaccination of rainbow trout, Oncorhynchus mykiss (Walbaum), against infectious haematopoietic necrosis virus using PLGA [Poly(D,L-Lactic-Co-Glycolic Acid)] nanoparticles. J. Fish Dis. 35, 203–214. http://dx.doi.org/10.1111/j.1365-2761.2011.01338.x.

Agius, C., Horne, M.T., Ward, P.D., 1983. Immunization of rainbow trout, Salmo gairdneri Richardson, against vibriosis: comparison of an extract antigen with whole cell bacterins by oral and intraperitoneal routes. J. Fish Dis 6, 129–134. http://dx.doi.org/10.1111/j.1365-2761.1983.tb00060.x.

Altun, S., Kubilay, A., Ekici, S., Didinen, B.I., Diler, O., 2009. Oral vaccination against lactococcosis in rainbow trout (Oncorhynchus mykiss) using sodium alginate and poly (lactide-co-glycolide) carrier. Kafkas Univ. Vet. Fak. Derg. 16, 211–217. http://dx.doi.org/10.9775/kvfd.2009.1327.

Anuradha, K., Foo, H.L., Mariana, N.S., Loh, T.C., Yusoff, K., Hassan, M.D., Sasan, H., Raha, A.R., 2010. Live recombinant Lactococcus lactis vaccine expressing aerolysin genes D1 and D4 for protection against Aeromonas hydrophila in tilapia (Oreochromis niloticus). J. Appl. Microbiol. 109, 1632–1642. http://dx.doi.org/10.1111/j.1365-2672.2010.04789.x.

Aoki, M., Kondo, M., Nakatsuka, Y., Kawai, K., Oshima, S.I., 2007. Stationary phase culture supernatant containing membrane vesicles induced immunity to rainbow trout Oncorhynchus mykiss fry syndrome. Vaccine 25, 561–569. http://dx.doi.org/10.1016/j.vaccine.2006.07.047.

Appaiahgari, M.B., Vrati, S., 2015. Adenoviruses as gene/vaccine delivery vectors: promises and pitfalls. Expert Opin. Biol. Ther. 15, 337–351. http://dx.doi.org/10.1517/14712598.2015.993374.

Arijo, S., Brunt, J., Chabrillón, M., Díaz-Rosales, P., Austin, B., 2008. Subcellular components of Vibrio harveyi and probiotics induce immune responses in rainbow trout, Oncorhynchus mykiss (Walbaum), against V. harveyi. J. Fish Dis. 31, 579–590. http://dx.doi.org/10.1111/j.1365-2761.2008.00932.x.

Azad, I., Shankar, K., Mohan, C., Kalita, B., 1999. Biofilm vaccine of Aeromonas hydrophila – standardization of dose and duration for oral vaccination of carps. Fish Shellfish Immunol. 9, 519–528. http://dx.doi.org/10.1006/fsim.1998.0206.

Bakopoulos, V., Volpatti, D., Gusmani, L., Galeotti, M., Adams, A., Dimitriadis, G.J., 2003. Vaccination trials of sea bass, Dicentrarchus labrax (L.), against Photobacterium damsela subsp. piscicida, using novel vaccine mixtures. J. Fish Dis. 26, 77–90. http://dx.doi.org/10.1046/j.1365-2761.2003.00438.x.

Ballesteros, N.A., Rodriguez Saint-Jean, S., Perez-Prieto, S.I., 2014. Food pellets as an effective delivery method for a DNA vaccine against infectious pancreatic necrosis virus in rainbow trout (Oncorhynchus mykiss, Walbaum). Fish Shellfish Immunol. 37, 220–228. http://dx.doi.org/10.1016/j.fsi.2014.02.003.

Ballesteros, N.A., Alonso, M., Saint-Jean, S.R., Perez-Prieto, S.I., 2015a. An oral DNA vaccine against infectious haematopoietic necrosis virus (IHNV) encapsulated in alginate microspheres induces dose-dependent immune responses and significant protection in rainbow trout (Oncorrhynchus mykiss). Fish Shellfish Immunol. 45, 877–888. http://dx.doi.org/10.1016/j.fsi.2015.05.045.

Ballesteros, N.A., Rodriguez Saint-Jean, S., Perez-Prieto, S.I., 2015b. Immune responses to oral pcDNA-VP2 vaccine in relation to infectious pancreatic necrosis virus carrier state in rainbow trout Oncorhynchus mykiss. Vet. Immunol. Immunopathol. 165, 127–137. http://dx.doi.org/10.1016/j.vetimm.2015.04.001.

Ballesteros, N.A., Saint-Jean, S.S.R., Encinas, P.A., Perez-Prieto, S.I., Coll, J.M., 2012. Oral immunization of rainbow trout to infectious pancreatic necrosis virus (Ipnv) induces different immune gene expression profiles in head kidney and pyloric ceca. Fish Shellfish Immunol. 33, 174–185. http://dx.doi.org/10.1016/j.fsi.2012.03.016.

Bangari, D.S., Mittal, S.K., 2006. Development of nonhuman adenoviruses as vaccine vectors. Vaccine 24, 849–862. http://dx.doi.org/10.1016/j.vaccine.2005.08.101.

Bauer, H., Horowitz, R., Levenson, S., Popper, H., 1963. The response of the lymphatic tissue to the microbial flora. Am. J. Pathol. 42, 471–483.

Behera, T., Swain, P., 2014. Antigen encapsulated alginate-coated chitosan microspheres stimulate both innate and adaptive immune responses in fish through oral immunization. Aquac. Int. 22, 673–688. http://dx.doi.org/10.1007/s10499-013-9696-8.

Böhles, N., Busch, K., Hensel, M., 2014. Vaccines against human diarrheal pathogens: current status and perspectives. Hum. Vaccin. Immunother. 10, 1522–1535. http://dx.doi.org/10.4161/hv.29241.

Bonaldo, A., Bonaldo, A., Thompson, K.D., Thompson, K.D., Manfrin, A., Manfrin, A., Adams, A., Adams, A., Murano, E., Murano, E., Mordenti, a L., Mordenti, a L., Gatta, P.P., Gatta, P.P., 2007. The influence of dietary beta-glucans on the adaptive and innate immune responses of European sea bass (Dicentrarchus labrax) vaccinated against vibriosis. Ital. J. Anim. Sci. 6, 151–164.

Bowden, T.J., Menoyo-Luque, D., Bricknell, I.R., Wergeland, H., 2002. Efficacy of different administration routes for vaccination against Vibrio anguillarum in Atlantic halibut (Hippoglossus hippoglossus L.). Fish Shellfish Immunol. 12, 283–285. http://dx.doi.org/10.1006/fsim.2001.0386.

Brudeseth, B.E., Wiulsrød, R., Fredriksen, B.N., Lindmo, K., Løkling, K.E., Bordevik, M., Steine, N., Klevan, A., Gravningen, K., 2013. Status and future perspectives of vaccines for industrialised fin-fish farming. Fish Shellfish Immunol. 35, 1759–1768. http://dx.doi.org/10.1016/j.fsi.2013.05.029.

Brugman, S., Liu, K.Y., Lindenbergh-Kortleve, D., Sansom, J.N., Furuta, G.T., Renshaw, S.A., Willemsen, R., Nieuwenhuis, E.E.S., 2009. Oxazolone-induced enterocolitis in zebrafish depends on the composition of the intestinal microbiota. Gastroenterology 137, 1757–1767. http://dx.doi.org/10.1053/j.gastro.2009.07.069.

Brugman, S., 2016. The zebrafish as a model to study intestinal inflammation. Dev. Comp. Immunol. http://dx.doi.org/10.1016/j.dci.2016.02.020.

Chakrabarti, I., Gani, M.A., Chaki, K.K., Sur, R., Misra, K.K., 1995. Digestive enzymes in 11 freshwater teleost fish species in relation to food habit and niche segregation. Comp. Biochem. Physiol. Part A Physiol. 112, 167–177. http://dx.doi.org/10.1016/0300-9629(95)00072-F.

Chang, C.-J., Sun, B., Robertsen, B., 2015. Adjuvant activity of fish type I interferon shown in a virus DNA vaccination model. Vaccine 33, 2442–2448. http://dx.doi.org/10.1016/j.vaccine.2015.03.093.

Chen, Y.M., Shih, C.H., Liu, H.C., Wu, C.L., Lin, C.C., Wang, H.C., Chen, T.Y., Yang, H.L., Lin, J.H.Y., 2011. An oral nervous necrosis virus vaccine using Vibrio anguillarum as an expression host provides early protection. Aquaculture 321, 26–33. http://dx.doi.org/10.1016/j.aquaculture.2011.08.035.

Cheng, S., Hu, Y.-H., Zhang, M., Sun, L., 2010. Analysis of the vaccine potential of a natural avirulent Edwardsiella tarda isolate. Vaccine 28, 2716–2721. http://dx.doi.org/10.1016/j.vaccine.2010.01.023.

Chikwati, E.M., Sahlmann, C., Holm, H., Penn, M.H., Krogdahl, Å., Bakke, A.M., 2013. Alterations in digestive enzyme activities during the development of diet-induced enteritis in Atlantic salmon, Salmo salar L. Aquaculture 402–403, 28–37. http://dx.doi.org/10.1016/j.aquaculture.2013.03.023.

Choi, S.H., Kim, M.S., Kim, K.H., 2011. Protection of olive flounder (Paralichthys olivaceus) against Edwardsiella tarda infection by oral administration of auxotrophic mutant E. tarda (Δalr Δasd E. tarda). Aquaculture 317, 48–52. http://dx.doi.org/10.1016/j.aquaculture.2011.04.021.

Companjen, A.R., Florack, D.E.A., Slootweg, T., Borst, J.W., Rombout, J.H.W.M., 2006. Improved uptake of plant-derived LTB-linked proteins in carp gut and induction of specific humoral immune responses upon infeed delivery. Fish Shellfish Immunol. 21, 251–260. http://dx.doi.org/10.1016/j.fsi.2005.12.001.

Couto, A., Kortner, T.M., Penn, M., Bakke, A.M., Krogdahl, Å., Oliva-Teles, A., 2014. Effects of dietary phytosterols and soy saponins on growth, feed utilization efficiency and intestinal integrity of gilthead sea bream (Sparus aurata) juveniles. Aquaculture 432, 295–303. http://dx.doi.org/10.1016/j.aquaculture.2014.05.009.

Cui, L.-C., Guan, X.-T., Liu, Z.-M., Tian, C.-Y., Xu, Y.-G., 2015. Recombinant lactobacillus expressing G protein of spring viremia of carp virus (SVCV) combined with ORF81 protein of koi herpesvirus (KHV): a promising way to induce protective immunity against SVCV and KHV infection in cyprinid fish via oral vaccination. Vaccine. http://dx.doi.org/10.1016/j.vaccine.2015.05.002.

Davison, A.J., Benko, M., Harrach, B., 2003. Genetic content and evolution of adenoviruses. J. Gen. Virol. 84, 2895–2908. http://dx.doi.org/10.1099/vir.0.19497-0.

de Clercq, E., 1979. Degradation of poly(inosinic acid). Poly(cytidylic acid) [(I)n·(Cn)] by human plasma. Eur. J. Biochem. 93, 165–172. http://dx.doi.org/10.1111/j.1432-1033.1979.tb12807.x.

de las Heras, A.I., Rodríguez Saint-Jean, S., Pérez-Prieto, S.I., 2010. Immunogenic and protective effects of an oral DNA vaccine against infectious pancreatic necrosis virus in fish. Fish Shellfish Immunol. 28, 562–570. http://dx.doi.org/10.1016/j.fsi.2009.12.006.

De Smet, R., Demoor, T., Verschuere, S., Dullaers, M., Ostroff, G.R., Leclercq, G., Allais, L., Pilette, C., Dierendonck, M., De Geest, B.G., Cuvelier, C.A., 2013. Beta-Glucan microparticles are good candidates for mucosal antigen delivery in oral vaccination. J. Control. Release 172, 671–678. http://dx.doi.org/10.1016/j.jconrel.2013.09.007.

Dhar, A.K., Manna, S.K., Thomas Allnutt, F.C., 2014. Viral vaccines for farmed finfish. Virusdisease 25, 1–17. http://dx.doi.org/10.1007/s13337-013-0186-4.

Duff, D.C.B., 1942. The oralimmunization of trout against Bacterium salmonicida. J. Immunol. 44, 87–94.

Ellett, F., Pase, L., Hayman, J.W., Andrianopoulos, A., Lieschke, G.J., 2010. mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. Blood 117, e49–e56. http://dx.doi.org/10.1182/blood-2010-10-314120.

Elson, C.O., Dertzbaugh, M.T., 2005. Mucosal adjuvants. In: Ogra, R., et al. (Eds.), Mucosal Immunology. Academic Press, New York, pp. 967–986.

Faria, A.M.C., Weiner, H.L., 2005. Oral tolerance. Immunol. Rev. 206, 232–259. http://dx.doi.org/10.1111/j.0105-2896.2005.00280.x.

FAO, 2014. The State of World Fisheries and Aquaculture. Food and Agriculture Oraganization of the United Nations, Rome, ISBN 92-5-105177-1.

Firdaus-Nawi, M., Yusoff, S.M., Yusof, H., Abdullah, S.Z., Zamri-Saad, M., 2013. Efficacy of feed-based adjuvant vaccine against Streptococcus agalactiae in Oreochromis spp. in Malaysia. Aquac. Res. 45, 87–96. http://dx.doi.org/10.1111/j.1365-2109.2012.03207.x.

Fischer, U., Koppang, E.O., Nakanishi, T., 2013. Teleost T and NK cell immunity. Fish Shellfish Immunol 35, 197–206. http://dx.doi.org/10.1016/j.fsi.2013.04.018.

Fredriksen, B.N., Sævareid, K., McAuley, L., Lane, M.E., Bøgwald, J., Dalmo, R.A., 2011. Early immune responses in Atlantic salmon (Salmo salar L.) after immunization with PLGA nanoparticles loaded with a model antigen and β-glucan. Vaccine 29, 8338–8349. http://dx.doi.org/10.1016/j.vaccine.2011.08.087.

Frey, A., Giannasca, K.T., Weltzin, R., Giannasca, P.J., Reggio, H., Lencer, W.I., Neutra, M.R., 1996. Role of the glycocalyx in regulating access of microparticles to apical plasma membranes of intestinal epithelial cells: implications for microbial attachment and oral vaccine targeting. J. Exp. Med. 184, 1045–1059.

Fryer, J.L., Rohovec, J.S., Garrison, R., 1978. Immunization of fish for the control of vibriosis. Mar. Fish. Rev. 40, 20–23.

Fuglem, B., Jirillo, E., Bjerkås, I., Kiyono, H., Nochi, T., Yuki, Y., Raida, M., Fischer, U., Koppang, E.O., 2010. Antigen-sampling cells in the salmonid intestinal epithelium. Dev. Comp. Immunol. 34, 768–774. http://dx.doi.org/10.1016/j.dci.2010.02.007.

Fujino, Y., Ono, S.-I., Nagai, A., 1987. Studies on uptake of rabbit's immunoglobulin into the columnar epithelial cells in the gut of rainbow trout Salmo gairdneri. Nippon. Suisan Gakkaishi 53, 367–370.

Fujkuyama, Y., Tokuhara, D., Kataoka, K., Gilbert, R.S., McGhee, J.R., Yuki, Y., Kiyono, H., Fujihashi, K., 2012. Novel vaccine development strategies for inducing mucosal immunity. Expert Rev. Vaccines 11, 367–379. http://dx.doi.org/10.1586/erv.11.196.

Georgopoulou, U., Sire, M.F., Vernier, J.M., 1986. Immunological demonstration of intestinal absorption and digestion of protein macromolecules in the trout (Salmo gairdneri). Cell Tissue Res. 245, 387–395.

Gerdts, V., Mutwiri, G.K., Tikoo, S.K., Babiuk, L.A., 2006. Mucosal delivery of vaccines in domestic animals. Vet. Res. 37, 487–510. http://dx.doi.org/10.1051/vetres:2006012.

Ghosh, B., Bridle, A.R., Nowak, B.F., Cain, K.D., 2015. Assessment of immune response and protection against bacterial coldwater disease induced by a live-attenuated vaccine delivered orally or intraperitoneally to rainbow trout, Oncorhynchus mykiss (Walbaum). Aquaculture 446, 242–249. http://dx.doi.org/10.1016/j.aquaculture.2015.04.035.

Gilbert, S.C., 2015. Adenovirus-vectored Ebola vaccines. Expert Rev. Vaccines 14, 1347–1357. http://dx.doi.org/10.1586/14760584.2015.1077122.

Gomez-Casado, E., Estepa, A., Coll, J.M., 2011. A comparative review on European-farmed finfish RNA viruses and their vaccines. Vaccine 29. http://dx.doi.org/10.1016/j.vaccine.2011.01.097, 2657–2571.

Gomez, D., Sunyer, J.O., Salinas, I., 2013. The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. Fish Shellfish Immunol. 35, 1729–1739. http://dx.doi.org/10.1016/j.fsi.2013.09.032.

Gray, C., Loynes, C.A., Whyte, M.K.B., Crossman, D.C., Renshaw, S.A., Chico, T.J.A., 2011. Simultaneous intravital imaging of macrophage and neutrophil behaviour during inflammation using a novel transgenic zebrafish. Thromb. Haemost. 105, 811–819. http://dx.doi.org/10.1160/TH10-08-0525.

Green, C.A., Scarselli, E., Sande, C.J., Thompson, A.J., De Lara, C.M., Taylor, K.S., Haworth, K., Del Sorbo, M., Angus, B., Siani, L., Di Marco, S., Traboni, C., Folgori, A., Colloca, S., Capone, S., Vitelli, A., Cortese, R., Klenerman, P., Nicosia, A., Pollard, A.J., 2015. Chimpanzee adenovirus – and MVA-vectored respiratory syncytial virus vaccine is safe and immunogenic in adults. Sci. Transl. Med. 7, 1–17. http://dx.doi.org/10.1126/scitranslmed.aac5745.

Griffiths, S.G., Melville, K.J., Salonius, K, 1998. Reduction of Renibacterium salmoninarumculture activity in Atlantic salmon following vaccination with avirulent strains. Fish Shellfish Immunol. 8, 607–619. http://dx.doi.org/10.1006/fsim.1998.0169.

Gudding, R., Lillehaug, A., Evensen, Ø. (Eds.), April 2014. Fish Vaccination. Wiley-Blackwell, ISBN 978-0-470-67455-0, 404 pages.

Hansen, J.D., Landis, E.D., Phillips, R.B., 2005. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: implications for a distinctive B cell developmental pathway in teleost fish. Proc. Natl. Acad. Sci. U. S. A. 102, 6919–6924. http://dx.doi.org/10.1073/pnas.0500027102.

Hapfelmeier, S., Macpherson, A.J., 2010. In remembrance of commensal intestinal microbes. Commun. Integr. Biol. 3, 569–571. http://dx.doi.org/10.4161/cib.3.6.13011.

Hill, D.A., Artis, D., 2010. Intestinal bacteria and the regulation of immune cell homeostasis. Annu. Rev. Immunol. 28, 623–667. http://dx.doi.org/10.1146/annurev-immunol-030409-101330.

Hu, Y.H., Cheng, S., Zhang, M., Sun, L., 2011. Construction and evaluation of a live vaccine against Edwardsiella tarda and Vibrio harveyi: laboratory vs. mock field trial. Vaccine 29, 4081–4085. http://dx.doi.org/10.1016/j.vaccine.2011.04.025.

Huang, H., Ostroff, G.R., Lee, C.K., 2010. Robust stimulation of humoral and cellular immune responses following vaccination with antigen-loaded β -glucan particles robust stimulation of humoral and cellular immune responses following vaccination with antigen-loaded ␣ -glucan particles. MBio 1–7. http://dx.doi.org/10.1128/mBio.00164-10.

Huang, L.Y., Wang, K.Y., Xiao, D., Chen, D.F., Geng, Y., Wang, J., He, Y., Wang, E.L., Huang, J.L., Xiao, G.Y., 2014. Safety and immunogenicity of an oral DNA vaccine encoding Sip of Streptococcus agalactiae from Nile tilapia Oreochromis niloticus delivered by live attenuated Salmonella typhimurium. Fish Shellfish Immunol. 38, 34–41. http://dx.doi.org/10.1016/j.fsi.2014.02.017.

Iida, H., Yamamoto, T., 1985. Intracellular transport of horseradish peroxidase in the absorptive cells of goldfish hindgut in vitro, with special reference to the cytoplasmic tubules. Cell Tissue Res. 553–560.

Irie, T., Watarai, S., Iwasaki, T., Kodama, H., 2005. Protection against experimental Aeromonas salmonicida infection in carp by oral immunisation with bacterial antigen entrapped liposomes. Fish Shellfish Immunol. 18, 235–242. http://dx.doi.org/10.1016/j.fsi.2004.07.006.

Jang, M.H., Kweon, M.-N., Iwatani, K., Yamamoto, M., Terahara, K., Sasakawa, C., Suzuki, T., Nochi, T., Yokota, Y., Rennert, P.D., Hiroi, T., Tamagawa, H., Iijima, H., Kunisawa, J., Yuki, Y., Kiyono, H., 2004. Intestinal villous M cells: an antigen entry site in the mucosal epithelium. Proc. Natl. Acad. Sci. U. S. A. 101, 6110–6115. http://dx.doi.org/10.1073/pnas.0400969101.

Jenkins, P.G., Harris, J.E., Pulsford, A.L., 1991. Enhanced enteric uptake of human gamma globulin by Quil-A saponin in Oreochromis mossambicus. Fish Shellfish Immunol. 1, 279–295. http://dx.doi.org/10.1016/S1050-4648(05)80066-X.

Jenkins, P.G., Wrathmell, A.B., Harris, J.E., Pulsford, A.L., 1994. The effects of different adjuvants on intestinal antigen absorption and subsequent immune responses of the tilapian Oreochromis mossambicus. Fish Shellfish Immunol. 4, 167–177. http://dx.doi.org/10.1006/fsim.1994.1016.

Johnson, K.A., Amend, D.F., 1983. Efficacy of Vibrio anguillarum and Yersinia ruckeri bacterins applied by oral and anal intubation of salmonids. J. Fish Dis. 6, 473–476. http://dx.doi.org/10.1111/j.1365-2761.1983.tb00101.x.

Jones, B.B.D., Ghori, N., Falkow, S., 1994. Salmonella typhlrnurium initiates murine infection by\nPenetrating and destroying the specialized epithelial\nM Cells of the Peyer's patches. J. Exp. Med. 180.

Joosten, P.H.M., Engelsma, M.Y., van der Zee, M.D., Rombout, J.H.W.M., 1997. Induction of oral tolerance in carp (Cyprinus carpio L.) after feeding protein antigens. Vet. Immunol. Immunopathol. 60, 187–196. http://dx.doi.org/10.1016/S0165-2427(97)00124-4.

Jørgensen, J.B., 2014. The innate immune response in fish. Fish Vaccin. 85–103.

Josepriya, T.A., Chien, K.-H., Lin, H.-Y., Huang, H.-N., Wu, C.-J., Song, Y.-L., 2015. Immobilization antigen vaccine adjuvanted by parasitic heat shock protein 70C confers high protection in fish against cryptocaryonosis. Fish Shellfish Immunol. 45, 517–527. http://dx.doi.org/10.1016/j.fsi.2015.04.036.

Kavaliauskis, A., Arnemo, M., Kim, S.-H., Ulanova, L., Speth, M., Novoa, B., Dios, S., Evensen, Ø., Griffiths, G.W., Gjøen, T., 2015. Use of poly(I:C) stabilized with chitosan as a vaccine-adjuvant against viral hemorrhagic septicemia virus infection in zebrafish. Zebrafish 12, 421–431. http://dx.doi.org/10.1089/zeb.2015.1126.

Kawai, K., Yamamoto, S., Kusuda, R., 1989. Plankton-mediated oral delivery of Vibrio anguillarum vaccine to juvenile ayu. Nippon Suisan Gakkaishi 55, 35–40.

Kim, M.S., Kim, D.S., Kim, K.H., 2011. Oral immunization of olive flounder (Paralichthys olivaceus) with recombinant live viral hemorrhagic septicemia virus (VHSV) induces protection against VHSV infection. Fish Shellfish Immunol. 31, 212–216. http://dx.doi.org/10.1016/j.fsi.2011.05.003.

Kim, S.-H., Jang, Y.-S., 2014. Antigen targeting to M cells for enhancing the efficacy of mucosal vaccines. Exp. Mol. Med. 46, e85. http://dx.doi.org/10.1038/emm.2013.165.

Kiyono, H., McGhee, J.R., Wannemuehler, M.J., Michalek, S.M., 1982. Lack of oral tolerance in C3H/HeJ mice. J. Exp. Med. 155, 605–610.

Knudsen, D., Jutfelt, F., Sundh, H., Sundell, K., Koppe, W., Frøkiaer, H., 2008. Dietary soya saponins increase gut permeability and play a key role in the onset of soyabean-induced enteritis in Atlantic salmon (Salmo salar L.). Br. J. Nutr. 100, 120–129. http://dx.doi.org/10.1017/S0007114507886338.

Koff, W.C., Burton, D.R., Johnson, P.R., Walker, B.D., King, C.R., Nabel, G.J., Ahmed, R., Bhan, M.K., Plotkin, S.A., 2013. Accelerating next-generation vaccine development for global disease prevention. Science 340, 1232910. http://dx.doi.org/10.1126/science.1232910.

Kolkovski, S., 2001. Digestive enzymes in fish larvae and juveniles—implications and applications to formulated diets. Aquaculture 200, 181–201. http://dx.doi.org/10.1016/S0044-8486(01)00700-1.

Kondo, M., Kawai, K., Okabe, M., Nakano, N., Oshima, S.I., 2003. Efficacy of oral vaccine against bacterial coldwater disease in ayu Plecoglossus altivelis. Dis. Aquat. Organ. 55, 261–264. http://dx.doi.org/10.3354/dao055261.

Kovács, G.M., LaPatra, S.E., D'Halluin, J.C., Benko, M., 2003. Phylogenetic analysis of the hexon and protease genes of a fish adenovirus isolated from white sturgeon (Acipenser transmontanus) supports the proposal for a new adenovirus genus.

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

Virus Res. 98, 27–34.
Lamichhane, A., Azegami, T., Kiyono, H., 2014. The mucosal immune system for vaccine development. Vaccine 32, 6711–6723. http://dx.doi.org/10.1016/j.vaccine.2014.08.089.
Leal, C.A.G., Carvalho-Castro, G.A., Sacchetin, P.S.C., Lopes, C.O., Moraes, A.M., Figueiredo, H.C.P., 2010. Oral and parenteral vaccines against Flavobacterium columnare: evaluation of humoral immune response by ELISA and in vivo efficiency in Nile tilapia (Oreochromis niloticus). Aquac. Int. 18, 657–666. http://dx.doi.org/10.1007/s10499-009-9287-x.
Li, L., Lin, S.-L., Deng, L., Liu, Z.-G., 2013. Potential use of chitosan nanoparticles for oral delivery of DNA vaccine in black seabream Acanthopagrus schlegelii Bleeker to protect from Vibrio parahaemolyticus. J. Fish Dis. 36, 987–995. http://dx.doi.org/10.1111/jfd.12032.
Li, L.P., Wang, R., Liang, W.W., Huang, T., Huang, Y., Luo, F.G., Lei, A.Y., Chen, M., Gan, X., 2015. Development of live attenuated Streptococcus agalactiae vaccine for tilapia via continuous passage in vitro. Fish Shellfish Immunol. 45, 955–963. http://dx.doi.org/10.1016/j.fsi.2015.06.014.
Liew, P.S., Hair-Bejo, M., 2015. Farming of plant-based veterinary vaccines and their applications for disease prevention in animals. Adv. Virol. 2015, 1–12. http://dx.doi.org/10.1155/2015/936940.
Lillehaug, A., 2014. Vaccination strategies and procedures. In: Gudding, R., Lillehaug, A., Evensen, Ø. (Eds.), Fish Vaccination. Wiley Blackwell, pp. 140–152.
Lin, C.-C., Lin, J.H.-Y., Chen, M.-S., Yang, H.-L., 2007. An oral nervous necrosis virus vaccine that induces protective immunity in larvae of grouper (Epinephelus coioides). Aquaculture 268, 265–273. http://dx.doi.org/10.1016/j.aquaculture.2007.04.066.
Løkka, G., Koppang, E.O., 2016. Antigen sampling in the fish intestine. Dev. Comp. Immunol. http://dx.doi.org/10.1016/j.dci.2016.02.014.
Lorenzen, E., Einer-Jensen, K., Rasmussen, J.S., Kjær, T.E., Collet, B., Secombes, C.J., Lorenzen, N., 2009. The protective mechanisms induced by a fish rhabdovirus DNA vaccine depend on temperature. Vaccine 27, 3870–3880. http://dx.doi.org/10.1016/j.vaccine.2009.04.012.
Lorenzen, N., LaPatra, S.E., 2005. DNA vaccines for aquacultured fish. Rev. Sci. Tech. 24, 201–213. http://dx.doi.org/10.3354/dao056031.
Lu, L., Xu, H., He, Y., Li, J., 2011. Protection of grass carp, Ctenopharyngon idellus (Valenciennes), through oral administration of a subunit vaccine against reovirus. J. Fish Dis. 34, 939–942. http://dx.doi.org/10.1111/j.1365-2761.2011.01310.x.
Lycke, N., 2005. From toxin to adjuvant: basic mechanisms for the control of mucosal IgA immunity and tolerance. Immunol. Lett. 97, 193–198. http://dx.doi.org/10.1016/j.imlet.2004.12.008.
Mantis, N.J., Frey, A., Neutra, M.R., 2000. Accessibility of glycolipid and oligosaccharide epitopes on rabbit villus and follicle-associated epithelium. Am. J. Physiol. Gastrointest. Liver Physiol. 278, G915–G923.
Maurice, S., Nussinovitch, A., Jaffe, N., Shoseyov, O., Gertler, A., 2004. Oral immunization of Carassius auratus with modified recombinant A-layer proteins entrapped in alginate beads. Vaccine 23, 450–459. http://dx.doi.org/10.1016/j.vaccine.2004.06.022.
Mayer, L., Shao, L., 2004. Therapeutic potential of oral tolerance. Nat. Rev. Immunol. 4, 407–419. http://dx.doi.org/10.1038/nri1370.
Meeusen, E.N.T., Walker, J., Peters, A., Pastoret, P.-P., Jungersen, G., 2007. Current status of veterinary vaccines. Clin. Microbiol. Rev. 20, 489–510. http://dx.doi.org/10.1128/CMR.00005-07 table of contents.
Miyazaki, T., Yasumoto, S., Kuzuya, Y., Yoshimura, T., 2008. A primary study on oral vaccination with liposomes entrapping Koi Herpesvirus (KHV) antigens against KHV infection in carp. In: Bondad-Reantaso, M.G., Mohan, C.V., Crumlish, M., Subasinghe, R.P. (Eds.), Diseases in Asian Aquaculture. Asian Fisheries Society, Manila, pp. 99–184.
Beck, Benjamin H., Peatman, Eric (Eds.), 2015. Mucosal Health in Aquaculture. Elsevier.
Mulero, I., Pilar Sepulcre, M., Roca, F.J., Meseguer, J., García-Ayala, A., Mulero, V., 2008. Characterization of macrophages from the bony fish gilthead seabream using an antibody against the macrophage colony-stimulating factor receptor. Dev. Comp. Immunol. 32, 1151–1159. http://dx.doi.org/10.1016/j.dci.2008.03.005.
Munang'andu, H.M., Mutoloki, S., Evensen, Ø., 2015. A review of the immunological mechanisms following mucosal vaccination of finfish. Front. Immunol. 6, 427. http://dx.doi.org/10.3389/fimmu.2015.00427.
Mutoloki, S., Jørgensen, J.B., 2014. The adaptive immune response in fish. In: Gudding, R., Lillehaug, A., Evensen, Ø. (Eds.), Fish Vaccination. Wiley Blackwell, pp. 105–115.
Nayak, D.K., Asha, A., Shankar, K.M., Mohan, C.V., 2004. Evaluation of biofilm of Aeromonas hydrophila for oral vaccination of Clarias batrachus – a carnivore model. Fish Shellfish Immunol. 16, 613–619. http://dx.doi.org/10.1016/j.fsi.2003.09.012.
Neutra, M.R., Kozlowski, P.A., 2006. Mucosal vaccines: the promise and the challenge. Nat. Rev. Immunol. 6, 148–158. http://dx.doi.org/10.1038/nri1777.
Neutra, M.R., Mantis, N.J., Kraehenbuhl, J.P., 2001. Collaboration of epithelial cells with organized mucosal lymphoid tissues. Nat. Immunol. 2, 1004–1009. http://dx.doi.org/10.1038/ni1101-1004.
Noaillac-Depeyre, J., Gas, N., 1973. Absorption of protein macromolecules by the enterocytes of the carp (Cyprinus carpio L.). Ultrastructural and cytochemical study. Z. Zellforsch. Mikrosk. Anat. 146, 525–541. http://dx.doi.org/10.1007/BF02347181.
Nochi, T., Takagi, H., Yuki, Y., Yang, L., Masumura, T., Mejima, M., Nakanishi, U., Matsumura, A., Uozumi, A., Hiroi, T., Morita, S., Tanaka, K., Takaiwa, F., Kiyono, H., 2007. Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. Proc. Natl. Acad. Sci. U. S. A. 104, 10986–10991. http://dx.doi.org/10.1073/pnas.0703766104.
Nur-Nazifah, M., Sabri, M.Y., Siti-Zahrah, A., 2014. Development and efficacy of feed-based recombinant vaccine encoding the cell wall surface anchor family protein of Streptococcus agalactiae against streptococcosis in Oreochromis sp. Fish Shellfish Immunol. 37, 193–200. http://dx.doi.org/10.1016/j.fsi.2014.01.011.
Ogier de Baulny, M., Quentel, C., Fournier, V., Lamour, F., Le Gouvello, R., 1996. Effect of long-term oral administration of beta-glucan as an immunostimulant or an adjuvant on some non-specific parameters of the immune response of turbot Scophthalmus maximus. Dis. Aquat. Organ. 26, 139–147. http://dx.doi.org/10.3354/dao026139.
Ogwang, C., Kimani, D., Edwards, N.J., Roberts, R., Mwacharo, J., Bowyer, G., Bliss, C., Hodgson, S.H., Njuguna, P., Viebig, N.K., Nicosia, A., Gitau, E., Douglas, S., Illingworth, J., Marsh, K., Lawrie, A., Imoukhuede, E.B., Ewer, K., Urban, B.C., S Hill, A.V., Bejon, P., 2015. Prime-boost vaccination with chimpanzee adenovirus and modified vaccinia Ankara encoding TRAP provides partial protection against Plasmodium falciparum infection in Kenyan adults. Sci. Transl. Med. 7 http://dx.doi.org/10.1126/scitranslmed.aaa2373, 286re5.
Olsen, C.M., Pemula, A.K., Braaen, S., Sankaran, K., Rimstad, E., 2013. Salmonid alphavirus replicon is functional in fish, mammalian and insect cells and in vivo in shrimps (Litopenaeus vannamei). Vaccine 31, 5672–5679. http://dx.doi.org/10.1016/j.vaccine.2013.09.058.
Owen, J.L., Sahay, B., Mohamadzadeh, M., 2013. New generation of oral mucosal vaccines targeting dendritic cells. Curr. Opin. Chem. Biol. 17, 918–924.
Park, S. Bin, Aoki, T., Jung, T.S., 2012. Pathogenesis of and strategies for preventing Edwardsiella tarda infection in fish. Vet. Res. 43, 67. http://dx.doi.org/10.1186/1297-9716-43-67.
Pérez, O., Romeu, B., Cabrera, O., González, E., Batista-Duharte, A., Labrada, A., Pérez, R., Reyes, L.M., Ramírez, W., Sifontes, S., Fernández, N., Lastre, M., 2013. Adjuvants are key factors for the development of future vaccines: lessons from the Finlay adjuvant platform. Front. Immunol. 4, 1–12. http://dx.doi.org/10.3389/fimmu.2013.00407.
Petit, J., Wiegertjes, G.F., 2016. Long-lived effects of administering β-glucans: indications for trained immunity in fish. Dev. Comp. Immunol. http://dx.doi.org/10.1016/j.dci.2016.03.003.
Pietretti, D., Wiegertjes, G.F., 2014. Ligand specificities of Toll-like receptors in fish: indications from infection studies. Dev. Comp. Immunol. 43, 205–222. http://dx.doi.org/10.1016/j.dci.2013.08.010.
Plant, K.P., Lapatra, S.E., 2011. Advances in fish vaccine delivery. Dev. Comp. Immunol. 35, 1256–1262. http://dx.doi.org/10.1016/j.dci.2011.03.007.
Porta, C., Kotecha, A., Burman, A., Jackson, T., Ren, J., Loureiro, S., Jones, I.M., Fry, E.E., Stuart, D.I., Charleston, B., 2013. Rational engineering of recombinant picornavirus capsids to produce safe, protective vaccine antigen. PLoS Pathog. 9, e1003255. http://dx.doi.org/10.1371/journal.ppat.1003255.
Rajesh Kumar, S., Ishaq Ahmed, V.P., Parameswaran, V., Sudhakaran, R., Sarath Babu, V., Sahul Hameed, A.S., 2008. Potential use of chitosan nanoparticles for oral delivery of DNA vaccine in Asian sea bass (Lates calcarifer) to protect from Vibrio (Listonella) anguillarum. Fish Shellfish Immunol. 25, 47–56. http://dx.doi.org/10.1016/j.fsi.2007.12.004.
Read, A.F., Baigent, S.J., Powers, C., Kgosana, L.B., Blackwell, L., Smith, L.P., Kennedy, D.A., Walkden-Brown, S.W., Nair, V.K., 2015. Imperfect vaccination can enhance the transmission of highly virulent pathogens. PLoS Biol. 13, e1002198. http://dx.doi.org/10.1371/journal.pbio.1002198.
Rivas-Aravena, A., Fuentes, Y., Cartagena, J., Brito, T., Poggio, V., La Torre, J., Mendoza, H., Gonzalez-Nilo, F., Sandino, A.M., Spencer, E., 2015. Development of a nanoparticle-based oral vaccine for Atlantic salmon against ISAV using an alphavirus replicon as adjuvant. Fish Shellfish Immunol. 45, 157–166. http://dx.doi.org/10.1016/j.fsi.2015.03.033.
Rombout, J.H.W.M., van den Berg, A.A., Witte, P., Egberts, E., 1989. Immunological importance of the second gut segment of carp. 111. Systemic and/or mucosal immune responses after immunization with soluble or particulate antigen. J. Fish Biol. 179–186.
Rombout, J.H.W.M., Kiron, V., 2014. Mucosal vaccination of fish. In: Gudding, R., Lillehaug, A., Evensen, Ø. (Eds.), Fish Vaccination. Wiley Blackwell, pp. 56–67.
Rombout, J.H.W.M., Lamers, C.H.J., Helfrich, M.H., Dekker, A., Taverne-Thiele, J.J., 1985. Uptake and transport of intact macromolecules in the intestinal epithelium of carp (Cyprinus carpio L.) and the possible immunological implications. Cell Tissue Res. 239, 519–530. http://dx.doi.org/10.1007/BF00219230.
Rombout, J.H.W.M., van den Berg, A.A., 1989. Immunological importance of the second gut segment of carp. I. Uptake and processing of antigens by epithelial cells and macrophages. J. Fish Biol. 35, 13–22. http://dx.doi.org/10.1111/j.1095-8649.1989.tb03388.x.
Rombout, J.W.H.M., Blok, L.J., Lamers, C.H.J., Egberts, E., Immunology, C., 1986. Immunization of carp (*Cyprinus carpio*) with a *Vibrio anguillarum* bacterin: indications for a common mucosal immune system. Dev. Comp. Immunol. 10, 341–351. http://dx.doi.org/10.1016/0145-305x(86)90024-8.
Rombout Jan, J.H.W.M., Abelli, L., Picchietti, S., Scapigliati, G., Kiron, V., 2011. Teleost intestinal immunology. Fish Shellfish Immunol. 31, 616–626. http://dx.doi.org/10.1016/j.fsi.2010.09.001.
Salinas, I., 2015. The mucosal immune system of teleost fish. Biology (Basel) 4, 525–539. http://dx.doi.org/10.3390/biology4030525.
Salinas, I., Zhang, Y.-A., Sunyer, J.O., 2011. Mucosal immunoglobulins and B cells of teleost fish. Dev. Comp. Immunol. 35, 1346–1365. http://dx.doi.org/10.1016/

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024

j.dci.2011.11.009.
Savelkoul, H.F.J., Ferro, V.A., Strioga, M.M., Schijns, V.E.J.C., 2015. Choice and design of adjuvants for parenteral and mucosal vaccines. Vaccines 3, 148–171. http://dx.doi.org/10.3390/vaccines3010148.
Schuldt, N.J., Amalfitano, A., 2012. Malaria vaccines: focus on adenovirus based vectors. Vaccine 30, 5191–5198. http://dx.doi.org/10.1016/j.vaccine.2012.05.048.
Scott, C.L., Aumeunier, A.M., Mowat, A.M., 2011. Intestinal CD103+ dendritic cells: master regulators of tolerance? Trends Immunol. 32, 412–419. http://dx.doi.org/10.1016/j.it.2011.06.003.
Selvaraj, V., Sampath, K., Sekar, V., 2006. Adjuvant and immunostimulatory effects of β-glucan administration in combination with lipopolysaccharide enhances survival and some immune parameters in carp challenged with Aeromonas hydrophila. Vet. Immunol. Immunopathol. 114, 15–24. http://dx.doi.org/10.1016/j.vetimm.2006.06.011.
Selvaraj, V., Sampath, K., Sekar, V., 2005. Administration of yeast glucan enhances survival and some non-specific and specific immune parameters in carp (Cyprinus carpio) infected with Aeromonas hydrophila. Fish. Shellfish Immunol. 19, 293–306. http://dx.doi.org/10.1016/j.fsi.2005.01.001.
Seo, J.Y., Chung, H.J., Kim, T.J., 2013. Codon-optimized expression of fish iridovirus capsid protein in yeast and its application as an oral vaccine candidate. J. Fish Dis. 36, 763–768. http://dx.doi.org/10.1111/jfd.12037.
Shin, Y.J., Kwon, T.H., Seo, J.Y., Kim, T.J., 2013. Oral immunization of fish against iridovirus infection using recombinant antigen produced from rice callus. Vaccine 31, 5210–5215. http://dx.doi.org/10.1016/j.vaccine.2013.08.085.
Shoemaker, C.A., Klesius, P.H., Evans, J.J., Arias, C.R., 2009. Use of modified live vaccines in aquaculture. J. World Aquac. Soc. 40, 573–585. http://dx.doi.org/10.1111/j.1749-7345.2009.00279.x.
Shoemaker, C.A., Vandenberg, G.W., Désormeaux, A., Klesius, P.H., Evans, J.J., 2006. Efficacy of a Streptococcus iniae modified bacterin delivered using Oralject™ technology in Nile tilapia (Oreochromis niloticus). Aquaculture 255, 151–156. http://dx.doi.org/10.1016/j.aquaculture.2005.12.016.
Shoemaker, C.A., Klesius, P.H., 2014. Replicating vaccines. Fish Vaccin. 99–117. http://dx.doi.org/10.1007/978-3-0346-0277-8.
Sicinski, P., Rowinski, J., Warchol, J.B., Jarzabek, Z., Gut, W., Szczygiel, B., Bielecki, K., Koch, G., 1990. Poliovirus type 1 enters the human host through intestinal M cells. Gastroenterology 98, 56–58.
Siriyappagouder, P., Shankar, K.M., Naveen Kumar, B.T., Patil, R., Byadgi, O.V., 2014. Evaluation of biofilm of Aeromonas hydrophila for oral vaccination of Channa striatus. Fish Shellfish Immunol. 41, 581–585. http://dx.doi.org/10.1016/j.fsi.2014.09.021.
Skov, J., Kania, P.W., Holten-Andersen, L., Fouz, B., Buchmann, K., 2012. Immunomodulatory effects of dietary β-1,3-glucan from Euglena gracilis in rainbow trout (Oncorhynchus mykiss) immersion vaccinated against Yersinia ruckeri. Fish Shellfish Immunol. 33, 111–120. http://dx.doi.org/10.1016/j.fsi.2012.04.009.
Sommerset, I., Krossøy, B., Biering, E., Frost, P., 2014. Vaccines for fish in aquaculture. Expert Rev. Vaccines 4, 89–101.
Stroband, H.W., Debets, F.M., 1978. The ultrastructure and renewal of the intestinal epithelium of the juvenile grasscarp, Ctenopharyngodon idella (Val.). Cell Tissue Res. 187, 181–200.
Stroband, H.W.J., Kroon, A., 1981. The development of the stomach in Clarias lazera and the intestinal absorption of protein macromolecules. Cell Tissue Res. 215. http://dx.doi.org/10.1007/BF00239123.
Suanyuk, N., Itsaro, A., 2011. Efficacy of inactivated Streptococcus iniae vaccine and protective effect of β-(1,3/1,6)-glucan on the effectiveness of vaccine in red tilapia Oreochromis niloticus x O. mossambicus. Songklanakarin. J. Sci. Technol. 33, 143–149.
Sudo, N., Sawamura, S., Tanaka, K., Aiba, Y., Kubo, C., Koga, Y., 1997. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. J. Immunol. 159, 1739–1745.
Sun, Y., Hu, Y.H., Liu, C.S., Sun, L., 2012. A Streptococcus iniae DNA vaccine delivered by a live attenuated edwardsiella tarda via natural infection induces cross-genus protection. Lett. Appl. Microbiol. 55, 420–426. http://dx.doi.org/10.1111/j.1472-765X.2012.03307.x.
Sun, Y., Liu, C.S., Sun, L., 2010. Identification of an Edwardsiella tarda surface antigen and analysis of its immunoprotective potential as a purified recombinant subunit vaccine and a surface-anchored subunit vaccine expressed by a fish commensal strain. Vaccine 28, 6603–6608. http://dx.doi.org/10.1016/j.vaccine.2010.07.050.
Tafalla, C., Bøgwald, J., Dalmo, R.A., 2013. Adjuvants and immunostimulants in fish vaccines: current knowledge and future perspectives. Fish Shellfish Immunol. 35, 1740–1750. http://dx.doi.org/10.1016/j.fsi.2013.02.029.
Thim, H., Villoing, S., McLoughlin, M., Christie, K., Grove, S., Frost, P., Jørgensen, J., 2014. Vaccine adjuvants in fish vaccines make a difference: comparing three adjuvants (Montanide ISA763A Oil, CpG/poly I: C combo and VHSV Glycoprotein) alone or in combination formulated with an inactivated whole Salmonid Alphavirus antigen. Vaccines 2, 228–251. http://dx.doi.org/10.3390/vaccines2020228.
Thim, H.L., Iliev, D.B., Christie, K.E., Villoing, S., McLoughlin, M.F., Strandskog, G., Jørgensen, J.B., 2012. Immunoprotective activity of a Salmonid Alphavirus Vaccine: comparison of the immune responses induced by inactivated whole virus antigen formulations based on CpG class B oligonucleotides and poly I: C alone or combined with an oil adjuvant. Vaccine 30, 4828–4834. http://dx.doi.org/10.1016/j.vaccine.2012.05.010.
Tobar, I., Arancibia, S., Torres, C., Vera, V., Soto, P., Carrasco, C., Alvarado, M., Neira, E., Arcos, S., Tobar, J.A., 2015. Successive oral immunizations against Piscirickettsia Salmonis and infectious Salmon Anemia Virus are required to maintain a long-term protection in farmed salmonids. Front. Immunol. 6, 1–7. http://dx.doi.org/10.3389/fimmu.2015.00244.
Tobar, J.A., Jerez, S., Caruffo, M., Bravo, C., Contreras, F., Bucarey, S.A., Harel, M., 2011. Oral vaccination of Atlantic salmon (Salmo salar) against salmonid rickettsial septicaemia. Vaccine 29, 2336–2340. http://dx.doi.org/10.1016/j.vaccine.2010.12.107.
Toda, H., Saito, Y., Koike, T., Takizawa, F., Araki, K., Yabu, T., Somamoto, T., Suetake, H., Suzuki, Y., Ototake, M., Moritomo, T., Nakanishi, T., 2011. Conservation of characteristics and functions of CD4 positive lymphocytes in a teleost fish. Dev. Comp. Immunol. 35, 650–660. http://dx.doi.org/10.1016/j.dci.2011.01.013.
Toda, H., Shibasaki, Y., Koike, T., Ohtani, M., Takizawa, F., Ototake, M., Moritomo, T., Nakanishi, T., 2009. Alloantigen-specific killing is mediated by CD8-positive T cells in fish. Dev. Comp. Immunol. 33, 646–652.
Tu, F.P., Chu, W.H., Zhuang, X.Y., Lu, C.P., 2010. Effect of oral immunization with Aeromonas hydrophila ghosts on protection against experimental fish infection. Lett. Appl. Microbiol. 50, 13–17. http://dx.doi.org/10.1111/j.1472-765X.2009.02746.x.
Urán, P.A., Aydin, R., Schrama, J.W., Verreth, J.A.J., Rombout, J.H.W.M., 2008. Soybean meal-induced uptake block in Atlantic salmon Salmo salar distal enterocytes. J. Fish Biol. 73, 2571–2579. http://dx.doi.org/10.1111/j.1095-8649.2008.02091.x.
Valdez, Y., Brown, E.M., Finlay, B.B., 2014. Influence of the microbiota on vaccine effectiveness. Trends Immunol. 35, 526–537. http://dx.doi.org/10.1016/j.it.2014.07.003.
Vander Veen, R.L., Harris, D.L.H., Kamrud, K.I., 2012. Alphavirus replicon vaccines. Anim. Health Res. Rev. 13, 1–9. http://dx.doi.org/10.1017/S1466252312000011.
Villumsen, K.R., Neumann, L., Ohtani, M., Strøm, H.K., Raida, M.K., 2014. Oral and anal vaccination confers full protection against enteric redmouth disease (ERM) in rainbow trout. PLoS One 9, e93845. http://dx.doi.org/10.1371/journal.pone.0093845.
Vimal, S., Abdul Majeed, S., Nambi, K.S.N., Madan, N., Farook, M.A., Venkatesan, C., Taju, G., Venu, S., Subburaj, R., Thirunavukkarasu, A.R., Sahul Hameed, A.S., 2014. Delivery of DNA vaccine using chitosan-tripolyphosphate (CS/TPP) nanoparticles in Asian sea bass, Lates calcarifer (Bloch, 1790) for protection against nodavirus infection. Aquaculture 420–421, 240–246. http://dx.doi.org/10.1016/j.aquaculture.2013.11.017.
Wallace, K.N., Akhter, S., Smith, E.M., Lorent, K., Pack, M., 2005. Intestinal growth and differentiation in zebrafish. Mech. Dev. 122, 157–173. http://dx.doi.org/10.1016/j.mod.2004.10.009.
Wang, K., Deng, L., Huang, J., Fu, X., Chen, D., Geng, Y., 2011. Study on the immunogencity of poly (D, L-lactide-co-glycolide) (PLGA) microspheres-encapsulated vaccine preparation against Stenotrophomonas maltophilia infection in channel catfish (Ictalurus punctatus). Afr. J. Biotechnol. 10, 2751–2761. http://dx.doi.org/10.5897/AJB10.175.
Wang, M., Gao, Z., Zhang, Z., Pan, L., Zhang, Y., 2014. Roles of M cells in infection and mucosal vaccines. Hum. Vaccin. Immunother. 10, 3544–3551. http://dx.doi.org/10.4161/hv.36174.
Weber, E.S., 2014. A veterinary guide to the fish gastrointestinal tract. Vet. Clin. North Am. Exot. Anim. Pract. 17, 123–143. http://dx.doi.org/10.1016/j.cvex.2014.01.001.
Weiner, H.L., Wu, H.Y., 2011. Oral tolerance. Immunol. Res. 241, 241–259. http://dx.doi.org/10.1385/IR:28:3:265.
Weyts, F.A.A., Rombout, J.H.W.M., Flik, G., Verburg-Van Kemenade, B.M.L., 1997. A common carp (Cyprinus carpioL.) leucocyte cell line shares morphological and functional characteristics with macrophages. Fish Shellfish Immunol. 7, 123–133.
WHO, 2014. Adverse Events Following Oral Poliovirus Vaccine. Inf. Sheet Obs. Rate Vaccine React. Polio vaccines, pp. 1–5.
Wi, G.R., Hwang, J.Y., Kwon, M.-G., Kim, H.J., Kang, H.A., Kim, H.-J., 2015. Protective immunity against nervous necrosis virus in convict grouper Epinephelus septemfasciatus following vaccination with virus-like particles produced in yeast Saccharomyces cerevisiae. Vet. Microbiol. 177, 214–218. http://dx.doi.org/10.1016/j.vetmic.2015.02.021.
Wise, D.J., Greenway, T.E., Byars, T.S., Griffin, M.J., Khoo, L.H., 2015. Oral vaccination of Channel catfish against Enteric Septicemia of Catfish using a live attenuated Edwardsiella ictaluri isolate. J. Aquat. Anim. Health 27, 135–143. http://dx.doi.org/10.1080/08997659.2015.1032440.
Wolf, A., Hodneland, K., Frost, P., Hoeijmakers, M., Rimstad, E., 2014. Salmonid alphavirus-based replicon vaccine against infectious salmon anemia (ISA): impact of immunization route and interactions of the replicon vector. Fish Shellfish Immunol. 36, 383–392. http://dx.doi.org/10.1016/j.fsi.2013.12.018.
Xu, Z., Takizawa, F., Parra, D., Gómez, D., von Gersdorff Jørgensen, L., LaPatra, S.E., Sunyer, J.O., 2016. Mucosal immunoglobulins at respiratory surfaces mark an ancient association that predates the emergence of tetrapods. Nat. Commun. 7, 10728. http://dx.doi.org/10.1038/ncomms10728.
Yasumoto, S., Kuzuya, Y., Yasuda, M., Yoshimura, T., Miyazaki, T., 2006a. Oral immunization of common carp with a liposome vaccine fusing Koi Herpesvirus antigen. Fish Pathol. 41, 141–145. http://dx.doi.org/10.3147/jsfp.41.141.
Yasumoto, S., Yoshimura, T., Miyazaki, T., 2006b. Oral immunization of common carp with a liposome vaccine containing Aeromonas hydrophila antigen. Fish Pathol. 41, 45–49. http://dx.doi.org/10.3147/jsfp.41.45.

Please cite this article in press as: Embregts, C.W.E., Forlenza, M., Oral vaccination of fish: Lessons from humans and veterinary species, Developmental and Comparative Immunology (2016), http://dx.doi.org/10.1016/j.dci.2016.03.024