Birkhäuser Advances in Infectious Diseases

Alexandra Adams *Editor*

Fish Vaccines

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Chapter 1
Fish Disease Causing Economic Impact in Global Aquaculture

Hamish D. Rodger

Abstract One of the main hurdles to sustainable finfish aquaculture in many regions has been the management and control of infectious disease. The most significant diseases of salmonid, carp, catfish, tilapia and marine finfish farming are considered in this chapter by viral, bacterial, parasitic and fungal group. The level of impact caused by disease and methods for control or management are outlined.

Introduction

As finfish aquaculture expands globally, in terms of numbers and biomass production, species diversification, geographic regions and rearing methods, the challenges faced by the sector from disease and health issues also diversify and emerge. Climate change and evolving fish husbandry may also contribute to the balance or imbalance of pathogen, host, and environment interaction with novel pathogens being observed or isolated annually and more familiar diseases emerging in different global regions and species. Many of these diseases or pathogens have no recommended treatments, vaccines or management methods established or developed and hence remain a significant hurdle for the economic viability of aquaculture in certain regions and species. For many of the established aquaculture species such as carp, tilapia, salmonids as well as some of the marine species (sea bass, sea bream, grouper), there are commercial vaccines for a limited number of diseases and authorised treatments for specific pathogens, although there is considerable variation from country to country even within a geographic region. Many of the diseases causing significant economic impact in aquaculture are viral conditions with no treatments available and vaccines, if developed, of only partial efficacy in protection. The bacterial, parasitic and fungal diseases also have examples that can cause major economic and welfare challenges to aquaculture globally, although there are a number of effective vaccines against bacterial diseases. In this chapter

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the viral, bacterial, parasitic and fungal diseases of most significant impact for global aquaculture are discussed and outlined. Non-infectious diseases can also cause significant health challenges in aquaculture; however, these are out with the scope of this communication. This chapter is organised by aquaculture species group in the first instance (salmonids, carp, tilapia, catfish and marine species) and then by pathogen group (viruses, bacteria, parasites and fungi) within each aquaculture species group, where appropriate.

Salmonids

Global salmonid production continues to increase annually year-on-year and remains in the top ten species in terms of volume but is the number one finfish species (Atlantic salmon, *Salmo salar*) in terms of economic value (FAO 2012).

Viral Diseases of Salmonids

Infectious salmon anaemia virus (ISAV) is an enveloped single-stranded RNA virus in the family *Orthomyxoviridae* and has been classified as the type species of the genus *Isavirus* (OIE 2012). There are genetic variants of ISAV with two major groups, one European and one North American group, plus a non-pathogenic suggested precursor, the non-deleted highly polymorphic region (HPR) or HPR0. The HPR0 ISAV strain appears to be present periodically in both healthy farmed and wild salmon in the majority of salmon-farming regions (OIE 2012); however, infection with an HPR-deleted ISAV strain can result in a severe anaemic disease resulting in high mortality which can continue for many months. The impact from outbreaks of clinical ISA has been highly significant in the past where the reduction in Atlantic salmon production in Chile declined from 400,000 tonnes in 2005 to just above 100,000 in 2010 and in the Faroe Islands where the decline was from 47,000 tonnes in 2004 to 12,000 tonnes in 2006 (Asche et al. 2010). In both cases the production has increased again (or surpassed previous levels) since the institution of improved biosecurity, marked changes in fish husbandry and increased surveillance.

Outbreaks of ISA have been predominantly recorded in Atlantic salmon (*S. salar*), although coho salmon (*Oncorhynchus kisutch*) in Chile have also been affected (Kibenge et al. 2001). Asymptomatic infections of farmed rainbow trout (*O. mykiss*) in Ireland as well as detection by RT-PCR in sea trout (*S. trutta*), pollack (*Pollachius virens*) and cod (*Gadus morhua*) have also been confirmed, although in the cases of the gadoid fish, these were in pens of salmon affected by clinical ISA (Kibenge et al. 2004).

ISA is predominantly a disease of marine-farmed Atlantic salmon, and mortality may start at a very low level in one pen but then can spread from pen to pen with

Fig. 1.1 Atlantic salmon affected by ISA presenting with dark red (congested) liver and numerous petechiae in the pyloric caecal fat

rising mortalities, and in the worst case, pens can reach 90 % cumulative mortality if no action is taken. Clinical signs of the disease include lethargy, pale gills, exophthalmia, ascites and sometimes haemorrhages in the eye or ventrum. Internally fish may present with blood-tinged ascitic fluid in the abdomen, petechiae in the viscera or diffuse dark red, almost black liver and splenomegaly as well as congestion of the gastrointestinal tract (Fig. 1.1). Packed red blood cell volume is often less than 10, and histopathology may reveal characteristic multifocal and confluent hepatic haemorrhage and necrosis distant from hepatic vessels. The diagnosis of ISA is based on the presence of clinical signs, pathological changes consistent with ISA with detection of ISAV in tissues by the use of ISAV-specific antibodies on fixed sections or tissue imprints, plus isolation of ISAV in cell culture or detection by RT-PCR.

Control of clinical disease in most countries is through culling and disinfection on either a pen-by-pen or farm basis and complimented by strict biosecurity, livestock movement restrictions and site or waterbody fallowing. There are no treatments for the disease; however, commercial vaccines are in use in Chile, Faroe Islands, Eastern Canada and to a limited extent in Norway.

Salmonid alphaviruses (SAVs) have emerged to become one of the most significant viral groups of pathogens affecting salmonid farming in Northern Europe. Salmonid alphaviruses are associated with the condition known as pancreas disease (PD), affecting predominantly marine-stage Atlantic salmon, and sleeping disease (SD) of mainly freshwater-reared rainbow trout (McLoughlin and Graham 2007). From the first descriptions of cases from 1976 in Scotland, PD now affects salmon in Ireland, Norway and Scotland, and SD affects trout throughout mainland Europe and the UK. PD can have a major impact on salmon farms with the Irish industry estimated to have experienced a loss of turnover of 35 million euro in 2003 to 2004 (Ruane et al. 2008) due to the virus. The number of sites diagnosed with PD in Norway has increased from 10 in 1999 to 137 in 2012 (NVI 2012). Direct costs associated with a PD outbreak in a site in Norway stocked with 500,000 salmon (vs. a similar site without the disease) have been estimated at 14.4 million Norwegian kroner (Aunsmo et al. 2010). Alphaviruses have a single-stranded RNA genome and are enveloped, and it has been demonstrated that the salmonid alphaviruses have at least six subtypes with some distinct geographical distributions (Fringuelli et al. 2008). SAV has also

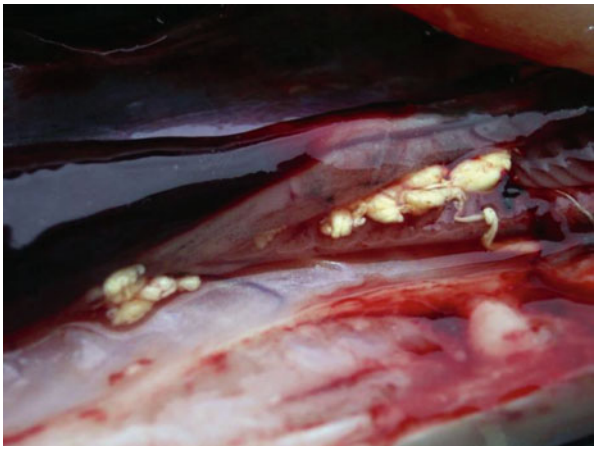

Fig. 1.2 Atlantic salmon affected by SAV infection and presenting with *pale yellow* casts in the lower intestine

been detected in wild flatfish fish distant from aquaculture operations off the coast of Scotland (SAV subtype 5) and Ireland (SAV subtype 1) (Snow et al. 2010; McCleary et al. 2014).

Clinical SAV infection in marine salmon causes lethargy, anorexia, tetany, variable mortality (from less than 1 % but up to 50 %) and in chronic cases the development of a subpopulation of poor condition, thin fish which have failed to thrive. In freshwater, trout mortalities can also be variable, and clinically high numbers of fish may appear lying on their sides (hence the use of the name sleeping disease) but following a period of weeks may fully recover with no mortalities. Internally fish can present with petechiae in the caecal fat in the early stages of the disease and cream, pale yellow to white-coloured casts in the intestine (Fig. 1.2). Histopathology in the early stages consists of acinar pancreatic necrosis, and in the majority of fish, the pancreatic tissue appears to regenerate; however, a population of fish are left with pancreatic fibrosis and no functional acinar tissue. Focal to diffuse myopathy of the myocardium is also present, usually concurrent with pancreatic pathology. Skeletal myopathies, initially in the red (aerobic) muscle fibres, then later in the white (anaerobic) bundles, develop to varying degrees. These myopathies may then have an impact on fillet quality, increasing further the economic impact of a case of PD in a farm (Lerfall et al. 2012).

Diagnosis is through a combination of clinical signs, gross pathology and laboratory tests including histopathology, serology, virology and RT-PCR. Routine monitoring of fish stocks in marine sites is often centred on monthly blood or tissue samples for SAV screening by virology and/or PCR. Control of the disease varies from country and region from Northern Norway where a cull and disinfect regime is in place to Ireland and Scotland where the virus appears endemic, and biosecurity and low-stress management can minimise the clinical impact. One commercial vaccine is available and in use in salmon farms in Ireland, Norway and Scotland.

Cardiomyopathy syndrome (CMS) was first described in farmed Atlantic salmon in Norway (Ferguson et al. 1990) and then subsequently in the Faroe Islands

Fig. 1.3 Atlantic salmon affected by CMS exhibiting dermal congestion and oedema

(Poppe and Seierstad 2003), Scotland (Rodger and Turnbull 2000) and Ireland (Rodger et al. 2013). The infectious nature of the disease has been demonstrated by Fritsvold et al. (2009), and a *Totivirus*, the piscine myocarditis virus (PMCV), has been identified as the infectious agent involved (Løvoll et al. 2010; Haugland et al. 2011). The disease primarily affects farmed Atlantic salmon in their second year at sea where it has a significant economic impact due to mortality associated with large-size fish (Brun et al. 2003). CMS has been one of the major diseases in Norwegian aquaculture for the last decade, and viral RNA can be detected in farmed fish for months without any signs of clinical disease (Wiik-Nielsen et al. 2012). Although the disease has only been described in farmed Atlantic salmon, histopathology consistent with CMS was described in wild Atlantic salmon (Poppe and Seierstad 2003), and, in a recent study, PCMV was detected and sequenced from wild Atlantic salmon in Norway (Garseth et al. 2012).

Moribund fish affected by CMS present with congestion and oedema in the skin, occasional exophthalmia (Fig. 1.3), lethargy and internally with blood-tinged ascites, purple to grey livers with diphtheritic fibrinous membranes, petechiae in caecal fat as well as swollen, blood-engorged atria of the heart and haemopericardium. Histopathology reveals a severe diffuse myopathy of the spongey layer of the heart ventricle and multifocal hepatic necrosis. Diagnosis is through clinical signs, gross pathology plus histopathology and RT-PCR. There is no treatment nor is there any commercial vaccine for the condition, and control is through biosecurity and early or accelerated harvest in significantly affected pens.

Heart and skeletal muscle inflammation (HSMI) was first observed in Norway in 1999 (Kongtorp et al. 2004a), and since then the number of cases diagnosed peaked at 162 per annum (pa) in 2007 and 2011 (NVI 2012) but has remained above 130 cases pa since 2007. HSMI is usually observed as a clinical disease in marine-stage salmon 5–9 months after transfer to sea. Affected fish exhibit anorexia, lethargy and increased mortality which can vary from less than 1–20 % in affected pens. The pathology associated with the disease appears limited to the

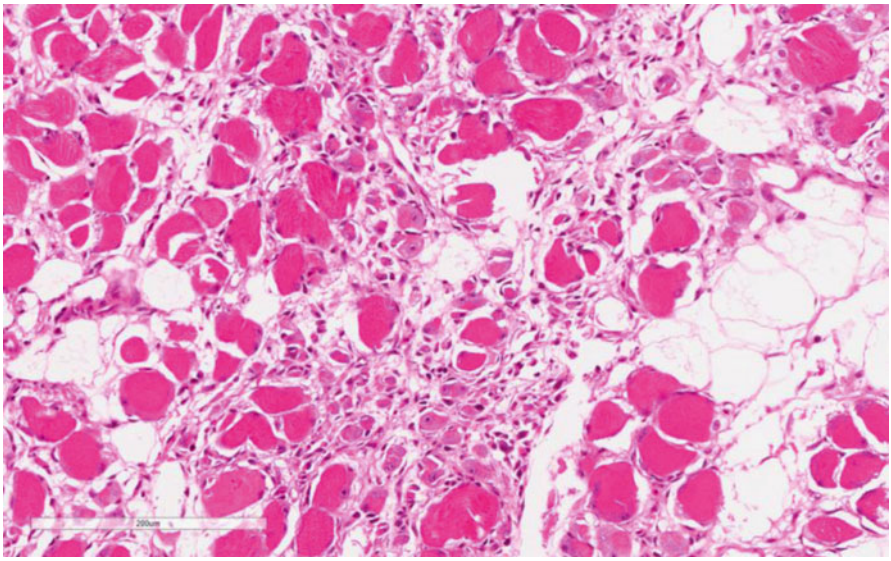

Fig. 1.4 Histopathological section of *red skeletal* muscle from Atlantic salmon affected by heart and skeletal muscle inflammation (HSMI)

heart and skeletal muscle where epicarditis, myocarditis, inflammation and degeneration of myocytes in the red skeletal muscle are the main findings (Fig. 1.4) (Kongtorp et al. 2004b).

HSMI has been demonstrated as an infectious disease and is strongly associated with the piscine *Orthoreovirus* (PRV), a recently discovered virus identified through high-throughput pyrosequencing of serum from HSMI-affected fish (Palacios et al. 2010). PRV is a double-stranded RNA virus and belongs to the *Reoviridiae* family and appears to be most close to the genus *Orthoreovirus*. HSMI as a disease has also been reported in Scotland (Ferguson et al. 2005) and Ireland but PRV appears widespread in farmed fish in Norway, Scotland, Ireland and Chile but is present in healthy farmed and wild salmon as well as the cases of HSMI in Norway and Scotland. As shown by Løvoll et al. (2012), the PRV load increases after transfer of smolts to sea, and the cases of HSMI in Norway are associated with high levels of virus, but the observations indicate that environmental factors associated with the seawater locations may be more important than PRV status. Recent research has also demonstrated that PRV resides in the erythrocytes of salmon with up to 50 % PRV positive in individual fish (Finstad et al. 2014). The appearance and investigations of PRV in the erythrocytes have shown strong similarities to the viral disease previously described as erythrocytic inclusion body syndrome (EIBS) (Leek 1987). There are no vaccines for HSMI nor any specific treatment, although there is one report of a reduction in impact of clinical disease through the feeding of tetradecylthioacetic acid (a synthetic fatty acid) (Alne et al. 2009).

Infectious pancreatic necrosis (IPN) is a highly contagious viral disease of young fish of salmonid species held under intensive rearing conditions. Susceptibility

Fig. 1.5 Salmon parr affected by IPN displaying a swollen intestine filled with mucus and catarrhal exudate

generally decreases with age and with resistance to the disease being reached at 1500° days except for Atlantic salmon smolts (Smail et al. 1992).

The first clinical sign in salmonid fry is frequently a sudden and usually progressive increase in daily mortality, particularly in the faster-growing individuals. Clinical signs include darkening pigmentation, a pronounced distended abdomen and a corkscrewing/spiral swimming motion. Cumulative mortalities may vary from less than 10 % to more than 90 % depending on a combination of various factors such as virus strain, host and environment. Internally the fish can display swollen intestine and catarrhal exudates in the lumen (Fig. 1.5). There may also be petechiae on the caecal fat and a pale liver. Histopathology of IPN affected fish involves focal necrosis of the acinar pancreatic tissue with necrotic areas replaced by a loose fibrous network and fat degeneration. Macrophages and leucocytes may infiltrate pancreatic and hepatic tissues. There may be necrosis and sloughing of the caecal endothelium. The causal agent is a double-stranded RNA virus of the family *Birnaviridae*, and there appear to be at least seven genogroups described so far based on molecular phylogenetic analyses (Munro and Midtlyng 2011). Histopathology and clinical signs can be diagnostic with confirmation conducted by cell culture and/or PCR. IPN historically emerged in North America and Europe and has been confirmed in most salmonids throughout the world; however, *Birnaviruses* appear to have a global distribution in both farmed and wild fish species and further in many non-salmonids and shellfish (Munro and Midtlyng 2011). There is a measurable antibody response to viral challenge, although in young fish this is limited. Commercial vaccines are available and are utilised in Norway, Scotland, Ireland and Chile. Prevention can be achieved by avoidance of fertilised eggs originating from IPN virus-carrier broodstock and the use of protected water supply. In outbreaks, a reduction in the stocking density may help reduce the overall mortality, or alternatively a short period of increased water temperature (>18 °C) also appears to be of benefit. Significant benefits have been observed through the use of genetics in recent years following the identification of two genomic quantitative trait loci (QTL) for IPN susceptibility or resistance (Houston et al. 2008).

Infectious haematopoietic necrosis (IHN) is an infectious disease, caused by a *Rhabdovirus*, of salmonids. It is of concern due to its clinical and economic consequences in trout and salmon farming and its effects in wild stocks. The disease was first reported in the early 1940s in North America (Pacific Rim states) but later spread to central and eastern USA, Canada, Japan and southern Europe. The economic impact from IHN can be very significant, and in the outbreak in British Columbia in 2001–2003, the cumulative mortality attributed to the disease in the 36 farms surveyed averaged 58 % with over 12 million Atlantic salmon lost either through mortality or culling (Saksida 2006).

Natural outbreaks of IHN are rare above 15 °C. Diseased fry are usually lethargic and hang at the areas of low water current. Whirling or flashing may also be seen. In older fish these signs may not be seen. Pale gills, dark skin, swollen abdomens, haemorrhages at the fin bases and opaque faecal pseudocasts trailing from the vent are frequently reported. Caecal fat petechiae and peritoneal haemorrhages may also be observed. Subdermal haemorrhage between the head and dorsal fin and spinal deformities in surviving sockeyes are quite common (Plumb and Hanson 2011). Histopathology of IHN-affected fish reveals multifocal degeneration and necrosis in the spleen and interstitial tissue of the kidney. Necrosis of the eosinophilic granular layer in the digestive tract is considered pathognomic. The aetiological agent is an enveloped RNA virus belonging to the family *Rhabdoviridae.* IHN affects members of the family *Salmonidae* in North America, Asia and Europe, but not in the Southern hemisphere, and in both fresh- and seawater. Reservoirs of IHNV are clinically infected fish and covert carriers from either cultured, feral or wild fish. The transmission of IHNV is horizontal and possibly vertical or egg associated. Strong antibody response in survivors is mounted to IHNV. Vaccination is widespread in salmon farming in British Columbia, Canada, since 2006, using a nucleic acid-based vaccine. Control methods in most countries for IHNV currently lie in official health surveillance schemes coupled with control policy measures. Thorough disinfection of eggs and incubation of eggs and rearing of fry and alevins in virus-free water supplies in premises completely separated from those harbouring possible virus carriers and free from possible contact with fomites are critical for preventing the occurrence of IHNV in a defined fish production site.

Viral haemorrhagic septicaemia (VHS) is an infectious disease caused by a cold-water *Rhabdovirus* which is of clinical and economic importance in rainbow trout and turbot farming in Europe. In North America VHS primarily affects wild fish species with several die-offs observed in recent years in the Great Lakes region with at least 28 species affected (OIE 2012).

Typical outbreaks result in acute to chronic disease among fingerling rainbow trout at temperatures generally below 14 °C. A wide range of possible disease signs are recorded including a profuse haemorrhaging, but in many fish a less dramatic pathology is noted. Fish may be lethargic and congregate at tank/pond sides or outlets, have pale gills, dark body colour, exophthalmos and in some cases intermittent periods of erratic spiralling swimming. Haemorrhage may be visible in the eyes and skin, within the muscle and internally in the viscera and intestine. In more chronic cases, some of the above signs may be obvious with abdominal distension due to oedema in visceral organs and ascites. The causal agent is an enveloped RNA virus belonging to the family *Rhabdoviridae* (Fig. 1.6), genus *Novirhabdovirus.* There

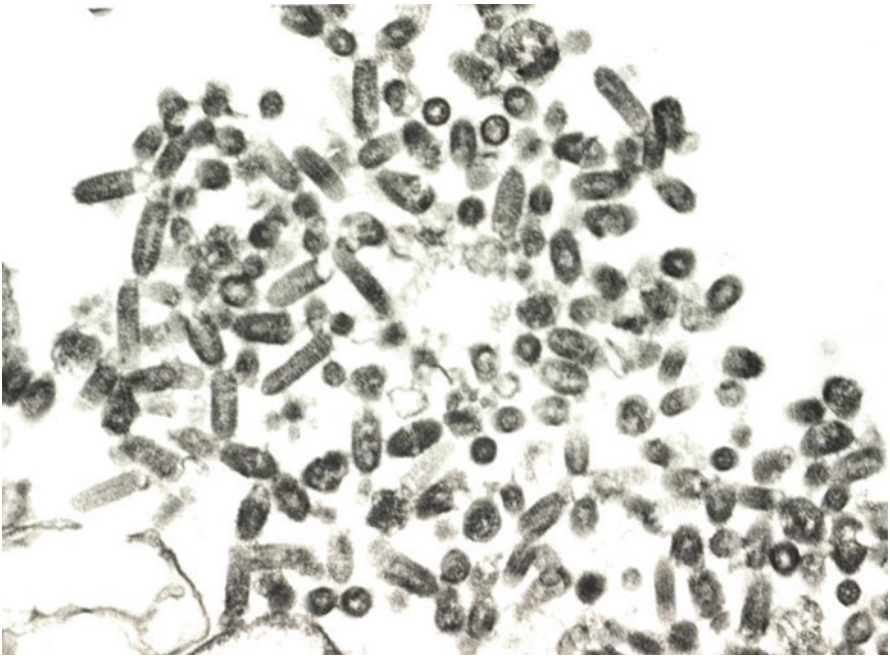

Fig. 1.6 Transmission electron micrograph of viral haemorrhagic septicaemia virus isolated from turbot (*Scophthalmus maximus*) in Scotland. Note the classical bullet-shaped virions (70–180 nm in size) (×43,000)

are four major genotypes (I to IV) of the virus, and these appear more associated with geographic origin than fish species; genotype I contains the European freshwater VHSV isolates and a group of marine isolates, and genotype IV contains the North American and Japanese/Korean isolates. Antibody response mounted to VHSV and fish serology could be of importance for detecting the carrier state among fish stocks, but has yet to be validated. Vaccine development has been ongoing for many years; however, no commercial vaccine is available. Control methods for VHS currently lie in official health surveillance schemes coupled with control policy measures, such as stamping-out procedures, and have resulted in eradication of the disease from several parts of Europe. Genetic approaches to selection of disease-resistant stock and intergeneric hybridisation are also being pursued.

Bacterial Diseases of Salmonids

Diseases caused by *Flavobacterium* spp. affect many farmed fish species in freshwater and conditions in salmonids are known as either:

(a) Bacterial coldwater disease (CWD), which is a serious septicaemic infection of hatchery-reared salmonids, also referred to as peduncle disease, is prevalent in

northwest American hatcheries during colder months of the year. *F. psychrophilum* is the bacterial species associated with this condition.

(b) Rainbow trout fry syndrome (RTFS) or rainbow trout fry anaemia is a freshwater systemic disease affecting trout (and to a lesser extent salmon) in Europe that results in high mortalities. *F. psychrophilum* is the causal agent of this disease.

(c) Bacterial gill disease which is commonly observed in freshwater salmonids and is associated with *F. branchiophilum*.

(d) Columnaris disease as induced by *F. columnare* is usually associated with warm water (20–25 °C) but can be observed in trout at lower temperatures (12–14 °C) (Starliper and Schill 2011).

CWD was first described in the USA in rainbow trout; however, juvenile coho salmon appear most susceptible. RTFS was described throughout Europe in the 1990s where due to its level of impact and persistent nature it has risen to become the most important disease problem for freshwater rainbow trout farming in Europe (Starliper and Schill 2011). Clinical signs of CWD include haemorrhage at the base of fins, pale gills, haemorrhagic ulceration in the muscle and tail rot. The disease usually appears in the spring with water temperatures 4–10 °C. If alevins are affected by yolk-sac erosions, mortalities can be 30–50%. Coagulated yolk sac may precede the disease. In RTFS high mortalities occur in trout fry with pale gills, swollen spleens, with blood-tinged caecal fat around the spleen, lethargy, darkened skin, ascites and exophthalmos (Fig. 1.7). Skin ulcerations or eroded/dissolving jaw may present in older affected fish. Bacterial gill disease presents with mortality and pale patches on the gills. These bacteria are Gram negative and filamentous and require extended growth (14 days) on Anaker and Ordal's media (or equivalent low-nutrient agar) at 15 °C. The diagnosis is based on clinical observations, fresh microscopy and histopathology with biochemical or serological characterisation of the isolated bacteria.

Natural reservoirs of the bacteria are uncertain; however, the disease can be transmitted vertically and horizontally. The bacterium is very robust, resisting some disinfectants which are normally used for egg cleaning (iodophors). Protection has

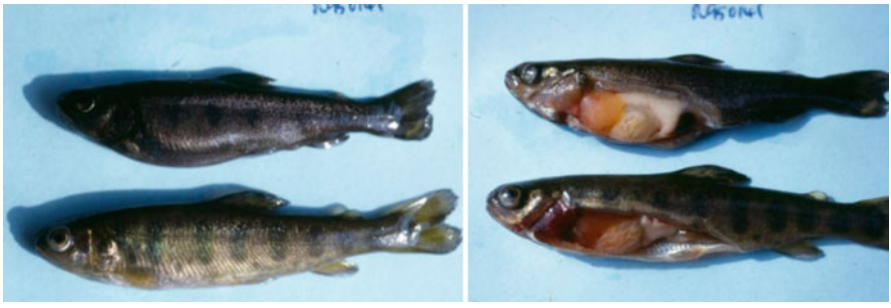

Fig. 1.7 Rainbow trout affected by *Flavobacterium psychrophilum* infection (*top fish in both images*) displaying dark colouration and abdominal swelling (*left upper*) and pale gills and swollen spleen typical in RTFS (*right upper*) with healthy trout for comparison

been demonstrated using a bacterin administered by injection or immersion. Autogenous vaccines are in use in some farm sites and although early stage development commercial *Flavobacterium* sp. vaccines are available in some countries, in general the level of protection afforded by these is less than that seen with other bacterial fish vaccines. Broad-spectrum antibiotics have been partially ineffective in controlling some outbreaks, but improving the environment through clearing a site (or part site) of livestock, deep cleaning and disinfection before restocking has shown benefits. Florfenicol appears effective at recommended dose regimes but other antibiotics have required to be used at three or four times recommended dose to demonstrate efficacy.

Bacterial kidney disease (BKD) is a serious disease of fresh- and seawater, farmed and wild salmonids that results in an acute to chronic systemic granulomatous disease. Those fish severely affected by the disease may show no obvious external signs or may show one or more of the following: pale gills, exophthalmia, abdominal distension (due to ascites), skin blisters (filled with clear or turbid fluid), shallow ulcers (the results of broken skin blisters), haemorrhages (particularly around the vent) and, more rarely, cavitations in the musculature, filled with blood-tinged caseous or necrotic material. Internally, there may be fluid in the abdomen, varying haemorrhage on the abdominal walls and viscera, a membranous layer on one or more of the visceral organs and, most characteristically, creamy-white granulomatous lesions in the kidney and less frequently in the liver and spleen (Fig. 1.8). Pacific salmon seem more susceptible to BKD than Atlantic salmon, and the granulomas are well encapsulated in the latter but less so in the former. The histopathology is chronic granulomatosis, principally of haematopoietic tissue, but extends to the liver, cardiac and skeletal muscle or indeed any organ. The granuloma is often large, with a central caseous zone bounded by epithelioid cells and infiltrating lymphoid cells. The presence of a capsule is variable, and a lack of encapsulation is often associated with more aggressive infections. The causal agent *Renibacterium salmoninarum* is a small, Gram-positive diplococcus that grows best at 15–18 °C and not at all at 25 °C (Evelyn 1993). It has a requirement for cysteine and serum or serum substitutes in bacteriological media. The diagnosis is based on clinical signs, Gram's smear, ELISA, FAT, IFAT, histopathology, isolation

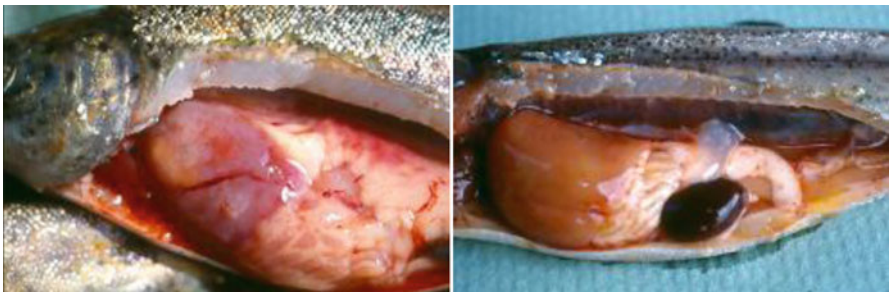

Fig. 1.8 Rainbow trout affected by BKD exhibiting ascites and diphtheritic membrane on the liver (*left*), and swollen spleen, ascites and petechiae in visceral fat (*right*)

(2–3 weeks at 15 °C) in cysteine-enriched media such as kidney disease medium (KDM2) or selective kidney disease medium (SKDM) with agglutination tests and PCR (Fryer and Saunders 1981).

Salmonids are clinically susceptible, especially the genus *Oncorhynchus* (Pacific salmon and rainbow trout), and the disease is reported in North America, Japan, Western Europe and Chile. No vaccines are commercially available, but are greatly needed. There is evidence that under some conditions the pathogen elicits an immune response in fish, and there are some reports of experimental vaccination. The protective ability of the vaccine is questionable, however, and one of the problems is the intracellular nature and vertical transmission of the agent. Chemotherapy (erythromycin) provides limited and only temporary relief. The bacteria can survive and multiply within phagocytic cells. Screening of farmed broodstock and regular testing of growing stock for agent combined with disinfection and movement controls have proven effective in the Europe.

Enteric redmouth disease (ERM) is a bacterial septicaemic condition of farmed salmonids, in particular the rainbow trout. There have been more recent reports of disease in channel catfish (*Ictalurus punctatus*). The disease was first associated with losses in rainbow trout in the Hagerman Valley, Idaho in the 1950s. A killed cell vaccine was in use before the organism was assigned a formal name in 1978. The disease is widespread in the USA and European trout farms with over 80 % estimated as affected. It is also present in Australia, South Africa and Chile; however, there are some variations in serotypes and biotypes of the bacteria (Barnes 2011). Gross external signs first described were lethargy, skin darkening and congestion around the mouth and operculum and at the base of the fins. Other signs seen include exophthalmos, ulceration and cutaneous petechiae. Internally the fish show signs of haemorrhagic septicaemia with congestion and petechiae throughout the peritoneum and visceral organs, in particular the caecal fat. Splenomegaly and fluid-filled stomach and intestine are also observed. *Yersinia ruckeri* is the causal agent, and the Gram-negative, motile rod-shaped bacterium is catalase positive and oxidase negative. Several serotypes have been identified including that described previously as EX5 and now as biotype 2 which appears widespread in Europe (Austin et al. 2003; Wheeler et al. 2009). Gross and histological signs are helpful but confirmation requires isolation on general nutrient agar (24 h at 22 °C) such as TSA or BHI. FAT and ELISA tests have also been used but bacterial isolation is necessary for antibiotic sensitivity. Authorised vaccines are in use globally. Protection with dip or immersion vaccines protects for up to 6 months. Broad-spectrum antibiotics are effective in controlling an outbreak, but increasingly antibiotic resistance is observed and sensitivity testing should be undertaken.

Furunculosis is a fatal epizootic disease, primarily of salmonids, caused by the bacterium *Aeromonas salmonicida*. This organism can also cause clinical disease in other fish species where it is named ulcer disease or carp erythrodermatitis. With the growth of salmon farming, particularly in Scotland and Norway in the 1980s, its effects were described in the marine environment where it became the dominant infectious disease until the development of commercial fish vaccines and management changes (Bernoth et al. 1997).

Fig. 1.9 Atlantic salmon post-smolts affected by typical furunculosis with dermal haemorrhage and liquefactive haemorrhagic lesions in the muscle

In a population affected by typical furunculosis, there will be examples of both chronic and acute forms of the disease. High mortalities, without external signs of infection, are often associated with acute furunculosis, although anorexia may be present. Other fish may appear dark in colour and lethargic with reddening at the fin bases. Internally there may be widespread petechiae in the viscera and a swollen spleen. In chronic furunculosis, usually seen in older fish, there may be similar clinical signs to a subacute form but with attempts at repair in the tissues. Liquefactive, haemorrhagic lesions may be present in the musculature with bloody discharge from the vent, and splenomegaly is also present (Fig. 1.9). Atypical furunculosis may cause lower-level mortalities and small skin ulcers with a dark, pigmented periphery. *Aeromonas salmonicida* is a Gram-negative, nonmotile short rod, and most strains produce a brown diffusible pigment on agar containing tryptone. Atypical furunculosis is caused by a slower-growing non-pigmenting isolate *A. salmonicida* ssp. *achromogenes.* Gross and histological signs are helpful for diagnosis but confirmation requires isolation on general nutrient agar (24–48 h at 22 °C) such as TSA or BHI, and isolation is vital for antibiotic sensitivity. Salmonids are principally affected in Europe and North America in both fresh- and seawater. Cyprinids (carps) and ornamentals are also affected in Europe and the USA where the disease manifests as skin ulceration. Salmonids (wild and farmed) can carry the organism, and when these fish are stressed, such as with high water temperatures or low oxygen levels, then clinical disease can break. Pioneering work started in 1940s on vaccines has now resulted in effective injectable oil-based vaccines, which are

widely used by the salmon-farming industry. Presmolts are usually injected 6–10 weeks prior to transfer to sea, and these vaccines provide protection for up to 12 months. Broad-spectrum antibiotics are effective in controlling an outbreak, but the increasing antibiotic resistance is observed, and sensitivity should be tested.

Piscirickettsiosis is a disease of salmonids caused by *Piscirickettsia salmonis* and is a significant disease problem in farmed marine salmonids. It was first reported in coho salmon in Chile in 1989 and remains a significant health challenge in salmon farming in that country. It has also been confirmed in northern hemisphere salmon-farming countries (Norway, Canada, Scotland and Ireland) where it is associated with a much lower clinical impact.

Clinical signs of the disease include skin lesions, dark skin, lethargy, anorexia, nervous signs in some cases and internally petechiae, peritonitis, ascites, white nodules in the liver and kidney (Fig. 1.10). Histopathology includes extensive necrosis of the haematopoietic tissues particularly the kidney with oedema, some fibrosis and an influx of inflammatory cells. Haemorrhage may be present in visceral organs, musculature and intestinal tract. Meningitis also reported in various fish species. Rickettsia may be observed within membrane-bound vacuoles using H & E or Giemsa stains in visceral organs or white blood cells. *Piscirickettsia salmonis* is a Gram-negative, acid-fast, nonmotile, spherical to coccoid, non-capsulated (although often pleomorphic) organism (Fryer and Lannan 1996). Diagnostic confirmation may be via immunohistochemistry, isolation in cell culture and PCR. Salmonids, particularly Pacific salmon, appear vulnerable, and in Chile the economic impact can be significant with over 10 million coho salmon mortalities attributed to piscirickettsiosis (estimated value of US$49 million) in 1995 (Smith et al. 1997). A variety of *P. salmonis* vaccines are used in Chile but oral broad-spectrum antibiotic therapy is also widely used.

Vibriosis is the term most commonly used to describe infections associated with *Vibrio anguillarum*, but *V. ordalii* and other *Vibrio* spp. may cause similar clinical

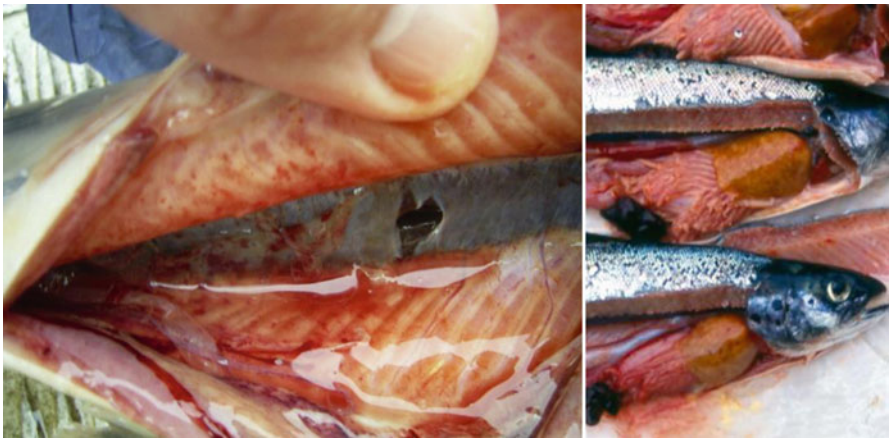

Fig. 1.10 Atlantic salmon affected by piscirickettsiosis exhibiting haemorrhage in the peritoneum and ascites (*left*), swollen spleens, congested caeca and mottled livers (*right*)

signs in wild and farmed fish in many parts of the world. *Vibrio anguillarum* was the first *Vibrio* isolated from fish, these being from eels in the Mediterranean. It is now known that there are many different *Vibrio* species in different parts of the world, and different species can cause significant and similar disease problems. In salmon mariculture, *V. anguillarum*, *V. ordalii* and *V. salmonicida* have proven to be the most seriously pathogenic (Hjeltnes and Roberts 1993).

Acutely affected fish are usually anorexic with pale gills and occasional periorbital oedema, and these signs correspond with rapidly rising mortalities. The fish may appear dark in colour, and dermal or subdermal skin lesions may ulcerate and release haemorrhagic fluid which contains numerous bacteria. Internally ascites with petechiae in the musculature and viscera are common. The genus *Vibrio* consists of Gram-negative, straight or slightly curved rods which are motile. Colony morphology, biochemical tests and the use of diagnostic keys will confirm the family *Vibrionaceae*. The organisms can be isolated on general nutrient agar plus sodium chloride (1.5 %). The diagnosis is based on a combination of clinical observations and biochemical or serological characterisation of the isolated bacteria (48 h at 20 °C for *V. anguillarum*, or 15 °C or less for *V. salmonicida*). Most marine species are susceptible to *Vibrio* sp., and wild fish carry the organisms. Killed commercial vaccines are available for *V. anguillarum*, *V. salmonicida* and *V. ordalii.* The injectable, oil-based vaccines are widely used and have been demonstrated to be effective, and immersion vaccines are also in use. Broad-spectrum antibiotics are effective in controlling an outbreak, but increasing antibiotic resistance is observed and sensitivity should be tested.

Moritella viscosa (previously known as *Vibrio viscosus*) is considered the main agent associated with winter ulcer which primarily affects marine salmonids at temperatures <10 °C (Løvoll et al. 2009). It causes severe skin ulceration (Fig. 1.11), septicaemia, low-level mortalities (usually less than 10 %) and downgrading at harvest; however, it remains the most significant bacterial disease in Norwegian salmon farming despite the widespread use of vaccines. Approximately 50 % of all antibiotic prescriptions in Norway can be attributed to efforts to control winter ulcer

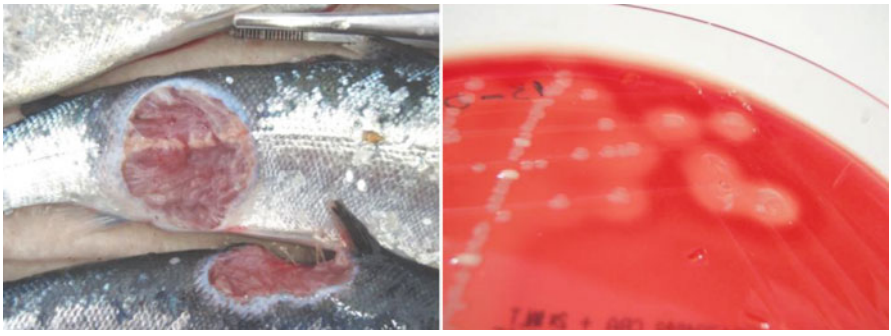

Fig. 1.11 Atlantic salmon affected by winter ulcer (*left*) and *M. viscosa* isolated on blood agar with 2 % salt demonstrating haemolytic zones surrounding cream-coloured colonies (*right*)

(Løvoll et al. 2009). The organism is a Gram-negative curved bacillus which grows on blood agar with 2% sodium chloride with a haemolytic zone around the cream-coloured colonies (Fig. 1.11). The disease is observed in salmonid farming throughout Northern Europe and Canada.

Parasitic Diseases of Salmonids

Sea lice have been a challenge to the culture of marine-farmed salmon in certain regions since husbandry began. The species of lice involved in marine salmonid farming are parasitic copepods and include *Lepeophtheirus salmonis*, the salmon louse which is circumpolar in the northern hemisphere and can occur on all salmonid species; *Caligus elongatus*, which is global in distribution and affects over 80 species of fish, and *C. rogercresseyi* which is widespread in southern Chile. Sea lice can affect the growth, reproduction and survival of the fish they infest. They graze on the skin and fins of the salmon and cause direct tissue damage. In addition the indirect effects of immunosuppression allow other pathogens to gain access to, or manifest more fully in, the fish, and there have been recorded associations or co-infections with other pathogens, such as the salmonid alphavirus (Rodger and Mitchell 2007), *P. salmonis* (Lhorente et al. 2014) and infectious salmon anaemia virus (Valdes-Donoso et al. 2013), in infested fish.

A recent review has calculated that sea lice cost the salmon-farming industry 10–20 cents/kg produced and that a large percentage of the cost (17–30%) is down to the purchase cost of the parasiticides. However, other significant costs include reduced fish growth, increased food conversion ratios, reduced marketability due to skin damage, stress and mortalities on the fish as a result of treatments, negative publicity as a result of infestations and losses due to secondary infections and fish mortality (Costello 2009). Regional estimates for the cost of sea lice ranged from 7 to 10% of production value for Scotland (Rae 2002) to approximately 3% for Ireland (which equates to 11 cents/kg produced) and 4% of production value for Atlantic Canada (Mustafa et al. 2001), and in the absence of effective treatment measures, the cost to the industry has been considered to be at least four times more. In Scotland the current impact of sea lice on the salmon-farming sector is estimated to be in the region of £30 million (€35.3 million) (Webster, personal communication). Using FAO production statistics for 2006, the total marine salmonid production was 1.7 million tonnes of fish which was worth US$8.4 billion. Medical treatment of salmon with parasiticides is widespread; however, where medical treatments are relied on for repeated treatments, the sea lice population will eventually develop resistance or tolerance of the chemicals, as has occurred with other terrestrial parasites. No commercial vaccines for sea lice exist although this has been the focus for research for over 20 years (Raynard et al. 2002).

Amoebic gill disease (AGD) has remained the most serious health and welfare challenge for marine-farmed salmonids in Australia since the 1980s, and the control of this disease contributes at least an additional 10–20% to the cost of production

Fig. 1.12 Amoebic gill disease (AGD) affected Atlantic salmon with white mucoid-like patches on the gills

for the Atlantic salmon-farming industry in Tasmania (Nowak 2012). AGD re-emerged in Europe in 2011 and 2012 to be the most serious infectious health challenge for the marine salmon-farming industry with Ireland, France, Norway and Scotland all affected (Rodger 2014). AGD can cause lethargy, loss in growth and high mortalities of up to 10 % of livestock per week reported in cases in Tasmania in the 1980s if untreated.

The aetiological agent of AGD is the protist *Neoparamoeba perurans*, a recently described parasitic and free-living amoeba (Young et al. 2008). Crosbie et al. (2012) cultured the pathogenic species of amoeba and were able to induce AGD from laboratory cultures for the first time, demonstrating that *N. perurans* was the causal agent of the disease. Presumptive diagnosis of AGD is based on the typical clinical signs (lethargy, elevated body position in the water column, increased respiratory rate and increased range of opercular movement with white or grey mucoid spots or patches on the gill surface) (Fig. 1.12) and the presence of amoeba on fresh gill smears by microscopy. Confirmation of AGD is through histopathology, where the criteria of hyperplasia, lamellar fusion, the presence of vesicles and the presence of amoeba (with parasomes) are considered the basis for the histological case definition. RT-PCR screening is also employed for surveillance. Treatment and control in Australia involve regular monitoring of gross gill appearance and prophylactic baths of the fish with freshwater for two to three hours. Fish in some regions of Tasmania may be treated up to 15 times during their marine grow-out cycle (S. Percival, personal communication). A plethora of alternative bath and in-feed remedies have been investigated but freshwater remains the treatment of choice in Australia. No commercial vaccine is available although screening of candidate antigens has been undertaken by various groups (Nowak 2012); however, a significant heritable component in AGD resistance has been demonstrated in Atlantic salmon in Tasmania.

Kudoa thyrsites is a cosmopolitan marine myxosporidean parasite which can result in post-mortem myoliquefaction and has resulted in an economic cost to Atlantic salmon producers in British Columbia, Canada, estimated at 50 million

Canadian dollars in lost revenues in 2002 alone (Funk et al. 2007). The control of this parasite presents many challenges to producers, and there is no effective treatment, and no vaccine. *K. thyrsites* is common in wild fish and there appears to be little host specificity.

Carp

Global production of cyprinid or carp species dominates finfish aquaculture production with China, India and Bangladesh highlighted as major carp-producing countries (FAO 2012). The main carp species farmed include grass carp (*Ctenopharyngodon idellus*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Hypophthalmichthys nobilis*), Indian major carp (*Cirrhinus mrigala*, *Catla catla*, *Labeo rohita*) and common carp (*Cyprinus carpio*). Infectious disease has a significant impact in some regions, and these can be grouped as viral, bacterial, parasitic or fungal in origin.

Viral Diseases of Carp

Grass carp haemorrhagic virus or grass carp reovirus (GCR) affects the aquaculture of *C. idellus* in China. Over 3.6 million tonnes of grass carp have been produced per annum in China since 2006; however, some farms may experience mortalities of 30–70 % as a result of this virus (Lu et al. 2011). Affected carp exhibit haemorrhages at fin bases, in the skin and mouth and have exophthalmia and pale gills. Internally they may have a dramatically haemorrhagic intestinal tract with further haemorrhaging in the viscera and muscle. The causative agent, an *Aquareovirus*, can be isolated in cell culture and detected by RT-PCR, and there are several serotypes. Most outbreaks occur during periods of elevated water temperature (25–28 °C) and affect fry and yearling fish. Autogenous killed vaccines are in use, and subunit vaccines have been developed (Jiang 2009; Lu et al. 2011).

Spring viraemia of carp (SVC) is an acute haemorrhagic and contagious viral infection, typically of cyprinids and more specifically of the common carp, in which the disease usually erupts in spring and causes mortality of the adults as well as the young. Viral outbreaks are often complicated with secondary bacterial infections; however, signs attributed only to the virus are as follows: lethargy, distended abdomen, petechiation on gills and the skin and around the eyes, oedematous vent and trailing mucoid casts, exophthalmia and internally ascites with focal haemorrhages in swim bladder and other visceral organs.

A *Rhabdovirus* named spring viraemia of carp virus (SVCV) is the aetiological agent and although traditionally considered a European virus has now been confirmed in North America, China and Brazil (Plumb and Hanson 2011). The impact of farms through mortality can be significant; however, the effect on trade will also be significant as SVC is notifiable in many regions.

Carp can mount an immune response to SVC, but response is influenced by temperature, route of immunisation, quantity, strain of virus and age and condition of the host. Vaccines have been developed but are not in commercial use. Control methods for SVC currently lie in official health surveillance schemes coupled with control policy measures, such as stamping-out procedures, and have resulted in eradication of the disease from several parts of Europe.

Koi herpes virus disease (KHV) is a herpesvirus infection capable of inducing a contagious and acute viraemia in common carp and varieties such as koi and ghost carp. The first reports of the disease were in Israel, the USA and Germany in the late 1990s, since when the disease is now known to have spread globally, predominantly with the trade in koi. All age groups of fish can be affected although younger fish are most susceptible and mortalities can be very significant. The skin appears pale or congested with a roughened appearance, and gills can appear pale. Gills may have necrotic patches present. The eyes appear sunken (enophthalmia), and haemorrhages may be obvious on the skin and base of fins with fin erosion. Fish appear lethargic, lose body coordination and may also show signs of hyperactivity. Koi herpesvirus (KHV) is in the family *Herpesviridae*. It is also known as cyprinid herpesvirus 3 (CyHV-3). Histopathology and clinical signs can be diagnostic with confirmation via PCR, IFAT or cell culture. KHV affects common carp and varieties such a koi, ghost carp and hybrids of these varieties and is present throughout Europe, including the UK, Asia and the USA. A live attenuated vaccine is currently licensed in Israel and is widely used in carp farms there. Methods to control and prevent disease should mainly rely on avoiding exposure to the virus coupled with good hygiene and biosecurity (Pokorova et al. 2005).

Bacterial Diseases of Carp

Bacterial haemorrhagic septicaemic conditions due to the bacterial species *Aeromonas* spp., *A. salmonicida* and *Y. ruckeri* are commonly encountered in cyprinid farming globally. Atypical *A. salmonicida* infections present as ulcer disease in carp, as in salmonids (Fig. 1.13), and *Y. ruckeri* is a significant challenge in silver carp farming. Infections with *Aeromonas* spp. in many cases may be as secondary opportunistic pathogens following stress or trauma.

Fungal Diseases of Carp

Saprolegniasis is the term most commonly used to describe infections in fish and fish eggs associated with the water mould of the fungus *Saprolegnia* spp. Lesions are focal, grey-white patches on the skin or gills of fish which, when examined underwater, have a cotton wool-like appearance where the hyphal filaments extend out into the water. The early lesions are often almost circular and grow by radial

Fig. 1.13 Goldfish (*Carassius auratus*) presenting with dermal ulceration as a result of *Aeromonas salmonicida* infection

Fig. 1.14 Roach (*Rutilus rutilus*) infected with *Saprolegnia* sp. fungi exhibiting brown fungal plaques on the skin and dermal haemorrhage

extension around the periphery until lesions merge. At this later stage, the patches are often grey or brown in colour as mud or silt becomes trapped by the mycelium (Fig. 1.14). Gills, mouth or branchial cavity can also be affected. Internal infections in the peritoneum or gastrointestinal tract in younger fry can also be seen and results in high mortalities. Freshwater fish eggs are also very prone to infection.

Saprolegnia parasitica-diclina complex is both a saprotroph and a necrotroph, typically feeding on waste from fish or other dead cells. Where they inhabit a live animal, the fungal infection is known as a mycosis. They are widely distributed in the freshwater aquatic habitat, produce motile biflagellate spores and undergo asexual reproduction by means of zoospores, produced in zoosporangium. The diagnosis is based on clinical signs, fresh smears for microscopy and histopathology. It is global and ubiquitous in distribution, affecting many temperate, freshwater fish species and their eggs. *Saprolegnia* sp. fungi are ubiquitous in freshwater

Fig. 1.15 Indian major carp affected by epizootic ulcerative syndrome (EUS) presenting with extensive *brown-black* haemorrhagic lesion on the flanks

and will tolerate brackish water and even moist soil. It has long been considered that the fungi responsible for saprolegniasis are secondary pathogens, and lesions are commonly seen after handling or trauma which abrade the skin and allow invasion by fungi. Overcrowding and poor water quality may also give rise to infection (Roberts 2012).

Epizootic ulcerative syndrome (EUS) is considered to be an infection with the oomycete known as *Aphanomyces invadans* and is characterised histologically by penetrating hyphae surrounded by granulomatous inflammation. It is an epizootic condition of wild and farmed freshwater and estuarine fish. EUS is also known as red spot disease (RSD), mycotic granulomatosis (MG) and ulcerative mycosis (UM). The first outbreak is considered to have been in Japan in 1971, and at least 24 countries in four continents are now known to be affected. Red spots may be observed on the body surface, head, operculum or caudal peduncle. Large, red or grey shallow ulcers, often with a black-brown necrosis, are observed in the later stages (Fig. 1.15). EUS causes mortality in farmed and wild fish worldwide. Around 94 species of fish have been confirmed by histopathological diagnosis to be naturally infected by EUS (OIE 2012). Some species including common carp (*Cyprinus carpio*), tilapia (*Oreochromis niloticus*) and milkfish (*Chanos chanos*) appear naturally resistant. The disease is widespread in Asia, North America, Southern Africa and Australia. There is no protective vaccine, and no effective treatment although to minimise fish losses in infected ponds water exchange should be stopped and lime or hydrated lime and/or salt be applied.

Bacterial and Viral Diseases in Tilapia

Tilapia (various species of *Oreochromis*, *Sarotherodon* and *Tilapia*) are now the third largest group of fish in terms of global aquaculture production (after carp and salmon) with over 3.5 million tonnes farmed in 2010 (FAO 2012). China, Egypt, Indonesia and the Philippines are the world's largest producer countries, and there are a number of bacterial diseases that pose some challenges for tilapia production.

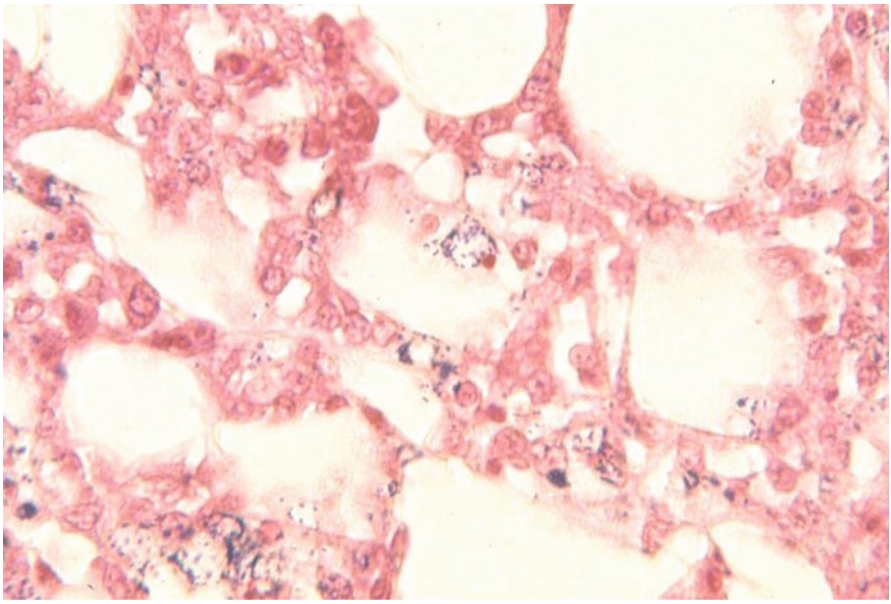

Fig. 1.16 Histological section of retrobulbar tissue in tilapia (*Oreochromis* sp.) affected by streptococcosis showing Gram-positive bacteria (*purple staining*) scattered throughout (Grams ×400)

Streptococcosis is a global disease problem in a variety of farmed and wild fish species in freshwater, estuarine and marine environments. *Streptococcus iniae* is the main species involved and is a Gram-positive (Fig. 1.16) facultative anaerobe which often occurs in long chains of cocci. *S. agalactiae* has also emerged as a pathogen for tilapia. Affected fish have exophthalmia, petechiae and congestion at fin bases (Amal and Zamri-Saad 2011). Histologically lesions are principally intravascular leading to meningitis, peritonitis and pericarditis. The disease has become particularly significant in recirculation units. Broad-spectrum antibiotic therapy can assist in short-term treatment, and commercial vaccines have been developed.

Francisellosis is the term used to describe infection associated with *Francisella noatunensis* which has emerged as a major pathogen of farmed cod as well as *F. noatunensis* subsp. *orientalis* (also termed *F. asiatica*) in tilapia species (Birkbeck et al. 2011). Affected fish appear emaciated and may have raised haemorrhagic nodules in the skin, uni- or bilateral ocular pathology including opacity and corneal perforation. Internally there may be minor or extensive white, partly protruding nodules in the spleen, heart, kidney and liver. The kidney and spleen may be swollen, and a thickened intestinal mucosa may also be present. Sero-haemorrhagic ascites may also be observed. Extensive chronic granulomatous inflammation with multiple granulomas in visceral organs and heart, white muscle, gills and eye.

The bacteria are Gram-negative, intracellular coccobacilli and can be grown on cysteine heart agar with 5 % sheep blood added and appear low convex, whitish and mucoid. Clinical signs, pathology and histopathology give presumptive diagnosis,

and the confirmation is by culture and PCR. There is no effective treatment due to the intracellular nature of the infection, and experimental vaccines are in development. Removal of affected fish and disinfection of premises/equipment and fallowing are the main means for reducing impact from the disease.

Tilapia is affected by other bacteria including *Edwardsiella tarda* (see later), *Nocardia seriolae* (see under marine fish) and *Flavobacteria* spp. (Evans et al. 2011; Labrie et al. 2008); however, no commercial vaccines are available.

Tilapia species are also susceptible to *Iridovirus* and present with mortality, ascites, paleness of gills and internal organs. Tissue smears or histopathology reveals hypertrophied cells in the spleen, kidney, liver and other organs (Ariel and Owens 1997; McGrogan et al. 1998). Mortalities can be high in larval fish and also affect growers, and a commercial injectable vaccine is licensed in some countries.

Bacterial and Viral Diseases in Catfish

Catfish species form a significant percentage of freshwater aquaculture with over 1.5 million tonnes of *Ictalurus punctatus* (channel catfish) and *Clarias* sp. farmed and another 1.5 million tonnes of *Pangasius* sp. catfish farmed in Asia (mainly Vietnam).

Although some viruses have been isolated and described to affect catfish aquaculture, such as the channel catfish virus (CCV), the impact in *I. punctatus* farming in the USA is relatively minor at present with only 1–2 % of total disease losses attributed to the herpesvirus (Camus 2004); however, in the past the virus was attributed with high mortalities in juvenile fish, and a more recent report in cage-cultured *P. hypophthalmus* in Malaysia isolated CCV from fish with mortality rates of 30–40 %; however, concurrent bacterial involvement was considered the most likely cause of the high mortality (Siti-Zahrah et al. 2014).

Bacterial diseases appear of more impact in catfish farming with a number of species causing significant losses, namely:

(a) *Edwardsiella ictaluri*, which causes bacillary necrosis disease in *Pangasius* sp. and is a significant challenge for catfish farms in Vietnam (Crumlish et al 2002). High mortalities in juvenile fish have necessitated antibiotic treatments, and there are reports of bacterial resistance (Crumlish et al. 2002); however, a commercial injectable vaccine has been introduced and is now increasingly being used in Vietnam. *E. ictaluri* was originally described as the cause of enteric septicaemia of channel catfish (ESC) and remains one of the most significant diseases in catfish farming in the USA with estimates of impact from the disease ranging from US$ 20–60 million annually (Evans et al. 2011). Vaccination (live, attenuated) is being utilised by catfish farms in the USA to counter this disease.
(b) Columnaris disease as induced by *F. columnare* is usually associated with warm water (20–25 °C) and can affect all ages of catfish. Columnaris disease is one of the most significant infectious diseases affecting the catfish industry, in terms of

mortality, but also in downgrading of fish for market, reduced growth rate and cost of treatments (Starliper and Schill 2011). The disease can be treated with antibiotics, and a commercial live attenuated vaccine is available in the USA.

(c) *E. tarda* is also observed in channel catfish, where it can cause clinical edwardsiellosis (also known as emphysematous putrefactive disease of catfish), and is also a challenge for many marine (and other freshwater) aquaculture species and is discussed in a later section.

(d) Motile *Aeromonas* spp. bacteria, predominantly *A. hydrophila* or *A. sobria*, can cause a haemorrhagic septicaemic condition in farmed catfish. In many cases systemic infection often follows a stressful event or sudden change in the environment; the classical bimodal appearance of the disease in spring and autumn with rising or falling water temperatures is an annual observation in farms in the USA, but mortalities are usually low level or of a chronic nature (Plumb and Hanson 2011).

Parasitic Disease in Catfish

Proliferative gill disease (PGD) of channel catfish is the third most significant disease affecting the US industry, and mortalities can be very high (close to 100 %) in fingerlings. The disease is caused by the myxozoan *Henneguya ictaluri*, and exposure of the fish to the actinospore stage of the parasite results in significant gill damage (Wise et al. 2008). Affected fish exhibit red and white mottled, swollen gills which are fragile and bleed easily. The only effective means to reduce losses is through efficient management, and a qPCR monitoring tool is used by farms to assess levels of *H. ictaluri* actinospores in the pond prior to stocking, and a decision can then be made on the safety of stocking. Genetics may hold some promise for reducing the impact as there appears to be a significant difference in susceptibility between the channel catfish and the blue catfish (*I. furcatus*) to PGD with the blue catfish rarely exhibiting clinical PGD, or if infected have only a transient infestation (Pote et al. 2012). There are neither effective treatments nor any vaccines for this condition.

Marine Fish Aquaculture

The number and volume of marine fish being successfully farmed continue to increase annually on a global basis, and from 1990 to 2010, there was an increases in quantity of 9.3 % pa (FAO 2012). In addition to marine salmonids, the species being farmed include amberjacks, sea breams, sea basses, croakers, grouper, drums, mullets, turbot and other flatfishes, snappers, cobia, pompanos, cods, puffers and tunas. Infectious diseases pose a threat and continual challenges in; many of these species and examples include viral, bacterial and parasitic pathogens.

Viral Disease in Marine Finfish

Viral encephalopathy and retinopathy (VER) or viral nervous necrosis (VNN) is a disease which results in a vacuolating encephalopathy and retinopathy of many farmed marine fish species. The causal agent is a *Betanodavirus* within the family *Nodaviridae* and is a non-enveloped, icosahedral, RNA virus, 25–30 nm in diameter. VNN was first described in Japanese parrotfish in 1990 and then appeared in many species in mariculture around the world, most notably in Japan and the Mediterranean, where hatcheries infected by the disease experienced 90–100 % mortalities. The disease has now been reported from all continents, with the exception of South America, and is one of the most important limiting factors in successful fry production of marine finfish species (Munday et al. 2002; Sano et al. 2011) and has been especially difficult in the European sea bass and Asian grouper aquaculture sectors (Harikrishnan et al. 2011).

Clinical signs of the disease include uncoordinated swimming, corkscrewing, whirling, dark colouration, darting across water surface and anorexia. Fish may also appear blind by swimming into tank walls. Internally the only abnormalities may be overinflation of the swim bladder and an empty intestine. Histopathology reveals extensive vacuolation in nervous tissue including the brain, spinal cord and nervous layer of the retina (Fig. 1.17). Malacia and gliosis are also observed, and occasionally intracytoplasmic inclusions are observed.

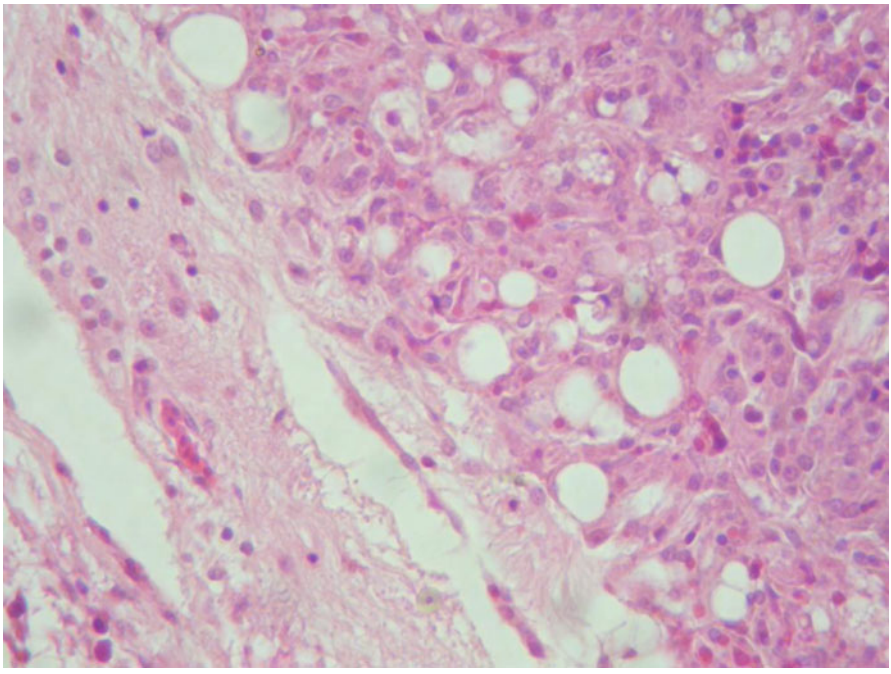

Fig. 1.17 Histological section of the brain of sea bass (*Dicentrarchus labrax*) affected by *Nodavirus* and exhibiting vacuolation of the telencephalon and focal gliosis (H & E ×400)

The isolation of the virus in SSN-1 cell culture (Frerichs et al. 1996) was epoch making, as reported by Sano et al. (2011), as there was then a dramatic acceleration of research activities into VNN including genotyping, phenotyping, infectivity and host specificity of the virus as well as transmission mechanisms, diagnosis and control measures. A correlation with antibody response and protection against the virus has been demonstrated and although no commercial vaccine is currently available, clinical trials are on-going. Sterilisation of water coming into hatcheries and disinfection of hatcheries after an outbreak are important for the prevention and control of VNN. Screening of juveniles prior to purchase and avoidance of wild fish as broodstock are also important. There is some evidence for vertical transmission (as well as horizontal), and hence broodstock can also be screened by PCR for the virus.

In the family *Iridoviridae*, the genera *Ranavirus*, *Lymphocystivirus* and *Megalocytivirus* are all known to cause disease in finfish. Of these the *Megalocytivirus* is the most significant, in terms of economic impact, and includes the agents of red sea bream iridoviral disease (RSIVD) and the infectious spleen and kidney necrosis virus (ISKNV). RSIV and ISKNV group viruses have been reported throughout Asia and affect at least 40 marine species (for RSIV) and 4 for ISKNV (OIE 2012) (Fig. 1.18). Mortality rates vary dramatically but can be up to 90 % in red sea bream, rock bream and grouper species (Sano et al. 2011; OIE 2012). Affected fish are lethargic and anaemic, may have enlarged spleens and on histopathology present with hypertrophied enlarged cells in the spleen, heart, kidney, liver, intestine, buccal

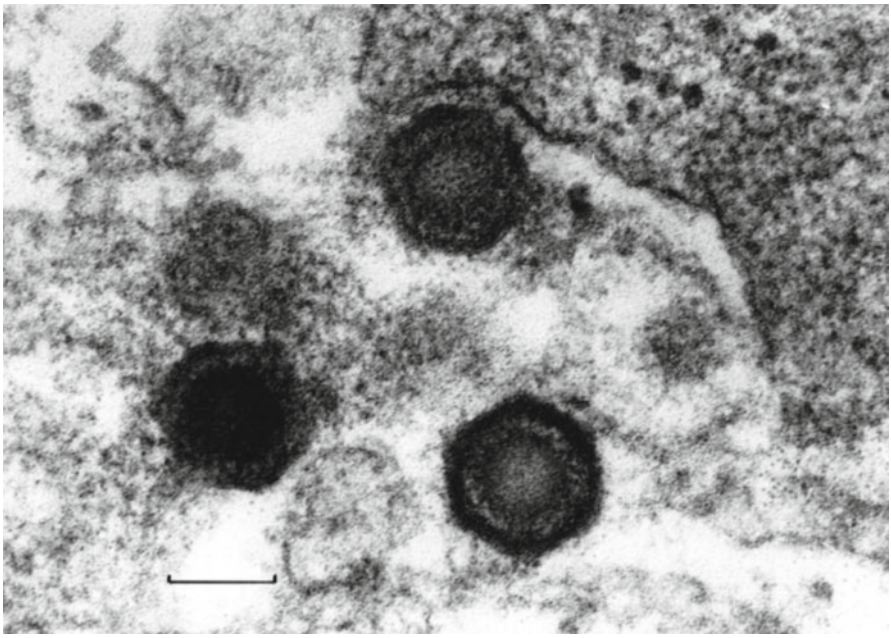

Fig. 1.18 Transmission electron micrograph of iridovirus from the spleen of *Pterophyllum scalare* affected by systemic *Iridovirus* infection. Bar = 200 nm

cavity or gill (Rodger et al. 1997), and these cells react to anti-RSIV monoclonal antibody in immunohistochemistry. The viruses can be cultured in cell culture and detected by PCR. Control of the disease through good hygiene and biosecurity as well as avoidance of poor rearing environments will be of benefit. Formalin-killed vaccines are available for red sea bream, jack and grouper in Japan.

Bacterial and Parasitic Disease in Marine Fish

Vibrio spp. have a global presence in the bacterial diseases that challenge marine finfish aquaculture. *V. anguillarum*, *V. ordalii* and *V. salmonicida* have been previously referred to in the salmonid section but up to 13 *Vibrio* species have been recorded to cause fish disease with examples such as *V. carchariae*, *V. harveyi* and *V. alginolyticus* causing some of the most serious infectious disease losses for grouper aquaculture (Harikrishnan et al. 2011). Haemorrhagic septicaemias with lethargy, ascites, skin lesions, internal organ petechiae and congestion with pale gills and sometimes intramuscular haemorrhage are common findings with systemic vibriosis in marine species (Fig. 1.19). Antibiotics have been used in the past; however, commercially available mono- and multivalent vaccines are available for

Fig. 1.19 Farmed Atlantic cod (*Gadus morhua*) affected by vibriosis (due to *V. anguillarum* O2β) exhibiting skin ulceration and haemorrhagic dermal lesions

Fig. 1.20 Captive moribund goldsinny wrasse (*Ctenolabrus rupestris*) presenting with haemorrhagic lesion around the vent and anal fin due to *V. tapetis* infection

administration by injection or immersion and are routinely used in the Mediterranean for sea bass (*D. labrax*), for various flatfish (*Paralichthys* sp.) species globally, for grouper species in Southeast Asia, for yellowtail (*Seriola* sp.) in Japan and most recently for protection of wrasse (*Labridae* sp.) fish farmed for sea lice control in Atlantic salmon (Fig. 1.20).

Edwardsiellosis, as caused by *E. tarda*, is highly significant economically in many marine aquaculture species including flatfish species, (such as olive flounder (*Paralichthys olivaceus*) and turbot (*Scophthalmus maximus*), red sea bream (*Pagrus major*), Japanese eel (*Anguilla japonica*) and yellowtail (*Seriola quinqueradiata*). At least 40 species of fish are known to have been infected), and the pathogen may also affect invertebrates and terrestrial species including humans (Evans et al. 2011). The economic importance of *E. tarda* in olive flounder and red sea bream aquaculture is significant with mortality ranging from 5 to 70 %, especially in South Korea where flounder is the most important aquaculture species with production valued at 489 billion Korean won (28 million euro) (Park et al. 2012). The disease can often be a chronic one and affects all sizes of fish including those close to market size. The disease can present with skin ulceration, haemorrhages and rectal protrusion, and histopathology in flounder and bream may reveal granulomatous inflammation. *E. tarda* can be cultured on general nutrient agar. Although many experimental vaccines have been tested in different fish species, no commercial vaccine is available for this disease (Park et al. 2012).

Pasteurellosis, as caused by *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*), causes systemic disease in farmed yellowtail in Japan and sea bass and sea bream (*Sparus aurata*) in the Mediterranean (Romalde 2002). This pathogen has also been associated with high mortalities in wild fish and has been isolated from over 20 fish species (farmed and wild). High mortalities may result from infection, and internally the fish may present with white granulomatous lesions throughout the visceral organs which accounts for the alternate name for the disease, pseudotuberculosis. Broad-spectrum antibiotic therapy had been widely used in the past to control the disease; however, commercial vaccines are now used throughout the Mediterranean and Japan for sea bass, sea bream, sole (*Solea solea*), red porgy (*Pagrus pagrus*) and yellowtail (Daly and Aoki 2011).

Nocardiosis is emerging in some regions as a significant disease of both freshwater and marine species. *Nocardia asteroides*, *N. salmonicida* and *N. seriolae* are the

Fig. 1.21 Atlantic salmon affected by tenacibaculosis presenting with haemorrhagic flank lesion and yellow colouration due to *T. maritimum* colonisation

three main species pathogenic in fish, and outbreaks have been reported in yellowtail, amberjack (*S. dumereli*) in Japan but also in pompano, threadfin, trevally, snapper and grouper in Southeast Asia (Labrie et al. 2008). The first case of nocardiosis in farmed fish in the Mediterranean was recently recorded in meagre (*Argyrosomus regius*) which presented with typical granulomatous lesions in the skin, ulceration and internally numerous white nodules on visceral organs (Elkesh et al. 2013). Antibiotic treatment appears of limited benefit, and methods for control rely on elevated husbandry. There are no vaccines available for these pathogens.

Infection of marine fish by *Tenacibaculum maritimum* is common in many marine-farmed fish species. The bacterium appears opportunistic, commonly infecting fish after minor epidermal or epithelial trauma or irritation, and can rapidly colonise such tissue. These bacteria are toxigenic (previously known as *Flexibacter maritimus*) and are Gram-negative, slender bacilli which multiply in mats on the damaged tissue. A yellowish colouration on lesions or damaged areas is characteristic of infection with this bacterium (Fig. 1.21). Mouth rot, tail rot, skin and fin lesions as well as gill, gill raker and intestinal colonisation are often seen (Avendaño-Herrara et al. 2006). Oral treatment with broad-spectrum antibiotics is generally successful if fish are maintained in a low-stress environment. Commercial vaccines are available in some regions.

Scuticociliatosis is a parasitic disease caused by a group of ciliates of the order Scuticociliatida and is found worldwide in marine aquaculture facilities (Jung and Woo 2012). *Miamiensis avidus*, *Philasterides dicentrarchi* and *Uronema marinum* are the three species of this group that have been associated with the most serious impact in global marine aquaculture. Mortality is particularly high for flatfish species such as olive flounder and turbot and results in significant economic loss in Europe, Japan and

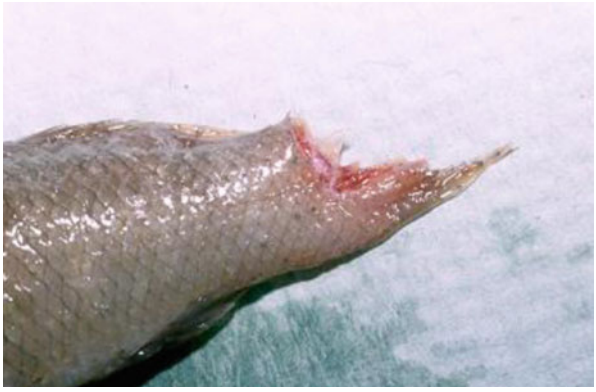

Fig. 1.22 Goldsinny wrasse (*Ctenolabrus rupestris*) infested with *Uronema marinum* with tail erosion and necrosis

South Korea with mortalities varying from 30 to 100 % in some units (Jung and Woo 2012). Affected fish are dark in colour, have congestion at fin bases and around the mouth, skin ulcers and erosion deep into the muscle may appear (Fig. 1.22). Abnormal swimming patterns, exophthalmos and ascites are also observed. Improving the environmental conditions in the rearing units, improved hygiene and bath treatments with various chemicals (formalin, hydrogen peroxide, antibiotics) all may help reduce the impact of infestation. No vaccines are available for these parasites.

Conclusions

All finfish aquaculture sectors are affected by infectious disease to varying degrees, and as new species are cultured and alternative rearing environments explored and developed, disease conditions emerge and re-emerge which present hurdles and challenges to sustainable aquaculture. Viral diseases present many of the most significant infectious disease challenges for the salmonid and marine finfish sectors, and there are only a limited number of effective vaccines available commercially for these. Bacterial pathogens present some major challenges for the carp, tilapia, marine and catfish industries, and there are an increasing number of effective, commercial vaccines available although some significant gaps remain. There are no vaccines available at present for the parasitic or fungal diseases which challenge all the finfish aquaculture groups. We would do well to consider the words of Sir Winston Churchill in 1941 “Give us the tools and we will finish the job.”

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Chapter 2
Overview of the Fish Adaptive Immune System

Chris J. Secombes and Rodrigo Belmonte

Abstract This chapter describes the immune responses that are elicited by vaccination of fish and that are key to providing protection against future encounter with these same pathogens. The cells and molecules involved are outlined, and the different functions they have within the immune system are detailed. The potential to use these responses as markers of protection is discussed and areas for future study are suggested.

Introduction

The term fish refers to three paraphyletic groups of vertebrates, comprising the jawless vertebrates (lampreys and hagfish), the cartilaginous fish and the bony fish. In the context of farmed fish, it is the bony fish that dominate in aquaculture, and indeed most species belong to the teleosts, an infraclass of the Actinopterygii. Teleost fish are characterised by having the maxilla and premaxilla fully movable, enabling it to protrude the jaw outward from the skull when opening the mouth. Another feature shared by all teleosts is a homocercal tail fin, with two symmetric lobes and a spine ending at the caudal peduncle, distinguishing this group from those in which the spine extends into the upper lobe of the caudal fin. Examples of teleost fish that are farmed around the globe are salmon, trout, eels, carp, sea bass and mullets. This review will focus on what is known about the immune system in teleosts, in terms of the responses that are elicited by vaccination.

As in all vertebrates, teleost fish possess both innate and adaptive immune responses, and the latter is the key component of the immune system that provides protection following vaccination. In jawed vertebrates these adaptive immune responses are mediated by lymphocytes (T cells and B cells), a particular type of white blood cell (leucocyte) that has special receptors on their surface to detect foreign molecules. Uniquely, the repertoire of these receptors is huge, but those on an individual cell have only a single specificity. When these receptors bind their ligand (or antigen),

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this triggers a proliferation of the cells that undergo clonal expansion giving a larger population able to fight off any potential microbial invaders. In addition, some of the cells remain long term as memory cells, so that a subsequent contact with the same antigen can elicit a faster, specific and more effective immune response, leading to protection. This is the basis of vaccination: prime the animals with a harmless antigen that can elicit this immunological memory so that if an encounter with the real pathogen occurs the responses give protection against the aforementioned pathogen.

It has been long known that teleost fish possess cells that look like lymphocytes, circulating in the blood and within immune competent tissues such as the thymus, kidney, spleen, gills and gut. More recently molecular evidence has been forthcoming to verify that these cells are indeed classical lymphocytes. They are relatively small compared to other leucocytes and have little cytoplasm in the resting state. They exist as two main types, T cells and B cells, that differ in several ways. They are known to differentiate at different sites, with T cells being produced in the thymus and migrating to other tissue sites to induce responses. B cells, in contrast, are made at different sites in different vertebrate groups, and in teleost fish, the kidney appears to be the most important site, akin to the bone marrow in mammals, although in zebrafish they are first seen, during development, between the dorsal aorta and posterior cardinal vein. The antigen receptors in T and B cells are formed from different genes and have a distinct structure, although the genetic mechanism that generates receptor diversity is similar. Interestingly, the T-cell antigen receptor (TCR) on most T cells requires antigen presentation, in the form of processed peptides from the original protein, delivered by major histocompatibility complex (MHC) molecules. B cells, on the other hand, can recognise soluble antigens and bind to them directly via their B-cell antigen receptor (BCR). In addition, B cells can produce a soluble form of this receptor that is secreted as an antibody or immunoglobulin (Ig) that can either act directly against the pathogen (neutralising antibody) or help in the internalisation of a pathogen by a phagocyte (opsonising antibody).

MHC Class I and Class II Molecules

Effective vaccines require efficient antigen recognition and presentation by the host immune cells (Fig. 2.1). This critical step for mounting an adaptive immune response in vertebrates is performed through MHC molecules and is dependent on the antigen origin. The MHC is a set of cell-surface proteins encoded by a family of genes that show a high degree of polymorphism between individuals, which allows different repertoires of peptides to be presented by different individuals in a population. This is one of the many genetic mechanisms that govern differential disease resistance within a species. MHC class I (MHC-I) and class II (MHC-II) molecules have been cloned in teleost fish (Wegner 2008) and, as expected, also possess a high degree of polymorphism. This inherent variety translates into individuals being capable of recognising different antigen subsets that can either lead to a protective response or not. Therefore, there is the potential to use these molecules as markers

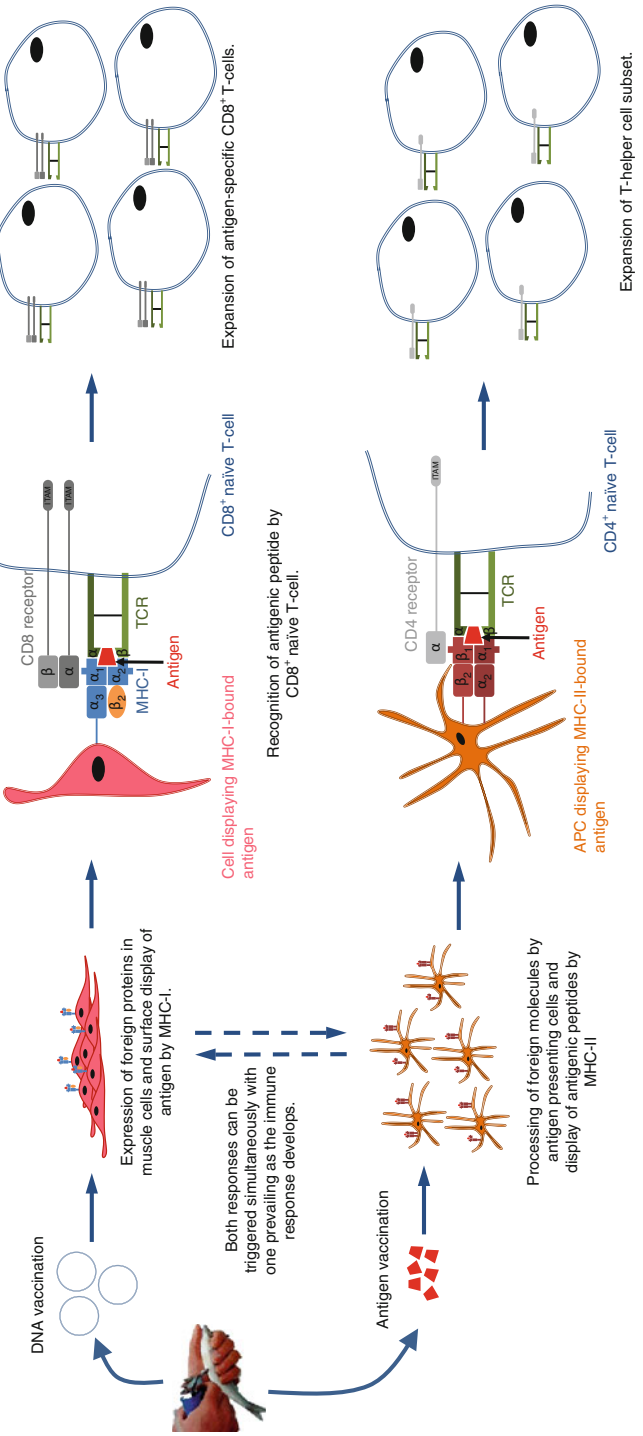

Fig. 2.1 Diagram illustrating the T cell responses elicited by vaccination. The various cell surface molecules depicted are not to scale

for selective breeding of fish for resistance to particular pathogens. In other vertebrates, the class I and class II molecules are linked in the genome but not in teleost fish. This feature allows for selection of disease-resistant individuals, either based on the MHC-I or the MHC-II diversity, in a simpler way.

Most cells express MHC-I molecules and are capable of presenting antigens to cytotoxic T cells (Tcs). Tcs, when activated, kill the target cell that is presenting a peptide with sufficient binding affinity. Therefore MHC-I monitors intracellular pathogens like viruses and the occurrence of malignant tumours (Janeway 2001). Class I molecules consist of a heavy chain with three extracellular domains (α_{1-3}) and a β_2-microglobulin light chain where peptides, usually nine residues, are bound in the peptide-binding region formed between the polar α_1 and α_2 domain (Wegner 2008). Only cells able to internalise extracellular microbes/proteins express MHC-II molecules, and these include macrophages, dendritic cells and B cells, the so-called professional antigen-presenting cells (APCs). Class II molecules are heterodimers of two peptide chains, the α- and β-chain, where processed peptides originated from antigen endocytosis are presented to a subset of T cells named helper T cells (Ths). In this case 9–12 amino acid residues of an antigenic protein are bound to the pocket formed between the distal α_1 and the β_1 domain and displayed on the APC surface (Wegner 2008). Recognition of this antigen-MHC-II complex will trigger the activation of Th starting a complex signalling cascade. Vaccine antigens are usually presented through MHC-II molecules to T cells, and it is the outcome of the interaction between an MHC-II molecule harbouring an antigen and a TCR that will determine whether the adaptive immune response will be activated and is a decisive step in adaptive immunity.

T-Cell Receptors

The interaction between antigens bound to MHC molecules and T cells occurs via a complex of cell-surface receptors that include the TCR and co-receptors that aid this interaction, like the proteins CD4 and CD8. T cells are characterised by the expression of the TCR on their surface and are effectors of the so-called specific cell-mediated immunity. The TCR exists as a heterodimer of two peptides chains, either the α- and β-chain or the γ- and δ-chain, linked by a disulphide bond, forming a single antigen recognition site. Most T cells possess the α/β-TCR in mammals, with γ/δ-T cells representing only ~2 % of T cells that are found mainly in the gut as intraepithelial lymphocytes (IELs). How such γ/δ T cells are activated is largely unknown, and it is possible that this TCR can recognise soluble proteins since in many cases MHC presentation of peptides is not required (Ma et al. 2013). All TCR chains (α, β, γ, δ) have been found to be present in teleost fish (Rast et al. 1995; Partula et al. 1995, 1996; Imai et al. 2005), and so in principle bony fish possess both subtypes of T cells.

The α/β TCR-bearing T cells in mammals are further subdivided into $CD4^+$ and $CD8^+$ subsets. This is an important distinction, especially in terms of the functional roles of these cells, and both molecules have been cloned in teleost fish (Laing et al. 2006; Hansen and Strassburger 2000). CD4 is a TCR co-receptor for MHC-II

molecules that in mammals is found as a single protein with four extracellular Ig-like domains and a cytoplasmic tail responsible for intracellular signalling (Laing and Hansen 2011). $CD4^+$ T cells are stimulated by peptides presented by the MHC-II molecules derived from extracellular proteins that are ingested through endocytosis, degraded in the lysosomes and then after interaction with the MHC-II molecules in the endoplasmic reticulum are displayed on the cell surface of APC. CD8 is a TCR co-receptor for MHC-I molecules that can be a homodimer formed by two α-chains or a heterodimer formed by an α- and β-chain. Both chains contain an extracellular Ig domain but are structurally diverse having only around 20 % homology between their primary sequences (Cole and Gao 2004). $CD8^+$ T cells are the Tc that can be stimulated via peptides derived from endogenous (cytosolic) proteins that interact with the MHC-I molecules in the endoplasmic reticulum and are then transported to the cell surface for presentation.

Curiously further CD4-related molecules have been discovered in teleost fish (named CD4L or CD4 REL) that encode proteins with either two (Laing et al. 2006) or three (Edholm et al. 2007) Ig-like domains. Through analysis of the syntenic relations of similar genes in other teleost and non-teleost genomes, it has been proposed that this CD4 REL molecule may represent an ancestral form of the mammalian CD4 molecules, before a duplication event of the Ig domains occurred (Laing et al. 2006).

Recent work has raised antibodies to CD4 and CD8 for the first time in fish (Toda et al. 2009, 2011; Takizawa et al. 2011), and these antibodies have been used to identify the cell populations expressing these molecules to begin to study their functions. For example, $CD4^+$ cells isolated from fugu do not express CD8 or Ig and so appear to be a distinct subpopulation. In ginbuna carp $CD4^+$ cells will proliferate in response to specific antigen restimulation, a characteristic feature of lymphocyte responses, and using adoptive transfer of sorted $CD4^+$ cells, it has been possible to show they play a role in protection against bacterial and viral pathogens. In trout, cells displaying CD8 on their surface were found in high ratios in the thymus, gill and intestine but are scarce in the head kidney (the anterior region of the kidney), spleen and blood. These cells do not express IgM on their surface, whilst transcripts for CD8-α/β and TCR-α could be detected, indicating a similar picture to that observed with $CD8^+$ cells in mammals.

$CD4^+$ T cells are the Th cells that release cellular mediators, termed cytokines, which activate, promote and regulate the responses elicited when an antigen is encountered. Whilst it is still early days in the characterisation of teleost Th, most of the cytokines known to be released from different subpopulations of Th in mammals exist in teleost fish (as outlined below), and the transcription factors associated with their differentiation are also known.

$CD8^+$ Tc are cytotoxic because of their ability to secrete cytotoxins like perforin and granzymes that will affect a target cell, initiating a controlled death process named apoptosis. Tc require that the target cell displays a high-affinity antigen through MHC-I in order to be activated. This antigen usually originates from an intracellular pathogen, such as viruses and some bacteria, or from abnormal cells undergoing tumorous growth. Several research groups have described lymphocytes with the characteristics of $CD8^+$ cells in fish, and these will be further detailed below.

T-Helper Cell-Associated Cytokines

In mammals, when $CD4^+$ T cells are activated, they undergo massive proliferation and differentiate into one or more of at least four different Th subsets: Th1, Th2, Th17 and T regulatory (Treg). Each cell subset expresses a unique set of signature cytokines, and the profile and magnitude of cytokines are dependent on the type of foreign organism detected and on other signals produced by APC and $CD4^+$ T cells themselves. The main cytokines of a Th1 response are interferon (IFN)-γ and interleukin (IL)-2 that drive a cell-mediated immune response that kills intracellular pathogens such as viruses and bacteria (Kidd 2003). The main cytokines of a Th2 response are IL-4, IL-13 and IL-20 amongst others, driving humoral immunity, upregulating antibody production to fight multicellular organisms, like parasitic worms, whilst also limiting the inflammatory response (Kidd 2003). The Th17 subset was described more recently, and these cells release IL-17A, IL-17F and IL-22 that have a major role in tissue inflammation and defence against extracellular bacteria and fungi, autoimmune diseases and cancer (Tato and O'shea 2006). Treg cells produce TGF-β1 and IL-10 to control the inflammatory response, especially the one elicited by Th17 cells, as uncontrolled inflammation leads to tissue damage and hampered immunity. Although the existence of Th cells and their potential subsets still needs to be confirmed in fish, the main cytokine players have already been described (Wang and Secombes 2013). The main cytokines of Th1 (IFN-γ), Th2 (IL-4), Th17 (IL-17) and Treg (IL-10) responses will be further described below.

The IFN molecule was discovered in 1957 during experimental work on embryonated chicken eggs (Isaacs and Lindenmann 1957) but the molecular analysis of non-mammalian IFN molecules only started several decades later, after most types of mammalian IFN had been studied in great detail, because the low homology to the mammalian sequences hindered their discovery (Schultz et al. 2004). IFNs constitute a large group of cytokines that are known for their ability to confer cellular resistance to viral pathogens, whilst playing a critical role in the response to microbial infections by modulating the innate and adaptive immune system, and are divided into Type I, Type II and Type III IFN. Type I IFN form a still-growing family of cytokines that in mammals comprises IFN-α, IFN-β, IFN-δ, IFN-k, IFN-ω, IFN-τ and limitin (reviewed in Vilcek (2003)). All Type I IFNs bind to the same heterodimeric receptor (IFN-α/βR). Type II IFN comprises a single molecule denominated IFN-γ that is produced after antigen or mitogen stimulation of certain T-cell populations, natural killer (NK) cells, B cells and dendritic cells (Fukao et al. 2000; Ohteki et al. 1999; Yoshimoto et al. 1998). They also bind to a single heterodimeric receptor, the IFN-γR. Type III IFN, also named IFN-λ, is a recently described family that is structurally related to Type I IFN but are found clustered in a different chromosome, and their genes contain several exons, in contrast to the single exon found in Type I IFN sequences, and they bind to a different receptor (Vilcek 2003).

In addition to the ability of IFN to confer viral resistance to cells, these cytokines have important roles in innate and adaptive immunity, in tumour surveillance and defence and in modulation of immune cell function (de Weerd and Nguyen 2012).

Type I IFN are well known in fish and exist as two major groups containing either two or four cysteine residues (Zou and Secombes 2011), with each further subdivided into three subgroups in salmonids (Zou et al. 2014). Multiple genes can exist within the subgroups, but not all teleost species possess all subgroups, with salmonids apparently having the most complex Type I IFN system known to date. IFN-γ molecules have been sequenced in several fish species including Atlantic salmon (Robertsen 2006), rainbow trout (Zou et al. 2005) and fugu (Zou et al. 2004). In Atlantic salmon and rainbow trout, at least two copies (paralogues) of IFN-γ (IFN-γ_1 and IFN-γ_2) exist (Zou and Secombes 2011). In the mammalian immune system, T cells and NK cells are the major sources of IFN-γ production (Milev-Milovanovic et al. 2006). In teleost fish, IFN-γ is upregulated following stimulation with virus-like molecules (Zou et al. 2005) and is a powerful activator of macrophages, with studies showing that this cytokine can boost MHC-I and MHC-II expression, suggesting an important role in antigen presentation (Martin et al. 2007), and can upregulate genes for increased microbicidal activity such as inducible nitric oxide synthase (Zou and Secombes 2011; Grayfer et al. 2010). Whilst in other vertebrates the Type II IFN family contains a single (IFN-γ) molecule, fish possess an additional cytokine named IFN-γ related (IFN-γ rel) (Igawa et al. 2006). The IFN-γ relgene is found in tandem to IFN-γ sharing the same genomic structure, indicating a common origin from a gene duplication event. IFN-γ rel presents a low comparative sequence identity to IFN-γ, suggesting that this duplication occurred very early in teleost evolution. Expression analyses of these genes suggest that common carp IFN-γ is expressed in T cells when stimulated with T-cell stimulants such as phytohaemagglutinin, whilst IFN-γ rel is expressed in lipopolysaccharide-/endotoxin-stimulated IgM^+ (B-cell enriched) cells (Stolte et al. 2008). Further functional studies are required to understand the role of this bony fish-specific IFN. To date no Type III IFN genes have been discovered in fish.

IL-4 belongs to the IL-2 family of cytokines, and genes with homology to mammalian IL-4 were only recently discovered in teleost fish (Li et al. 2007; Ohtani et al. 2008), although it is still debatable if they are true orthologs of mammalian IL-4 or IL-13 and therefore have been named IL-4/13A and IL-4/13B. Several putative receptors for IL-4/13A have been characterised with a strong similarity to the mammalian counterparts (Wang et al. 2011a), indicating a probable conserved signalling pathway. Little information is available on the functional aspects of IL-4/13, but in zebrafish injection of rIL-4/13A increases the surface expression of *DC-SIGN* (a possible dendritic cell marker) (Lin et al. 2009) and increases the percentage of IgZ^+ B cells (see below) in peripheral blood (Hu et al. 2010). In rainbow trout, recombinant IL-4/13A has been shown to modulate the expression of a number of genes in vitro but has no clear effect on expression of Ig isoforms (Wang and Secombes 2013).

The IL-17 family has several members (A to F) which are structurally related in mammals, with IL-17A and IL-17F being the two with the highest amino acid sequence similarity (Wang and Secombes 2013). In fish the homologues have been named IL-17A/F, and at least three paralogues (IL-17A/$F_{1–3}$) have been found in zebrafish (Gunimaladevi et al. 2006). The expression of these genes appears to be

different in each tissue and in response to bacterial infection (Monte et al. 2013). In rainbow trout rIL-17A/F_2 was found to induce the expression of the antimicrobial peptide β-defensin-3 and the pro-inflammatory cytokines *IL-6* and *IL-8* in splenocytes (Monte et al. 2013), suggesting a role in antibacterial defences.

IL-10 gives its name to the IL-10 cytokine family that comprises many molecules with diverse activity that have anti-inflammatory, pro-inflammatory and antiviral effects (Commins et al. 2008). In most species of fish, only a single IL-10 gene can be found, whilst in rainbow trout, two genes (IL-10_a and IL-10_b) have been described (Harun et al. 2011). IL-10 plays a critical anti-inflammatory role as its expression is induced after the pro-inflammatory mediators and has as its main activity the reduction of immune reactions that otherwise could cause tissue damage. In rainbow trout the expression of *IL-10* has been shown to be induced upon stimulation with pathogen-associated molecules and to be modulated by IL-21 and IFN-γ (Harun et al. 2011; Wang et al. 2011b). Regarding activity, IL-10 has been demonstrated to suppress the expression of pro-inflammatory cytokines such as IL-1β, IL-8, TNF-α and itself (Grayfer et al. 2011).

Cytotoxic T Cells and Antiviral Immunity

The presence of cell-mediated immunity was first demonstrated in teleost fish by the phenomenon of graft rejection which consists of the immune system of a host attacking and destroying incompatible transplanted tissue from a donor (Nakanishi and Ototake 1999). Further evidence for the presence of Tc in fish was obtained from experiments with rainbow trout where previously sensitised IgM negative peripheral blood leucocytes were capable of destroying allogeneic (from another fish) cells in vitro (Fischer et al. 2003). The activity of these cells, although similar in outcome to that of non-specific NK cells, contrasts in terms of requirements for activation and memory. Tc require activation and maturation processes that occur due to the reaction between the CD8 and TCR chains and an MHC-I-bound antigen, whilst NK cells can directly induce apoptosis if a target cell fails to display self-antigens, which indicates an altered state.

Tc play a crucial role in fighting off viral infections in fish. This has been demonstrated in experiments where leucocytes expressing CD8 and TCR, obtained from virus-infected ginbuna carp, displayed high levels of cytotoxicity against virus-infected cells in vitro (Somamoto et al. 2006). In vivo this has been demonstrated by the fact that viral protection could be achieved by adoptive transfer of sensitised leucocytes (Somamoto et al. 2002): specific cell-mediated cytotoxicity of ginbuna carp leucocytes against crucian carp haematopoietic necrosis virus (CHNV)-infected cells was observed 8 days after infection when specific cytotoxic activity reached a peak. This cytotoxicity was virus specific and MHC restricted, in a manner similar to what is observed in mammals. The effectiveness of the virus-specific cytotoxicity was transferable, since hosts receiving leucocytes from donors that had been previously infected did not exhibit clinical signs of the infection and also presented lower viral titres.

Tc are likely to be involved in the protection that DNA-based vaccines induce. These vaccines use naked DNA to cause host cells, usually the muscle cells, to express a viral protein in high amounts and therefore trigger an immune response. In the case of the salmonid rhabdoviruses VHSV and IHNV, it has been demonstrated that a single injection of plasmids containing viral glycoprotein genes leads to a fast but long-lasting antiviral response that provides protection by 4 days post-vaccination. This early response is non-specific and transient, probably due to the activity of secreted IFN and NK cells (Einer-Jensen et al. 2009). However, later a specific antiviral response is induced, leading to the protection of immunised individuals when challenged with the viruses months postvaccination.

Helper T-Cell Activation and Memory

The event of a naïve T cell recognising an antigen presented by an MHC molecule in an immune tissue is rare, and the majority of Th cells will re-enter the bloodstream. Only a TCR that binds the MHC-presented antigen with sufficient affinity will trigger cell activation. Once this event has happened, the Th cell will differentiate and expand into one of the aforementioned subsets (in mammals). Each of these subsets has a specific function: Th1 cells are crucial for activating macrophages, whilst Th2 cells are potent activators of B cells. Th17 cells induce a strong inflammatory tissue response to fight off pathogens that are not cleared by a Th1 or Th2 response. At the beginning of an adaptive immune response, the cytokine profile of T cells might not be entirely polarised, with cytokines being produced from different subsets, but chronic stimulation will eventually lead to a terminal unequivocal phenotype where only a single subset will prevail.

Once the immune system has successfully cleared a pathogen from the body, the majority of the population of Th cells will die, but a small percentage will convert into memory T cells. The reason why some T cells will enter the memory pool and some will be destroyed is still not clear in mammals. Effector, memory and naïve T cells present different levels of surface receptors like the CC chemokine receptor 7 (CCR7) that is highly expressed on naïve and memory cells, directing their migration towards immune sites such as lymph nodes, whilst a low expression in effector cells allows these cells to migrate towards the site of infection (Badovinac and Harty 2006). Other receptors have also been shown to be differentially expressed on T cells at different states of activation. For example, downregulation of CD27, CD28 and CD62L is detected in effector cells, whilst CD11a, CD44 and CD95 are upregulated (Badovinac and Harty 2006; Hamann et al. 1996; Hedlund et al. 1995). Another characteristic that differentiates these three types of T cells is the expression of different splice variants of receptor proteins, as seen with CD45 where CD45RA is expressed on naïve and effector T cells, whilst the CD45RO variant is expressed on memory T cells (Hamann et al. 1996; Clement 1992).

The CCR7 gene has been described in several fish species indicating that this chemokine receptor arose early in vertebrate evolution (Liu et al. 2009), but to date there is no proof of a role of this receptor in T-cell trafficking. CD45 has also been

described in several fish species (Fujiki et al. 2000; Diaz del Pozo et al. 2000) with splice variants expressed depending on the immune activation status of B and T cells (Kountikov et al. 2004, 2005, 2010). Although this evidence points to a similar picture in teleost fish and mammals, CD45 cell specificity and function has still to be determined in fish. Nevertheless it is possible that such molecules could be used to identify T cells at different stages of differentiation in bony fish, and this could be an interesting read-out of adaptive immunity status that provides more information than just measurements of specific antibody titres. Following the T-cell differentiation process may help determine whether an antigen is capable of triggering T-cell differentiation and whether memory cells have arisen from the immune stimulation.

B-Cell Activation

B cells are the major component of humoral adaptive immunity. Their main role is to produce high-affinity antibody against antigens and to present antigens as an APC. B-cell activation is initiated in response to specific antigen binding by the BCR followed by antigen internalisation, processing in specific endosomes and presentation in a complex with MHC-II molecules to elicit T-cell recruitment for further development (Lanzavecchia 1985; Rock et al. 1984). The BCR is a complex comprised of membrane Ig heavy and light chains, in association with the Ig-α/β heterodimer (Reth 1989). The latter are crucial for signalling as they have the only significant cytoplasmic portion of the complex, with each chain harbouring an immunoreceptor tyrosine-based activation motif (ITAM) (Janeway 2001). When an antigen binds to the BCR, the tyrosines in the ITAM become phosphorylated triggering an intracellular signalling cascade involving a variety of cellular processes, including regulation of gene expression, reorganisation of the cytoskeleton and BCR-mediated internalisation of antigen complexes (Harwood and Batista 2008). Once activated B cells start the process of maturation leading to the production of plasma cells (see below) that are capable of producing and secreting high amounts of antibodies and memory B cells leading to the elimination of the antigen and long-lasting protection from subsequent exposure to the same pathogen. Consequently, B-cell activation is dependent on complex intracellular signalling pathways and intercellular communication with Th cells. Like the previously mentioned antigen receptors on T cells (TCR), BCR diversity relies on the rearrangement of the Ig superfamily genes by the recombination-activating genes (RAGs) responsible for the somatic recombination of the Ig genes, leading to the possible recognition of a vast array of antigenic epitopes (Secombes and Ellis 2012). For example, a recent study in zebrafish calculated that there are approximately 5×10^3 different heavy-chain sequences in a single fish, and if this is also true for the light chain repertoire, this gives an estimated 2.5×10^7 different antigen-binding sites that could be formed (Boehm et al. 2012).

B-Cell Maturation

Following antigen stimulation, B cells begin the process of proliferation and maturation into plasma cells. Initially plasmablasts arise that are actively proliferating but capable of producing limited amounts of antibodies. These cells can then differentiate into either short-lived plasma cells (SLPC) or long-lived plasma cells (LLPC) that can no longer proliferate and are now committed to antibody secretion alone. The cell cycle inhibitor hydroxyurea (HU) can be used to distinguish between plasmablasts and plasma cells: HU reversibly arrests cells at a certain stage of the cell cycle (G1/S) stopping antibody secretion of plasmablasts whilst plasma cells are unaffected (Golding et al. 1988; Bouchard et al. 1994). Using HU it has been possible to show that in trout only plasmablasts are produced in the blood, whilst ~20 % of antigen-stimulated B cells can develop into plasma cells in the spleen and head kidney (Ye et al. 2011). When these cell types are isolated using Percoll density gradients, it is apparent that the expression of the transcription factor B lymphocyte-induced maturation protein-1 (Blimp-1) is associated with the transformation into a plasma cell and is known to shift Ig mRNA to the secretory form.

SLPC arise quickly after antigen induction when naïve B cells encounter an antigen. In mammals, if this is a high-affinity interaction, the cells differentiate in situ (Tarlinton et al. 2008), whilst lower-affinity B cells will migrate to "germinal centres" in immune tissues where they undergo somatic mutation and affinity maturation to reach the plasma cell stage. These cells will then migrate into the bone marrow becoming LLPC that can persist for years (Elgueta et al. 2010; Oracki et al. 2010). In teleost fish, as in mammals, B-cell populations are not uniformly distributed amongst all immune tissues. Lymphopoiesis occurs in the head kidney, but evidence suggests that, after reaching maturity, antigen-responsive B cells exit this tissue via the circulatory system and enter sites such as the posterior kidney and/or the spleen where a higher potential for plasmablast and plasma cell generation is observed (Ye et al. 2011). Immune tissues do not possess germinal centres in fish, but there is some evidence that melano-macrophage centres have a similar function in spleen and kidney tissues (Saunders et al. 2010). LLPC that settle back in the head kidney can remain functional for a long period of time secreting antibodies without the need for antigen restimulation. Achieving the LLPC stage is the endpoint of B-cell maturation and is key for the humoral adaptive immune response. If a vaccine can induce the production of LLPC, it is likely that specific antibodies against that antigen will be maintained at high levels for many weeks, helping to protect fish against a pathogen challenge. It is interesting to note that teleost IgM half-life is relatively short (approximately 2 days) in salmonids (Ye et al. 2010), and this means that antibody titres should tightly parallel the total antibody-secreting cell numbers.

Immunoglobulins (Ig) and B-Cell Populations

Antibodies are Ig molecules secreted by plasma B cells and are the final step of their response. The presence of abundant specific Ig in the serum of fish is often used as a diagnostic for vaccine efficacy. As described above, the presence of these antibodies indicates that the whole process of antigen recognition, presentation, activation of Th, maturation and expansion of B cells has occurred. Whilst in mammals five isotypes of Ig exist (IgA, IgD, IgE, IgG and IgM), three isotypes have been described in teleost fish: IgM, IgD and IgT (also called IgZ in zebrafish). IgM was initially thought to be the only Ig class that responded to antigenic challenge in both systemic and mucosal tissues until the discovery of IgT, which appears to be associated with mucosal immune responses (Zhang et al. 2010, 2011). A recent study looking at the repertoires of the IgM, IgT and IgD (i.e. heavy chain) variable domains elicited in response to a virus infection (VHSV) found that in splenocytes, there was a highly diverse IgM response, a smaller but clear IgT response, but the IgD response was barely detectable (Castro et al. 2013). This indicates that both IgM and IgT responses are involved in the response to systemic infection, whilst the function of IgD remains elusive.

In rainbow trout, B cells expressing the membrane-bound version of IgM ($_{m}IgM^{+}$) are also found to be $_{m}IgD^{+}$ but $_{m}IgT^{-}$, whilst $_{m}IgT^{+}$ B cells do not express the other two immunoglobulin types (Hansen et al. 2005; Salinas et al. 2011). This demonstrates that two separate populations of B cells exist. This scenario is different to that in mammals where naïve B cells express both IgM and IgD, with subsequent class switching leading to their downregulation and expression of one of the other isotypes (i.e. IgG, IgA or IgE) on the surface of mature B cells (Yuan 1984). An additional population of $_{m}IgD^{+}/_{m}IgM^{-}$ cells has been described in catfish (Edholm et al. 2010) and rainbow trout (Castro et al. 2014) recently, but the roles of these cell types in adaptive immunity are still to be understood.

In rainbow trout IgT^{+} B cells account for 16–27 % of all B cells in the systemic immune organs, with the remainder being $_{m}IgM^{+}$ B cells (Ye et al. 2011). However, in gut the proportions are more equal (54 % $_{m}IgT^{+}$ and 46 % $_{m}IgM^{+}$), suggesting IgT is more important for mucosal immunity. Still, the amount of IgT present in the (gut) mucus is still very low (~7 μg/mL) in comparison to the amount of IgM (75 μg/mL in gut mucus and 2.5 mg/mL in blood) (Zhang et al. 2010, 2011).

Memory B Cells

It is well known that the numbers of cells secreting specific antibody and serum-specific antibody titres both increase following a booster immunisation, indicating a memory response. The memory B cells are mainly circulating in the blood but need to migrate to immune tissues (as outlined above) to mature. One fascinating feature of the secondary response is that the B cell clone size that occurs following antigen restimulation is similar to that seen following primary immunisation, unlike

the situation seen in mammals. Thus it appears to be the greater number of antigen-specific B cells generated in the primary response that drive a subsequent secondary response in fish. Also, since this increase in antibody-secreting cell number does not correlate with an exponential increase in antibody titres, this suggests that an increased number of cells rather than a qualitative change into more productive (antibody-secreting) cells is being generated. A qualitative difference that does exist is in the number of plasmablasts that develop into plasma cells during secondary responses (Ye et al. 2011).

Affinity maturation of the antibodies generated is known to occur during these responses, coincident with the appearance of the LLPC in the head kidney (~12–15 weeks after immunisation in salmonids). Initially the low-affinity antibodies are replaced by intermediate-affinity antibodies and eventually by high-affinity antibodies (Ye et al. 2011). An enzyme involved in somatic hypermutation of the Ig heavy-chain (IgH) gene in mammals, called activation-induced cytidine deaminase (AICD), exists in fish and has been demonstrated to have this function when expressed in mammalian cells (Wakae et al. 2006). Thus selection of high(er)-affinity B cell clones following (at least a degree of) somatic hypermutation as the responses progress likely accounts for this change in the secreted Ig. The high-affinity antibodies are maintained during secondary responses, even as the antibody levels eventually decline. Interestingly the half-life of the high-affinity antibody that appears late in the immune response is longer than that of low-affinity antibody. This appears to be associated with increased polymerisation of the (IgM) antibody molecule relative to that seen in low-affinity antibodies, rather than due to the increased strength of binding of the antigen-binding site (Ye et al. 2010).

Markers of Protection

Besides the testing of vaccine efficacy using survival as a read-out post-exposure to a lethal dose of pathogen, other methods can be employed when such a strategy is unavailable due to the lack of a reliable challenge model or other constraints. This can include monitoring of the pathogen level present in the fish post-exposure, typically by PCR or bacterial/viral titration. Protected fish would be expected to have a reducing pathogen load compared to control fish if their immune system is winning the fight for host survival. Methods based on measurement of immune molecules can also be employed to some degree, with assay of B cell/Ig responses the most studied to date. This can be the evaluation of the numbers of specific antibody-secreting cells present in immune tissues or determination of specific antibody titres in the blood or mucus. However, a correlation with antibody production is not in itself evidence of a protective response per se, since the antibody target may not be vital for pathogen survival. However, there are examples where this is the case, as seen with virus-neutralising antibodies that stop cells from becoming infected. One conclusive means of demonstrating the protective capabilities of specific antibodies is via passive immunisation. This method consists of antibody transfer to a naïve

fish, which receives the antibodies from a sensitised donor. The fish are then challenged with the pathogen used to raise the antibodies, and if they survive this exposure, then the protective effect can be clearly attributed to the administered antibodies.

More recently attempts have been made to find markers of the T-cell responses that are expected to make an important contribution to protection. With the cloning of many cytokine genes, it is now possible to analyse by PCR the relative expression of these molecules and see if they correlate with vaccine-induced protection. Unfortunately the results are not yet clear cut, but at least one marker (IL-22 expression) is looking promising in a model of *Vibrio anguillarum*-vaccinated haddock where vaccinated fish presented a higher IL-22 expression in gill tissue following a challenge that was correlated with protection provided by the vaccine (Corripio-Miyar et al. 2009).

Conclusions

With the large body of knowledge that now exists for the immune genes present in fish, we can measure with precision some of the responses elicited by vaccination and infection. This gives a huge advantage when attempting to develop or improve fish vaccines and will likely yield a new generation of vaccines in the not too distant future. However, there are still aspects of the immune responses in fish that we know little about, and one of these is key – do Th exist? This will likely be a focus for future studies in the next few years. Once we know what populations are present and their role in eliciting disease resistance to different pathogen types, this will give another means to ensure future vaccines are fit for purpose and stimulate protective response to the pathogen of interest.

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Chapter 3
Development of Fish Vaccines: Focusing on Methods

Øystein Evensen

Abstract Sustainable aquaculture is not possible without disease prevention, and vaccination has become the single most important tool. There has been a dramatic reduction in the use of antibiotics in Norwegian salmon farming since the introduction of oil-based vaccines. Today, it is an industry standard in all salmon-producing countries, and we are seeing a similar approach being adopted in other countries producing high-value fish species, e.g. Japan. Fish can be vaccinated by immersion and via the oral route; however, the protection falls short using these methods compared to injection vaccination. Interesting new technologies have emerged over the last 5 years, particularly injection of a single dose of naked DNA into the fish muscle. New technologies are promising, but it is more likely that there will be improvements of existing vaccines than completely new technologies taking over the fish vaccination scene in the next 5–10 years.

Introduction

Sustainable development of aquaculture relies on disease prevention. We see an intensification of the production for several aquacultured fish species and, with this, an increased risk of disease and also spread of infectious diseases through transport and/or trade.

There is a profound and consistent positive attitude towards vaccines. Vaccines stimulate the immune system to help fight off diseases, and vaccination is of growing importance to control infectious diseases. This article summarises the development in fish vaccinology with focus on methods applied and discusses possibilities and limitations regarding the use of vaccination for control of infectious diseases in commercial fish farming.

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Immunological Basis

Fish possess, as mammals, a defence system, which enables them to survive and maintain their integrity in a hostile environment. The major lymphoid tissues in teleost fish are the (head) kidney, thymus, spleen and mucosa-associated lymphoid tissues (Press and Evensen 1999), including the skin (Xu et al. 2013), the gills (Haugarvoll et al. 2008) and the nostrils (Tacchi et al. 2014). An obvious difference from the mammalian immune system is that fish lack bone marrow and lymph nodes (Press and Evensen 1999).

Immunity and Vaccination of Fish

Vaccines aim at stimulating the adaptive immune system to mount a response against a pathogen or rather against defined structures of the pathogen, the immunogenic parts. Vaccination has been used as a prophylactic means in aquaculture for decades, and it has been estimated that ten percent of all cultured aquatic animals are lost because of infectious diseases alone, amounting to >10 billion USD in losses annually at global scale. High-value species like Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*) are vaccinated against a variety of diseases (Gudding et al. 1999). Administration of vaccines to aquatic animals represents obvious technical challenges different from what is encountered in terrestrial production systems. Modalities used are injection, immersion or oral delivery.

The Start

The history of fish vaccination dates back to an early publication by Duff in 1942 working on vaccinating against *Aeromonas salmonicida* infection in cutthroat trout (Duff 1942), and oral immunisation strategies were used to protect against the disease. This research and studies came out of the Pacific Biological Station in British Columbia, Canada. Many of the pioneers in the fields of fish vaccination were people with a scientific background that combined good theoretical knowledge with excellent understanding of practical fish farming. Many companies were involved at early stages, and Wildlife Vaccines (in Colorado) included Guy Tebbit, John Rohovec and Thomas Goodrich as main contributors (Gudding and Goodrich 2014). In 1976, this company was the first to manufacture and license a vaccine for fish. The second vaccine for fish was licensed by a subsidiary of Johnson & Johnson (Tavolek Inc.). Vaccine development for farmed fish gained momentum when Biomedical Research Laboratories in Bellevue outside Seattle, Washington, established their research and development programmes. Pioneers were Stephen Newman, Tony Novotny, Robert Busch and James Nelson. Their focus was production of bacterins, and Biomed Inc. was the first company to launch an oil-based

vaccine against furunculosis in Norway in 1992–3. Other companies were Aqua Health Inc. working out of Charlottetown, Canada (today part of Novartis), and Aquaculture Vaccines Ltd. in the UK (today Merck). All these companies and people played an important role in the early days of fish vaccination. In the mid-1980s, Apothekernes Laboratorium (AL; a Norwegian pharmaceutical company with focus on generic pharma) established their fish vaccine and therapeutics activities, and they acquired Biomed in the mid-1990s. AL's vaccine production started in Tromsø and later moved to Overhalla in mid-Norway, and they later changed name to ALPHARMA AS. PHARMAQ AS was later "created" through management buy-out in 2004, today with global presence. Yet another Norwegian company was Norbio, established in 1985 (in Bergen) and recruited scientists from the university in the region. Norbio was later sold to Intervet (1993), became part of Schering-Plough (2007), and in 2009 their vaccine division merged with the Merck group. Finally, Novartis is also involved in the fish vaccine business and acquired Aqua Health Inc. in 2000 on which they have later built their aquabusiness. The animal health business of Novartis was taken over by Elanco in 2014. Over the last decade, we have seen small companies growing in emerging markets, in Europe serving the Mediterranean region (Fatro Inc.), the Chilean salmon market (Centrovet Inc., Veterquimica) and also towards carp production in Israel (KoVax Inc.).

Current Vaccines in Different Markets

The majority of vaccines currently available for farmed fish are prepared by conventional methods, i.e. typically a suspension-based fermentation of bacteria or virus harvested from cell culture. Inactivation methods typically include the use of formalin or alkylating compounds, and downstream methods can include filtration ("washing"), concentration of antigens or purification of antigen preparations.

Vaccines available in the salmon markets (Norway, Faroe Islands, the UK, Ireland, Canada, the USA and Chile) are oil-adjuvanted, injectable vaccines and are provided by the main manufacturers (Centrovet, Elanco, Merck and PHARMAQ). Oil-based vaccines for injection are available for use in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) in the Mediterranean countries against photobacteriosis (Sommerset et al. 2005; Hastein et al. 2005). Vaccines for use in the salmon markets are multivalent and can contain as many as seven different antigens. While salmon transferred to sea was previously vaccinated with a monovalent, oil-adjuvanted vaccine against pancreas disease (PD) in Norway; this antigen has now been included in a 7-valent vaccine (manufactured by MSD). In Canada (British Columbia), Atlantic salmon are vaccinated against infectious haematopoietic necrosis (IHN) with a plasmid (DNA) vaccine (intramuscularly) (Alonso and Leong 2013) in addition to vaccines given intraperitoneally.

An oil-adjuvanted vaccine against enteric septicaemia of catfish (*Pangasionodon hypophthalmus*) is licensed in Vietnam (by PHARMAQ AS), an inactivated vaccine based on whole cell preparations. Vaccines against *Streptococcus agalactiae*

infections in tilapia (*Oreochromis niloticus/mossambicus*) are available (from Merck) for use in several Asian countries and in Brazil. This vaccine is based on biotype 1 of *S. agalactiae*. There are also vaccines available against *Lactococcus garvieae* infections for use in rainbow trout in the Mediterranean region and for amberjack (*Seriola dumerili*) and yellowtail (*Seriola quinqueradiata*) in Japan, also combined with *Photobacterium damsela* sp. *piscicida* and *Vibrio anguillarum* (Sommerset et al. 2005; Hastein et al. 2005).

An inactivated, whole virus vaccine against red sea bream iridovirus infection (Nakajima et al. 1999) is available in Japan, combined with *Lactococcus garvieae* and *Vibrio anguillarum* (serotype J-O-3) (Kyoritsu Seiyaku Corp.). A monovalent iridovirus vaccine (against megalocytivirus) is available (from Merck) for use in Asian markets (tilapia). These vaccines are non-adjuvanted or oil adjuvanted.

Inactivated, immersion vaccines are available for vaccination against *Photobacterium damselae* sub-species *piscicida* in European sea bass and bream (from Merck). Similarly, an inactivated vibriosis vaccine is available for the same species (from Merck). An oral ERM (enteric redmouth) vaccine for use in trout is also available from the same supplier.

There is currently one commercial subunit (peptide; VP2) vaccine available for use against IPN in Norway (Merck) and one recombinant vaccine against infectious salmon anaemia (Centrovet) in Chile. The antigens are expressed in *Escherichia coli* (IPNV VP2 protein) or in *Saccharomyces cerevesiae* (ISAV HE and F proteins). Live attenuated vaccines against enteric septicaemia (*Edwardsiella ictaluri*) and *Flavobacterium columnare* infections of catfish (*Ictalurus punctatus*) are licensed in the USA (Klesius and Pridgeon 2014).

Vaccine preparations intended for injection are delivered at 25, 50 or 100 µl per fish per vaccine preparation, irrespective of fish size. The vaccine is delivered intraperitoneally and injected in the midline, approximately 1.5 cm cranial to the caudal fins. DNA vaccines are injected intramuscularly at 0.1 ml per fish. Live attenuated vaccines are delivered to small fish by immersion.

Development Trends

The development of fish vaccines goes along three major trends: (i) *Mode of delivery*, i.e. vaccination via mucosal surfaces (immersion or oral) or injected. (ii) *Nature of the antigen*, i.e. non-replicating or replicating vaccines, which are still the two poles of vaccine technology developed by Louis Pasteur more than 100 years ago (Pasteur 1880). This cover classical inactivated bacterial or viral vaccines. (iii) *Recombinant technologies* where purified or designed subunit, protein-based vaccines are used. Recombinant DNA-based technologies have taken this further where antigens are expressed in vector viruses, and in designed, attenuated virus and bacteria. Ultimately. one can use the animal to "produce" the antigens, via injection of plasmids encoding defined antigenic parts of the pathogen. The different trends will be discussed in more detail below, and advantages and disadvantages for the different delivery modes are summarised in Table 3.1.

Table 3.1 Summary of different routes of administration for vaccines for farmed finfish

Route of administration	Type of formulation/ modality	Advantages	Disadvantages
Injection (non-replicating; replicating vaccines)	Often comes with adjuvant; oil-based (water-in-oil, oil-in-water or w/o/w); aluminium salts; experimental liposomal vaccines	Most potent, little waste of vaccine Allows the use of adjuvants Cost-effective method for high-value species Mass vaccination is possible, but costly and time-/resource demanding	Stressful to the fish Impractical for fish <15 g (depending on species) Labour intensive and costly Injection-site reactions
Immersion (non-replicating; replicating vaccines)	Limited used in salmon Marine fish (smaller) more frequent use Live attenuated vaccines Vector vaccines	Large-scale application possible Moderate stress to the fish Moderately labour intensive Allows mass vaccination of immunocompetent fish High efficacy using live, attenuated vaccines	A large amount of vaccine is needed, can be cost prohibitive Low to moderate efficacy for inactivated vaccines, depending on antigen Inferior to injection delivery in terms of efficacy Cost prohibitive for large fish
Oral delivery	Top-dressing Incorporation into pellet (post extrusion) Formulation in PLGA; micromatrix systems (experimental)	Imposes no stress to the fish Moderate to high cost All fish sizes can be vaccinated when immunocompetent Usually safe – primes mucosal immunity (external surfaces) Used in combination with injection vaccines or immersion	Usually moderate to low efficacy when administered alone Better prospects as "boost" strategy Can be cost prohibitive for larger fish

w/o/w water-in-oil-in-water

Modalities: Mucosal Vaccines

Vibrio anguillarum and *Yersinia ruckeri* bacterins induce good protection following immersion vaccination of rainbow trout (Johnson and Amend 1983) and also Atlantic salmon and several marine fish species (Hastein et al. 2005). The protective antigen(s) are LPS for both pathogens (Croy and Amend 1977a), particularly the O-antigens (Welch and LaPatra 2016). The antigens are taken up across mucosal surfaces (gill, skin, stomach or gut) (Joosten et al. 1997) and likely induce local

immunity (mucosal) and/or a systemic immunity sufficient to protect the animal against lethal challenge. For other diseases, it has been difficult to obtain a sufficient level of protection using immersion or oral delivery, typical examples being furunculosis (*Aeromonas salmonicida*), pasteurellosis (*Photobacterium damselae* spp. *piscicida)* in sea bass and *Edwardsiella ictaluri* infections in catfish and pangasius. There are exemptions to the rule (Villumsen and Raida 2013; Thinh et al. 2009), but the use of mucosal vaccines against furunculosis and pasteurellosis are not frequently used under commercial conditions (Hastein et al. 2005). The explanation for mucosal vaccines falling short compared to injection vaccines are not understood in detail. The induction of an immune response after mucosal immunisation is dependent on either local responses (in the mucosa) or uptake of antigens from the external surfaces and/or the gut lumen and systemic distribution to head kidney and/or spleen. In higher vertebrates, proliferating and dead particulate antigens (as well as soluble antigens) are taken up through a specialised follicle-associated epithelium, the so-called M ("membrane") cells, and with subsequent transepithelial transport to underlying lymphoid tissue, the Peyer's patches (Brandtzaeg et al. 1987). Regular M cells are not found in fish, but cells with antigen-sampling capabilities have been identified in the gut epithelium of Atlantic salmon (Fuglem et al. 2010). Their involvement in particle uptake in association with oral vaccination is not known.

Despite the observation that vaccine efficacy in fish is limited after oral delivery, there are very few studies that address the uptake and transepithelial transport in enterocytes of soluble versus particulate antigens. The morphological or functional characterisation of enterocytes is also scant, yet there are indications for a regional specialisation of the gut epithelium with regard to uptake of macromolecules, and the hindgut enterocytes are considered important in this respect (Georgopoulou et al. 1985), and possibly sampling cells located between epithelial cells (Fuglem et al. 2010). Trinitrophenylated-lipopolysaccharides (TNP-LPS) and biologically active proteins like horseradish peroxidase are absorbed from the gut into the circulatory system (Doggett et al. 1993). Studies of uptake of particulate antigens in stomachless carp (*Cyprinus carpio*) using a bacterin of *Vibrio anguillarum* have shown that the bacteria are taken up by epithelial cells in the second gut segment and are later identified in intraepithelial macrophages (Rombout and van den Berg 1989). However, no attempts were made to distinguish between soluble (such as LPS) and particulate components of the antigen preparation, and thus no conclusion could be made with regard to the transport of particulate versus soluble antigens across the epithelial cells (Rombout and van den Berg 1989).

Nakanishi and coworkers (Nakanishi et al. 2002) explored some of the underlying factors that could possibly explain the importance of particle uptake across the dermis; this is for *Streptococcus iniae* vaccines in trout. They punctured the skin of the fish prior to vaccination allowing for percutaneous delivery when the fish were submerged in vaccine solutions (non-replicating) of α-haemolytic *Streptococcus sp.* It was shown that the puncture method facilitated uptake of antigens into the skin (and subcutaneous tissue), and the protection achieved was comparable to injection-vaccinated groups, while immersion gave no protection (Nakanishi et al. 2002). Skin puncture will result in a high number of particulate antigens being taken up for systemic distribution to lymphoid tissues, thereby inducing protective immune

responses. There will be no antigen uptake (to systemic distribution) in non-punctured fish, and thus the immune responses will be weak and non-protective. There are two important things to learn from this study. First, protection against *Streptococcus iniae* cannot easily be obtained through mucosal delivery. Second, protection against *S. iniae* is reliant on systemic distribution of particulate antigens likely involving the kidney/spleen in immune induction. This contrasts the protection obtained with bacterin preparations of *Vibrio* or *Yersinia* antigens delivered on mucosal surfaces, aligning with an understanding that protective antigens are LPS associated (Welch and LaPatra 2016; Croy and Amend 1977b) and likely soluble. Further to this, one can ask when local immunity versus systemic immunity would be required to obtain protection against mortality/disease?

It is known that the mucosa harbours a large community of commensal microbes, and the mucosal immune system deploys a wide variety of cells (locally) that creates a complex regulatory network with the aim to establish a balance between surveillance for pathogens and immunological tolerance (Chen and Cerutti 2010). Although there is limited knowledge of the interaction between the commensal flora and the immune cells in general and in fish in particular, one can anticipate that mucosal vaccine would have to leverage the functions of these immune cells and regulatory components. There is an asymmetric principle observed in mice where vaccines delivered parenterally induce strong systemic responses but fail to induce mucosal immunity (Belyakov et al. 1998). In contrast, mucosal vaccines elicit a local response and at the same time induce systemic immunity. These phenomena have not been studied to a very limited extent in fish (Zhang et al. 2010). In a recent study, we showed that salmon vaccinated against IPN had circulating antibodies and upregulated transcript levels of IgM mRNA in the kidney, while the hindgut was negative (Chen et al. 2015). When these fish were boosted orally with IPNV antigen, we saw upregulation of IgM mRNA in the kidney but also in the gut. These findings mirror the asymmetry described for immune responses in mice, and the practical importance of these findings needs to be explored in more detail in future studies.

The importance of the commensal flora for postnatal maturation of the immune system has not been studied to any detail in fish, but it is known to play an important role for postnatal maturation of mucosal immune systems in higher vertebrates (Artis 2008; Macpherson and Uhr 2004). It has been shown that intestinal IgA responses are directed against a minor proportion of the commensal microbial species (Chen and Cerutti 2010; Cerutti et al. 2011). It is further known that IgA immune responses in the intestine have a high threshold, and 10^8–10^9 bacteria are needed to elicit a response, below which there is no IgA production. Further, IgA responses lack the classical memory features seen with priming and boost, and another interesting finding is the attrition phenomenon observed for IgA responses, i.e. subsequent challenges with different antigens diminish the response to previous antigenic challenges. It thus seems as if IgA responses in the gut are programmed for strength control, and thereby IgA levels adapt to the microbiota of the gut. This is conceivable under a concept where high abundance of microbial species is more likely to breach the epithelial barrier. This brings an interesting concept to the recently discovered mucosal Ig in zebrafish and trout where gut bacteria were found covered with IgT (in trout)

under normal conditions (Zhang et al. 2010). There are several studies on the importance of delivery of antigens to different compartments in the gut, and long before IgT was described, it was shown that anal and to a lesser extent oral delivery of bacterins of *Yersinia ruckeri* will result in a high level of protection (Johnson and Amend 1983). Similarly, previous studies in other fish species have shown that bacterial antigens of *Vibrio anguillarum* were identified in the hindgut epithelium, but no transport to the circulation was observed (Tatner et al. 1984; Nelson et al. 1985). Interestingly, the LPS moiety of the bacterial cell is considered an important component of the protective antigens (Croy and Amend 1977b), and it is possible that induction of local immunity is sufficient to protect against lethal challenge with *V. anguillarum*.

The role of gut IgT (or skin) is still to be explored in more detail, and one role that should be subject of future research is the possibility that IgT plays a role in controlling the relative number of bacterial species in the gut and possibly also on the skin. Further studies are needed to understand these interactions in more detail.

Modalities: Injection Vaccines

Vaccination of Atlantic salmon against furunculosis and vibriosis/cold-water vibriosis with oil-adjuvanted vaccines results in the induction of long-lasting and protective immunity. The protection obtained is good and long-lasting, and salmon vaccinated with oil-adjuvanted vaccines against furunculosis will not result in clinical disease outbreaks after transfer to seawater (Gudding et al. 1999). Retention of antigens at the injection site is believed to be a prerequisite for long-term protection in fish, also known as the depot effect (Evensen et al. 2005). The antigens of a water-in-oil emulsion are located in the water droplets (mainly) and the distribution of antigens (schematically in Fig. 3.1). However, the

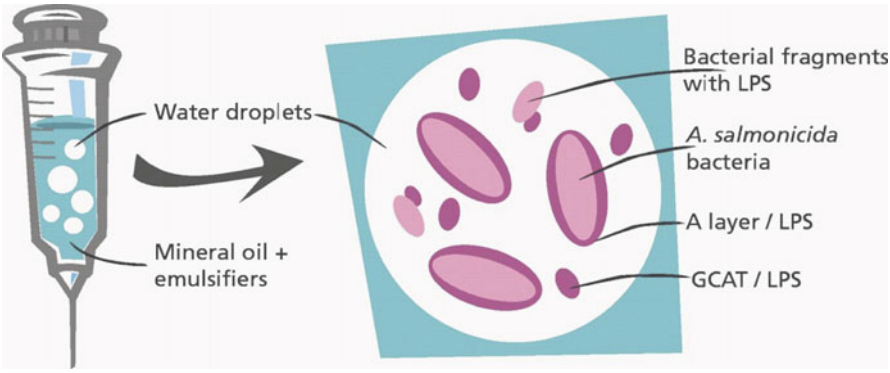

Fig. 3.1 Schematic presentation of a water-in-oil formulation. Water droplets are dispersed in a continuous oil phase, and antigens (here bacterial components) are found within the water droplet and at the interphase between water and oil, possibly also in the oil phase (Illustration by Ida Skaar)

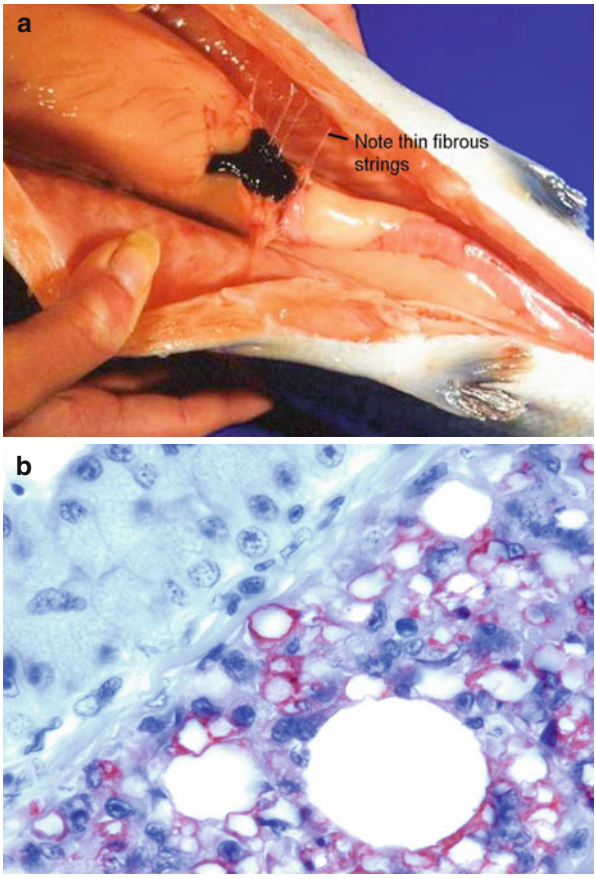

Fig. 3.2 (**a**) Mild intraperitoneal granulomas in Atlantic salmon following the use of oil-adjuvanted vaccines (photo by Trygve Poppe). (**b**) Immunohistochemical documentation of *Aeromonas salmonicida* LPS antigen (*red colour*) present in a peritoneal granuloma in Atlantic salmon vaccinated with oil-adjuvanted vaccine

drawback is that oil-adjuvanted vaccines result in the formation of visible injection-site lesions (Figs. 3.2a, b) that persist through to harvest size (Midtlyng 1997). Antigens (LPS) of *A. salmonicida* can be found as long as 18 months post vaccination using immunohistochemistry and in situ detection of antigens (Fig. 3.1b) (Evensen et al. 2005).

The side-effect may on some occasions also result in downgrading of fish at slaughter or after processing, and in fish with high side-effect scores, immune profiling indicates an autoimmune response (Mutoloki et al. 2010; Koppang et al. 2008). The intra-abdominal lesions are recognised grossly as melanisation and adhesions between internal organs or between the organs and the peritoneal wall (Mutoloki et al. 2004). Histomorphological examination shows the presence of granuloma with macrophages, epithelioid-like cells, and occasionally with formation of multinucleate giant cells and with varying numbers of lymphocytes and eosinophilic granular cells (EGC)/mast cells (Mutoloki et al. 2006, 2008).

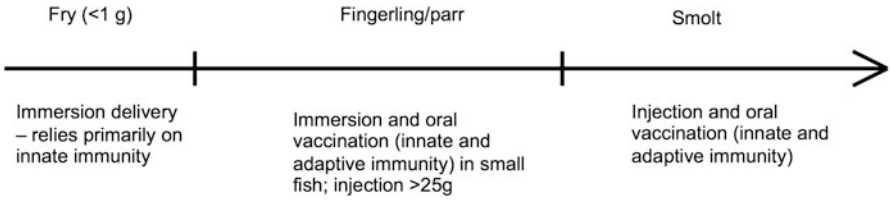

Fig. 3.3 Example from Atlantic salmon where size is given indicated relative to immunocompetence and vaccine modalities. In fry below 1 g, adaptive responses have not matured and any stimulation of the immune system would have to rely on an innate immune response. At a later stage and up to around 10 g, immersion or oral vaccination is preferred, while in larger fish, parenteral delivery is applied

Modalities and Fish Size

With intensification of fish farming, it has become prudent to protect the fish throughout the production cycle, including the early life cycle stages. Adaptive responses rely on partial or full immunocompetence, and the recommendation would be to postpone vaccination until the fish reach an age where they are able to mount an adaptive immune response. For salmonids, this will typically be around 0.5–1 g (Tatner and Horne 1983), while for other species, the animal can potentially have developed an ability to respond to vaccination at an even earlier stage, i.e. smaller sizes (Padros and Crespo 1996; Watts et al. 2003). Immersion and oral vaccines would have to meet these requirements, as would injection vaccines (Fig. 3.3).

Non-replicating or Nonlive Vaccines

The majority of fish vaccines intended for injection are inactivated, whole virus/bacteria vaccines, often prepared with an adjuvant like mineral or vegetable oil. Immersion vaccines are usually non-adjuvanted, and the majority of these vaccines are bacterin-based, while live vaccines, for injection or immersion, are used to a very limited extent. In line with the understanding that inactivated vaccines are B cell vaccines, i.e. elicit an antibody response, and that the oil formulation will function as a depot, injectable vaccines confer strong and long-lasting immunity towards infection with extracellular bacteria. Immersion vaccines will require repeated vaccinations to protect the fish throughout the production cycle.

Inactivated vaccines are less efficient against virus infections and diseases caused by intracellular bacteria. Examples are infectious pancreatic necrosis virus (IPNV), salmon pancreas disease virus (SPDV) and red sea bream iridovirus. Also it is difficult to obtain long-lasting immunity against salmon rickettsial syndrome (*Piscirickettsia salmonis* infections), while oral boost can strengthen the immune response to the pathogen (Tobar et al. 2015) and francisellosis (*Francisella noatunensis* infection). There is a need for alternative strategies to protect against intracellular

pathogens. Since the majority of fish is vaccinated by intraperitoneal injection, a future challenge in fish vaccinology is to develop vaccine formulations with lower injection-site reactions. Alternative delivery modalities are also needed, and vaccine efficacy must be improved when the antigen is delivered via mucosal surfaces.

Adjuvants and Principle of Action

The mode of action of adjuvants is in general poorly understood. Adjuvants aid in an early onset of immunity, long duration of effector responses, such as antibody formation or cytotoxic T cell activity, and make booster immunisations unnecessary (Singh and O'Hagan 2003). The mechanisms of adjuvanticity are complex and not fully understood. Adjuvants facilitate delivery of antigen (to the secondary lymphoid organs), which can be a time-dependent effect. Adjuvants provide a nonself microbial signal or a host-derived danger signal from stressed cells (Singh and O'Hagan 2003) and thereby increase the immune response to a given antigen and also prolong the immune responses, the latter being the depot effect. It is conceived as particularly important for fish for long-term immune protection (Evensen et al. 2005). Fish vaccines for parenteral delivery formulated with an adjuvant are typically a water-in-oil formulation with various emulsifiers and stabilisers added. Oils used are either of vegetable or mineral origin. Inactivated vaccines intended for immersion delivery come without an adjuvant and the same for live, attenuated vaccines and DNA vaccines.

Vaccines for salmonid fish, Atlantic salmon in particular, are for the most part administered parenterally and formulated with an adjuvant to enhance immunogenicity (and duration of immunity). Most vaccines currently available are non-replicating/nonliving vaccines. Non-replicating vaccines are preferred because of their safety in normal and even immunocompromised individuals, but they lack immunogenicity and require adjuvants being added.

Immunomodulation

Cytokines are involved in the regulation of immune response, both strength and profile. Cytokines can skew the immune response and will arm immune effector cells. T helper (Th) cells play a central role in the immune system by producing several cytokines that direct the immune responses into different categories of responses. Overall these are defined as Th1, Th2 and Th17, also in fish (Wang and Secombes 2013). Molecular tools allow in vitro production of cytokines that can be used as immunomodulators or stimulants in vaccine preparations. It should be noted though that cytokines serve in a fine-tuned network, and too high doses/concentrations can be deleterious to the host and promote disease rather than preventing it. Cytokines are also short-lived compounds, which can make it difficult to achieve prolonged effects.

Components added to vaccine preparations can also mimic pathogen "fingerprints" and interact with specific receptors of various cells, like toll-like receptors and cytosolic receptors recognising dsRNA compounds and triphosphate RNA sensors. Receptor ligands are potential compounds that can be used as immunomodulators in vaccines like dsRNA (poly I:C) (Kavaliauskis et al. 2015), CpG (Strandskog et al. 2011), and other synthetic compounds. The anticipation would be that we will see toll-like receptor ligands as immunomodulators in the vaccines before cytokines come into general use. These are particularly attractive for inactivated vaccines (Strandskog et al. 2011).

Molecular Technologies

Advances in molecular biology have provided many contributions to vaccine research particularly related to recombinant vaccine development (Kim et al. 2016). Techniques make it possible to knock out or insert genes in the pathogen genome; this can be used for study of virulence mechanisms (Kim et al. 2015) and pathogen components of importance for protective immunity. Techniques are used for development of vaccines candidates that carry certain advantages over conventional non-live or live vaccines, particularly when the pathogen is difficult to grow in culture (like piscine reovirus (Palacios et al. 2010) or piscine myocarditis virus (Haugland et al. 2011) of Atlantic salmon).

Recombinant Vaccines

New molecular technologies created expectations for new applications in recombinant vaccine technology; however, these were not fully met, and we have few recombinant vaccines for farmed fish and even so for warm-blooded animals or humans. The reasons are many. Recombinant, subunit vaccines have poor immunogenicity. For DNA vaccine technologies, we have seen that the dose needed to elicit an immune response is high (and thus there is a cost issue), and for many pathogens, we have seen poor efficacy (Evensen and Leong 2013), which again has discouraged many. Currently, one DNA vaccine is licensed for use in farmed fish against IHN in Atlantic salmon (for use in Canada (Alonso and Leong 2013).

Subunit Vaccines

Escherichia coli strains are used as competent cells for production of antigen at the end of the fermentation cycle. A classical example in fish vaccinology is the *E. coli*-based subunit vaccine against infectious pancreatic necrosis in Atlantic salmon,

licensed for the first time in 1995. *E. coli* is convenient for use, not the least because this bacterium is widely used in molecular biological work, and therefore many research laboratories have knowledge of the techniques and tools that are needed for proper expression of the transgene. Recombinant subunit vaccines have particular advantages if it is difficult to cultivate the disease-causing microorganism. This applies to some viruses and other micro-organisms. There is general agreement that recombinant subunit vaccines are safe for use but of inferior immunogenicity compared to inactivated, whole cell/virus vaccines (Webster and Laver 1966). Potent adjuvants are therefore needed to improve immunogenicity (see above).

Recombinant vaccines have also been produced in *Saccharomyces cerevisiae*, including a subunit vaccine against infectious salmon anaemia in Chile. Other vectors may also be used for production of recombinant of vaccines, like silkworms, cabbageworms, plants and insect cells. There are currently no commercial vaccines in the market based on any of these methods, but several experimental studies have been carried out, including IPN vaccines produced in cabbageworms (*Trichoplusia ni*) (Shivappa et al. 2005), plant-derived antigens expressing the capsid protein of nodavirus (Gomez-Casado et al. 2011), and the G-protein of viral haemorrhagic septicaemia virus has been produced in insect cells (Lorenzen and Olesen 1995; Lorenzen et al. 1993). All have been tested for their ability to induce immune responses and protective immunity and with variable results.

Plant-based vaccines are also attractive in the sense that they are potentially cheap to produce and can be maintained in well-defined environments. This is also referred to as molecular farming where whole plants or plant cells/tissues are cultured in vitro for the production of recombinant proteins (Schillberg et al. 2013). The system has been established as an economically viable alternative to mainstream production systems such as microbes and mammalian cells cultivated in large-scale bioreactors. Plants have several advantages compared with the traditional platforms for recombinant protein production; they are less expensive to maintain than cultured mammalian cells; they lack the undesirable components found in conventional systems, e.g. endotoxins in bacteria, and hyperglycosylated proteins produced by yeast, and there is no limit to the production scale and the cost of scaling up is low. This field is at an early stage for fish vaccines (Shin et al. 2013) but likely to develop in the near future.

Genetically Modified Vaccines

Live, attenuated vaccines generated through numerous in vitro passages have been used for many decades for vaccination of higher vertebrates. In vitro passaging results in accumulation of genome mutations that render the pathogen nonpathogenic, but the exact mechanisms of attenuation are usually not known. By the use of molecular techniques, it is possible to attenuate micro-organisms by removing/deleting specific genes or part of genes and thereby render the microorganism apathogenic. Live attenuated virus vaccine will replicate to a lower titre compared

to their pathogenic counterpart and will stimulate humoral (Munang'andu et al. 2013) and cellular immunity (Boudinot et al. 2001), although this is studied to a lesser extent in fish. They can also be used for induction of immunity at mucosal surfaces. Thus a broad immune response is one of the main benefits of live, attenuated vaccines as they are immunogenic and confer a high degree of protection against disease.

Reverse genetics is the method of choice for attenuated virus vaccines but of course not feasible for all virus species. There are examples where infectious haematopoietic necrosis virus and viral haemorrhagic septicaemia virus of the genus *Novirhabdovirus* have been rendered apathogenic by deleting the NV gene (Romero et al. 2008; Biacchesi et al. 2000; Thoulouze et al. 2004). These NV-knockout variants have then been used to immunise fish against IHN and VHS, and a high degree of protection has been obtained, for example, in Japanese flounder against VHS (Kim et al. 2011) and rainbow trout against IHN (Romero et al. 2008). Further avirulent variants of salmon pancreas disease virus (SPDV) made by reverse genetics have shown to give a high level of protection against sleeping disease in trout (Moriette et al. 2006). Mutations in the 3′-UTR of VHS virus have recently been used to develop a vaccine concept for use in Japanese flounder (Kim et al. 2016). The virus strain has attained some residual virulence in fry of flounder but induces a strong immunity.

Live attenuated bacterial vaccines can be made by recombinant technology where genes encoding specific enzymes are mutated or deleted. This can be enzymes required for production of certain amino acids and delta aromatic mutants (Δ*aroA*) of *Aeromonas salmonicida* have been used to vaccinate brown trout (*Salmo trutta*) against furunculosis with good protection (Vaughan et al. 1993; Marsden et al. 1996, 1998). The Δ*aroA* mutant proliferates in the kidney and is also retained for up to 12 weeks post vaccination (Grove et al. 2003). Similar findings have been obtained using an attenuated strain of *Edwardsiella ictaluri* against enteric septicaemia of catfish (Shoemaker et al. 2011).

Genetic stability is the key point when evaluating safety of live attenuated vaccines. The risk of "reversion to virulence" or "increase in virulence" is considered to be higher for vaccines attenuated by traditional methods (passage), than for vaccines attenuated by molecular methods. The reasoning is that vaccines attenuated by traditional passage will usually have point mutations in the genome, whereas vaccines generated by molecular techniques is better defined and can include "knock out" of the entire genes.

Vector Vaccines

A third type of recombinant vaccine is based on transfer of genes encoding one or more virulence factors and/or protective antigens of a pathogenic microorganism to a live avirulent microorganism, a vector. Vectors can be viruses and bacteria. A vector vaccine will stimulate a diverse immune response. Recombinant vector vaccines may stimulate humoral or cell-mediated immunity, which is usually not obtained for

inactivated vaccines. Vector vaccines have been tested to a very limited extent in finfish vaccinology. Immunisation with vector vaccines will also result in the development of an immune response against the vector/vector antigens. Pre-existing antibodies against the vector virus can neutralise or inhibit viral vector such that the immune response against the foreign antigens is reduced. Yet another type of vector vaccine is the replicon-based variant, where a gene of interest (GOI) has been cloned into an alphavirus-replicon, typically expressing the structural genes of the candidate alphavirus and the GOI (Vander Veen et al. 2012). A few studies have explored this potential for vaccines for farmed fish showing good level of protection using salmonid alphavirus-based replicons (Wolf et al. 2013, 2014). Additional studies have shown temperature sensitivity being associated with E2 protein expression (occurring only at temperatures between 10 and 15 C) is related to virion formation (Hikke et al. 2014) and would thus influence on immunity induced. Future studies should include other GOIs with the purpose to explore the applicability of this technology in general for finfish.

DNA Vaccines

DNA vaccination technology is rooted in gene therapy, the delivery of a therapeutic gene for expression in somatic tissue. It was shown relatively long ago that injection of naked plasmids into the muscle of mice can elicit an immune response (Ulmer et al. 1993). DNA vaccines will result in a transient expression of the gene of interest, and this is sufficient to evoke an immune response (Fig. 3.4).

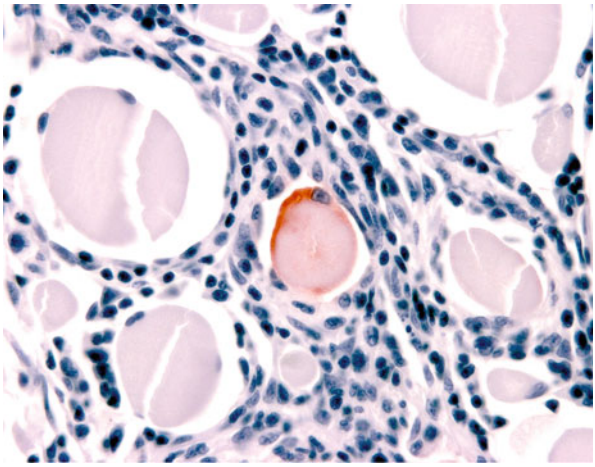

Fig. 3.4 Rainbow trout, skeletal muscle. Sample was collected 4 weeks post vaccination using a DNA vaccine encoding the G-protein of viral haematopoietic necrosis virus. Expression of the G-protein has been revealed by immunohistochemistry using a G-protein-specific rabbit serum (*red coloration*). There is a strong inflammatory response in the area of the muscle cell expressing the protein, dominated by lymphocytes

The efficacy of DNA vaccines is well documented for a number of fish pathogenic viruses and bacteria. More specifically, it has been demonstrated that DNA vaccines induce a strong and protective immunity to some viral infections in fish, particularly rhabdoviruses of rainbow trout and Atlantic salmon (Lorenzen and LaPatra 2005), for channel catfish herpesvirus infection (Nusbaum et al. 2002) and red sea bream iridovirus (Caipang et al. 2006). DNA vaccines also elicit protective immunity to bacterial kidney disease under experimental conditions (Gomez-Chiarri et al. 1996). DNA vaccines are, with a few exceptions (Ballesteros et al. 2012a, b, 2014), reliant on intramuscular injection for induction of protective immunity. For oral DNA vaccines, solid documentation of efficacy tested by in vivo challenge is meagre (Ballesteros et al. 2012b). The distribution to internal organs following i.m. vaccination has not been studied in detail, but it has been shown that a luciferase-encoding plasmid was distributed to internal organs and expression can be detected in organs shortly after administration (Romoren et al. 2004). Furthermore, luciferase expression in internal organs of fish has been observed over an extended period (up to 24 months) (Dijkstra et al. 2001). Cationic liposomes have been for delivery of DNA by the immersion route but have been met with severe toxicity problems (Romoren et al. 2002a, b, 2004). The mechanism of the acute toxicity is suggested to be an interaction between the cationic liposomes and anionic components of gill mucin, resulting in hypoxia and acute toxicity (Romoren et al. 2002b).

One important challenge for DNA vaccines is regulatory requirements and fish safety primarily related to genome integration. This applies to the vaccinated animal; the vaccine construct is not considered a GMO. Any such event is not likely to impact the health of the vaccinated animal, but such an event will have implications for food safety and the end user (Evensen and Leong 2013). Concerns have been raised as to production of anti-DNA antibodies in the vaccinated animal resulting in autoimmunity and also tumorigenicity, but studies so far lend very little support for this concern.

It has been demonstrated that retention and expression of antigens at the injection site appear for an extended time period, however, not beyond 4–5 weeks post vaccination (Lorenzen et al. 2005). The local reactions at the injection site are prominent and last for an extended period and longer than the actual antigen expression in situ. Strong inflammation and muscle cell degeneration and necrosis are seen at 3 and 12 weeks post vaccination (Lorenzen et al. 2005). There is currently one DNA vaccine for use in farmed fish against infectious haematopoietic necrosis virus in Atlantic salmon. This vaccine is licensed in Canada.

Marker Vaccines

Marker vaccines are used to distinguish between vaccinated and infected animals, also referred to as DIVA vaccine (differentiation between infected and vaccinated animals (Fu et al. 2011). Such vaccines are usually genetically modified, typically gene-deleted vaccines, or they lack an antigen against which the infected animal

will mount an immune response, while the infected animal will (van Oirschot 1999). All categories of recombinant vaccines may be used as marker vaccines. So far such marker vaccines (against a fish disease) have been tested in the lab (Enzmann et al. 1998) but are not available commercially.

Vaccines Against Parasitic Diseases

Parasites are causing major losses in aquaculture, worldwide, but there are currently no vaccines available for use in farmed fish. One reason is access to drugs for treatment and partly that it has proven difficult to vaccinate against parasites. Two main approaches have been explored for vaccination against parasites. These are based on live, attenuated parasites or they are based on subunit vaccines containing specific parasitic antigens or enteric origin and often produced by recombinant techniques, often referred to as concealed antigens (Wang and Nuttall 1999); the best studied is tick vaccine used in Australia against *Boophilus microplus* in cattle which is based on a protein, Bm86, as antigen (Jittapalapong et al. 2010; Nuttall et al. 2006; Willadsen and McKenna 1991). This protein is found in cell membranes in the intestine of the tick, and the host is not exposed to this protein when infested. The antigen is delivered as a subunit vaccine to the host, and blood feeding will result in circulating antibodies binding to the protein in the epithelial lining of the gut resulting in damage to intestinal functions of the parasite. The vaccine reduces losses and reduces the risk of other diseases transmitted by the parasite. A similar approach has been used for development of a sea lice vaccine in salmonids, *Lepeophtheirus salmonis* and *Caligus rogercresseyi* (Carpio et al. 2011). Studies report a significant reduction in the number of parasites per fish was observed at 24 days post challenge (Carpio et al. 2011), but such vaccines did not make it to field testing – at least not yet.

Future Directions

Multi enim sunt vocati, pauci vero electi "Many are asked to come, but only a few are chosen" (St. Matthews' Gospel, 22, 14). Oil-adjuvanted vaccines for fish are based on an "old technology", and while many studies have been carried out with an attempt to develop new and more advanced principles for immune induction, the light at the end of the tunnel is still dim. In humans, aluminium salts still remain the standard (Del Giudice et al. 2001), also an "old-fashioned" tool. There are few alternatives to oil adjuvants for many of the "difficult" fish pathogens. Many scientists and the industry have hopes for mucosal delivery systems, and major advancements have been done (Tobar et al. 2011, 2015), still with some limitations. Improvements of oil adjuvant delivery systems for fish have been seen over the last years, and reduced injection volume is a simple and effective way or reducing side-effect profiles. It is likely that we also for the future will make small steps rather than a giant

leap forward. My prediction would be that different modalities, prime-boost vaccination strategies and combination of modalities (injection prime and oral boost), where we have already seen the first products in the market (Tobar et al. 2015), will be the future also in fish vaccinology.

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Chapter 4
Adjuvants and Delivery Methods: Current and Novel

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Abstract Vaccination is the most appropriate method to control infectious diseases that threaten the aquaculture industry worldwide. Unfortunately, vaccines are usually not able to confer protection on their own, especially those vaccines based on recombinant antigens or inactivated pathogens. Therefore, the use of adjuvants or immunostimulants is often necessary to increase vaccine efficacy. Furthermore, an important additional problem that limits the entry of novel fish vaccines to the market is that many of the vaccines experimentally produced only work when injected (either intraperitoneally or intramuscularly). Therefore, the search for alternative methods of mass vaccine delivery (oral or immersion) should also be addressed in parallel. Unfortunately, it is probable that the search for a specific combination of antigen/adjuvant/delivery method has to be experimentally addressed for each pathogen/fish species, and only a few general conclusions can be drawn from each of these studies. In this chapter, we summarise previous studies performed with both traditional and new generation adjuvants as well as those studies that have explored methods for vaccine delivery alternative to injection.

Introduction

Disease prevention by vaccination is, on economic, environmental and ethical grounds, the most appropriate method for pathogen control currently available for the aquaculture production sector. Traditionally, vaccines comprise either live-attenuated, replicating pathogens or non-replicating, inactivated pathogens, or their subunits. In aquaculture, live vaccines are often not approved for safety reasons, and inactivated vaccines based on either killed pathogens or isolated non-replicating

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pathogen subunits are in many cases weakly immunogenic. Thus, adjuvants are required to ensure optimal immune responses and protection.

During the past, fish vaccines were made by a trial-and-error approach (conventional vaccine design) including pathogen identification, pathogen cultivation and vaccine formulation containing the complete inactivated pathogen, sometimes formulated in oil. Through using this strategy, vaccines based on whole inactivated extracellular bacterial pathogens were quite efficient – resulting in important reductions in mortalities and antibiotic usage in the aquaculture industry (Hastein et al. 2005). However, many of the economically important diseases as of today are due to intracellular pathogens, and for this type of pathogens, the production of effective vaccines has been quite challenging. Therefore, fish vaccine development strategies should be subjected to a rational vaccine design wherein a combination of a tailored adjuvant system with the most appropriate antigen(s) is used to create vaccines that may provide a more effective immune response against a specific pathogen with minimal side effects.

Additionally, injection vaccination is labour intensive, expensive and not feasible for early fish stages, even though it is precisely at these early stages when vaccination is often needed. Thus, exploring novel strategies for mass delivery (immersion or oral) of vaccines is an important field of research that still needs to be developed. In this sense, specific adjuvants may also contribute to increase the immunogenicity of vaccines delivered through these alternative routes.

One of the main limitations for the selection of an adequate antigen/adjuvant/delivery method is the fact that many aspects of fish immunology are still unknown, and we are far from close to understanding which are the exact immune correlates of natural or vaccine-mediated protection (Secombes 2008). Moreover, there are currently close to 22,000 different fish species, and most of them have their "immune peculiarities". Although the innate defence system of fish plays an important role in eradicating infectious agents, many pathogens resist innate defence mechanisms, and then an adaptive immune response, present for the first time in evolution in teleost fish, must come into play to fight these pathogens. The adaptive immune response is the basis for vaccinology and provides the vertebrate immune system with the ability to recognise and remember specific pathogens, to be able to mount stronger and faster responses each time this pathogen is encountered. In higher vertebrates, adaptive immunity to extracellular pathogens is generally mediated by humoral immune responses (antibodies), while immunity to intracellular pathogens (including viruses) often relies on cellular immune responses (cytotoxic T cells). In fish, and despite the fact that the main elements for an adaptive immune response are present in most species, the regulation of these elements greatly differs from mammalian systems and even among different species. Both immunoglobulin (Ig) or B cell receptor (BCR) and T cell receptor (TCR) genes are known among all lineages of gnathostomes (jawed vertebrates), but in fish Ig are expressed as only as three isotypes (IgM, IgD and IgT) with no isotype switching and with low affinity maturation (Hikima et al. 2011). Interestingly, there is a tight link between the innate and adaptive system that has not been much explored in fish immunology.

This link, governed by several innate receptors and signalling molecules such as cytokines and transcription factors, is key in the responses following activation by vaccine adjuvants, since recent advances in immunology have shown that the magnitude and specificity of the signals perceived by the innate immune cells following vaccination shape subsequent adaptive immune responses (Palm and Medzhitov 2009).

Principles of How Adjuvants Work

Adjuvants have traditionally been defined as helper substances that increase the magnitude of an adaptive response to a vaccine (potency) or ability to prevent infection and death (efficacy). But nowadays, scientists have acknowledged that adjuvants may become more important in the way they instruct or guide the type of adaptive response against a specific pathogen. Thus, adjuvants have been now defined as a group of structurally heterogeneous compounds able to modulate the intrinsic immunogenicity of an antigen (Guy 2007). They can be classed according to their chemical nature or physical properties; however, since even related compounds can have very different immunomodulating capacities, new classifications have focused on the immunological events they induce, even though for many of them the exact mechanism of action is unknown. At present, the classification of adjuvants that distinguishes between signal 1 facilitators and signal 2 facilitators has been widely accepted (Schijns 2001). According to this two-signal model, both the presentation of an antigen (signal 1) and the additional secondary signals (signal 2) are required for activation of specific T and B lymphocytes, which form the adaptive arm of the immune system (Ribeiro and Schijns 2010). The signal 1 facilitators influence the fate of the vaccine antigen in time, place and concentration, ultimately improving its immune availability, while signal 2 facilitators provide the co-stimulation signals during antigen recognition that will provide an adequate environment for the most adequate antigen-specific immune response.

Another important aspect of the immune response conferred by adjuvants is the fact that they mimic the recognition of microbes through the detection of conserved molecular patterns, designated as pathogen-associated microbial patterns (PAMPs) or microbe-mediated tissue damage through damage-associated molecular pattern molecules (DAMPs). These molecules activate pathogen recognition receptors (PRRs) that include Toll-like receptors (TLRs), C-type lectin receptors, NOD-like receptors, RIG-I-like receptors and peptidoglycan recognition proteins (PGRPs) that are predominantly found on cells of the innate immune system. Nowadays, this first recognition is considered critical in signal 2 induction and downstream activation of distinct T helper cell subsets; however, other authors make a distinction and refer to adjuvants that trigger PRRs as signal 0 adjuvants. In fact, most of the recent research on adjuvants has especially focused on different PRR ligands.

Signal 1 Adjuvants Used in Fish Vaccinology

To increase the immunogenicity of an antigen, a slow release is often achieved through the introduction of the antigen in the context of an emulsion. An emulsion is defined as a dispersion of a liquid, called the dispersed phase, and in a second liquid, called the continuous phase, with which the first one is not miscible. In vaccine formulations, these phases are water (often with added antigens) and oil. In order to stabilise the emulsions, surfactants are added. A surfactant is a compound containing a polar group that is hydrophilic and a non-polar group that is hydrophobic and often composed of a fatty acid residue. Surfactants can be defined by their hydrophilic-lipophilic balance (HLB) value which gives information on their relative affinity for both phases. According to the HLB value of the surfactant, different kinds of emulsions can be achieved (Aucouturier et al. 2001). Those having a low HLB value have a high affinity for oily phases and render W/O emulsions, whereas those with a high HLB value have a high affinity for the aqueous phase and render O/W emulsions, which are well tolerated but induce a shorter-term immune response. With certain specific surfactant systems, when the HLB value is intermediate, W/O/W emulsions can be achieved. In this case, the continuous phase is aqueous and the dispersed phase is oil. But inside the oil droplets, an entrapped aqueous phase with water-soluble antigens/suspended antigens is found. This type of emulsions has shown to generate long-term immune responses with various antigens.

Freund's Complete Adjuvant

Mineral oils are widely used in vaccine manufacturing processes and are a mixture of alkanes in the C_{15}–C_{40} range often obtained from non-vegetable sources. The most widely used and most effective adjuvant for experimental purposes has been Freund's complete adjuvant (FCA). FCA is composed of heat-killed mycobacteria and a mineral oil with surfactant (Opie and Freund 1937). Before injection, the antigen in an aqueous solution is mixed with the FCA producing a stable W/O emulsion. Immunisation with FCA and antigens results in strong Th1 and Th17 responses mostly via the MyD88 pathway. Unfortunately, the use of FCA has been associated with a variety of severe side effects including injection site granuloma; therefore, its use has been limited within animal research. Surprisingly, the use of FCA in fish has not always resulted in increase in immunogenicity or protection – as outlined below.

Pasteurellosis, caused by *Pasteurella piscicida*, also named *Photobacterium damselae* subsp. *piscicida* is one of the major diseases in many species of wild and farmed fish in Asia, the USA and Europe. In yellowtail (*Seriola quinqueradiata*), a susceptible species, vaccination against pasteurellosis has been assayed with a lipopolysaccharide (LPS)-mixed chloroform-killed bacterin that resulted in protection against challenge with the virulent bacterium. In this case, the inclusion of

FCA in the vaccine did not significantly enhance the protective effect (Kawakami et al. 1998).

Streptococcus iniae is a Gram-positive bacterium associated with disease in several commercial species including tilapia (*Oreochromis aureus* and *O. niloticus*), yellowtail, hybrid striped bass (*Morone saxatilis*), turbot (*Scophthalmus maximus*) and rainbow trout (*Oncorhynchus mykiss*). Vaccination of rainbow trout with a formalin-killed culture of *S. iniae* resulted in good protection against experimental challenge that was not significantly potentiated in the presence of FCA (Soltani et al. 2007).

Aeromonas salmonicida is the etiological agent for furunculosis. In a study in coho salmon (*Oncorhynchus kisutch*), formalin-killed *A. salmonicida* was interaperitoneally (i.p.) injected in the absence or presence of FCA. In this model, the best protection was found in the vaccine in which FCA was included with *A. salmonicida* compared to the antigen in saline. Interestingly, fish injected with FCA (without antigen) were partly protected even 90 days after challenge (Olivier et al. 1985). Thus, it seems that injection of inactivated *M. bovis* may induce innate defence mechanisms that may result a certain degree of protection to a heterologous pathogen, as shown by Kato et al. (2012) where Japanese flounder (*Paralichthys olivaceus*) were partially protected against nocardiosis with FCA exclusively. In a recent study, Zheng et al. (2012) compared naturally occurring adjuvants (astragalus polysaccharide and propolis) with FCA in a pentavalent vaccine. In that study, FCA outcompeted the other adjuvants despite the immunostimulant activities of the natural adjuvants.

Recently, a vaccine against *A. hydrophila* was prepared (LaPatra et al. 2010) using a bacterial lysate. The vaccine was administered i.p. in combination with FCA, and the efficacy of the vaccine was studied using a new challenge model optimised for rainbow trout in which *A. hydrophila* was injected into the dorsal sinus. The vaccine provided protection and this protection could be potentiated with FCA (LaPatra et al. 2010).

Flavobacterium psychrophilum is a widespread Gram-negative pathogen in freshwater causing rainbow trout fry syndrome (RTFS) and bacterial cold-water disease (BCWD) (Hogfors et al. 2008). In addition to rainbow trout, coho salmon is the most susceptible species together with other non-salmonid species which are also affected. Injection of a low molecular weight fraction emulsified in FCA resulted in an enhanced level of protection for rainbow trout (Hogfors et al. 2008).

Flavobacterium columnare is a Gram-negative bacterium responsible for columnaris disease. The disease was first described in 1917 in several warm-water fish species from the Mississippi river, and since has been isolated from freshwater fish species worldwide (Grabowski et al. 2004). Specific antibodies were found in tilapia plasma and mucus following i.p. injection of formalin-killed sonicated (disrupted cells with ultrasonic frequency) or whole cells of *F. columnare* in FCA within 2 weeks. After a secondary immunisation, the antibody response increased and remained elevated at 10 weeks post-immunisation. Antibodies were also observed in cutaneous mucus in fish i.p. immunised with formalin-killed sonicated cells in FCA 6 and 8 weeks post-immunisation (Grabowski et al. 2004).

Freund's Incomplete Adjuvant

Because of its high toxicity, the use of FCA has been widely replaced by Freund's incomplete adjuvant (FIA) that lacks the mycobacterial components of the emulsion, being therefore just a W/O emulsion. This adjuvant is still highly effective in vaccination with a significant reduction of toxicity; however, peritonitis is still a major side effect, as perfectly detailed for Atlantic cod (*Gadus morhua*) (Gjessing et al. 2012).

Edwardsiella tarda is a Gram-negative intracellular bacterium that can infect both marine and freshwater fish, including Japanese flounder. In order to develop effective vaccines against this pathogen, fish were i.p. injected with a vaccine containing a major antigenic protein of *E. tarda* in the absence or presence of FIA (Jiao et al. 2010a). Protection against experimental challenge achieved by the vaccine without adjuvant resulted in a relative percentage survival (RPS) of 34 % that was increased to 81 % in the presence of FIA. Moreover, vaccination with the oil-adjuvanted antigen stimulated the expression of a series of genes like complement component 3 (C3), major histocompatibility complex (MHC) class I and MHC class II, CD8α, CD40, Mx, interferon γ (IFN-γ), tumour necrosis factor α (TNF-α) and interleukin 6 (IL-6), whereas vaccination with the antigen alone resulted in increased expression of just IgM, MHC class I and class II and Mx (Jiao et al. 2010a).

Nocardia seriolae is a Gram-positive acid-fast bacterium that causes nocardiosis in cultured marine and freshwater fish in Taiwan, Japan and China. Although the disease results in considerable economic loss, no commercial vaccines are available. Recently, an oil-adjuvanted vaccine was developed and tested on protection against challenge with a virulent strain (Shimahara et al. 2010). Formalin-inactivated whole cell antigen was used as a vaccine with or without FIA; however, even though antibody levels increased, no protective effects were found.

Another Gram-positive bacterium that causes disease (lactococcosis) and mortality in rainbow trout is *Lactococcus garvieae*. In this case, the vaccine was prepared based on formalin-inactivated bacterin or bacterin suspended in FIA, fish were given i.p. injections and challenged by exposure to virulent bacteria 30, 75 and 125 days after vaccination (Kubilay et al. 2008). At 125 days after vaccination, the RPS in fish vaccinated with bacterin only was 54 %, whereas it was 85 % in fish vaccinated with bacterin and FIA.

Tenacibaculum maritimum is a marine bacterium that causes flexibacteriosis worldwide. In Australia, Atlantic salmon (*Salmo salar*) and rainbow trout are the most heavily affected species, and due to the lack of vaccines, so far the disease has been treated with trimethoprim and oxytetracycline with the subsequent negative impact on the environment (Van Gelderen et al. 2009). Salmon injected with formalin-inactivated bacteria mixed with FIA provided protection against challenge with *T. maritimum,* while the vaccine without the adjuvant could not provide sufficient protection against a moderate challenge of *T. maritimum*.

Infection with fungi oomycetes such as *Aphanomyces invadans* may cause heavy mortalities of fresh water and estuarine fish species as a result of granulomatous

inflammation. In catla (*Catla catla* Hamilton), a fungal extract combined with FIA showed to increase both the survival rate and the antibody response compared to non-adjuvanted vaccines (Saikia and Kamilya 2012).

Montanide

Mineral oil adjuvants registered under the trademark of Montanide by Seppic have been optimised in order to improve efficacy and stability of vaccine formulations and to reduce side effects. These adjuvants are based on either mineral oil, non-mineral oil or a mixture of both, as well as those made from specific surfactant chemistry using dianhydro-D-mannitol monooleate (e.g. Montanide ISA 720) and may be used to manufacture different type of emulsions, W/O, O/W or W/O/W, for use in both mammals and fish (Lawrence et al. 1997; Ravelo et al. 2006).

Philasterides dicentrarchi is a scuticociliate parasite that causes mortalities and significant economic losses in cultured turbot (Lamas et al. 2008). An important attempt to optimise a vaccine against this parasite was performed on the basis of antigenic dose, concentration of inactivating agent (formalin) and proportion of the adjuvant Montanide ISA763A (W/O, non-mineral oil) in the emulsion. The results of this study showed that a high concentration of antigen, 0.2 % formalin and 50 % adjuvant generated the longest time of survival after challenge 30 days after the second injection, and the highest levels of antibodies in the vaccinated fish (Lamas et al. 2008).

Pseudomonas plecoglossicida is a bacterium causing bacterial hemorrhagic ascites of cultured ayu (*Plecoglossus altivelis*). To develop a vaccine against the disease, formalin-killed *P. plecoglossicida* bacterin was emulsified with Montanide and injected i.p. The fish were challenged with an i.p injection of virulent *P. plecoglossicida* 22 and 52 days after vaccination (Ninomiya and Yamamoto 2001). The RPS of vaccinated fish was 17–58 % without adjuvant, 57–92 % with Montanide ISA711 and 65–86 % with Montanide ISA763A. Another study on the same disease and adjuvant (Montanide ISA 763A) concluded that there is a good correlation between antibody levels and protection against disease in a challenge test (Sitja-Bobadilla et al. 2008).

To study the efficacy of different adjuvants in Atlantic halibut (*Hippoglossus hippoglossus*), fish were injected i.p. with a model vaccine of human gamma globulin with either FCA or Montanide ISA711 as adjuvants (Bowden et al. 2003). Antibody responses and intraperitoneal adhesions were examined every month for up to 12 months. FCA produced the highest and fastest antibody response, since in the group injected with the Montanide adjuvant only 4 of 47 fish reached a titre of 1:1000 (on month 6) compared to 27 of 48 fish in the FCA group (after 2 months); however, FCA also induced the fastest intraperitoneal adhesions (Bowden et al. 2003).

In a recent study in carp (*Cyprinus carpio*), a recombinant S-layer protein of *A. hydrophila* was used to assess the ability to protect fish against six virulent isolates

of *A. hydrophila*. The recombinant S-layer protein of *A. hydrophila* was produced, diluted in phosphate buffered saline (PBS) and mixed with a Montanide adjuvant at a ratio of 30:70. Common carp were i.p. injected with the emulsion, and after 35 days, the fish were challenged with six different isolates of *A. hydrophila* (Poobalane et al. 2010). The RPS values varied between the different challenge isolates (40–75 %), but it was concluded that the S-layer protein together with Montanide adjuvant is a good candidate for an efficacious vaccine against this bacterium.

Furthermore, Montanide ISA-763 has also been used as an adjuvant in experimental bivalent vaccine for *L. garvieae* and *A. hydrophila* with high degree of efficacy in rainbow trout (Bastardo et al. 2012).

Other Mineral Oil Adjuvants

Moritella viscosa is the causative agent of winter ulcers in farmed fish like Atlantic salmon and Atlantic cod. Vaccination of Atlantic salmon against *M. viscosa* is performed with oil-adjuvanted polyvalent injection vaccines based on formalin-inactivated bacterial cultures, using an AJ-oil (Alphaject 5200) used in some vaccines commercialised by Pharmaq (Gudmundsdottir and Bjornsdottir 2007). However, a multivalent commercial salmon vaccine containing *M. viscosa* as one of five bacteria mixed in a mineral oil adjuvant (Alphaject 5200) did not protect turbot against challenge (Bjornsdottir et al. 2004), whereas moderate intra-abdominal adhesions were detected in vaccinated fish.

Other commercial oil-adjuvanted vaccines have been shown to give protection in Atlantic salmon against bacterial diseases like vibriosis, cold-water vibriosis and furunculosis for a long time. However, side effects and retardation in growth have been clearly demonstrated (Midtlyng and Lillehaug 1998; Midtlyng et al. 1996). Mutoloki and coworkers investigated the intraperitoneal lesions induced by an oil-adjuvanted vaccine against infection with *A. salmonicida* and *M. viscosa* in Atlantic salmon (Mutoloki et al. 2010). The cellular composition was typical of granulomas containing large macrophages, eosinophilic granular cells, lymphocytes and multinucleate cells.

Oil-adjuvanted vaccines are also used to control sea bass (*Dicentrarchus labrax*) against bacterial diseases like vibriosis and pasteurellosis. Sea bass is one of the most used fish species in the Mediterranean area, and suffers from infection by *V. anguillarum* and *Photobacterium damselae* subsp. *piscicida*. Oil-adjuvanted vaccines against these diseases have been prepared and injected i.p., but despite their effectiveness, granulomatous peritonitis was also recognised (Afonso et al. 2005).

The major bacterial disease of farmed Atlantic cod is classical vibriosis (Samuelsen et al. 2006). Cod vaccinated by injection with mineral oil-adjuvanted vaccines against both *V. anguillarum* and atypical *A. salmonicida* were very well protected against homologous challenges (Mikkelsen et al. 2004). In this model, even without adjuvant, the fish were protected against *V. anguillarum*, but not against atypical *A. salmonicida* challenge.

Signal 2 Facilitators and TLR Ligands as Adjuvants or Immunostimulants

In general, signal 2 facilitators do not influence the concentration and distribution of antigen between injection site and presentation site, but provide co-stimulatory signals during the antigen recognition phase, thus increasing the immune response or skewing it to provide the most suitable immune environment for the establishment of protection. This category of vaccine adjuvant has dominated the literature on vaccine research in the last decade, and includes "stranger" and "danger" molecules, as well as inflammatory cytokines.

"Stranger" and "danger" signals are recognised by innate receptors such as TLRs. Teleost fish species may possess close to twice the number of different TLRs compared to mammalian species, presumably due to an ancient genome duplication event. This may open up new possibilities adding signal 2 facilitators into fish vaccines. However, the existing polyvalent fish vaccines may already contain a high number of different PPR agonists that complicate a further improvement by using rational vaccine development. Several up-to-date reviews on immune relevant genes including TLR-like receptors in fish have been recently published (Palti 2011; Zhu et al. 2013), providing an excellent overview of the current knowledge on fish TLR. In general, those TLRs that, after ligand binding induce the production of IL-12, favour a Th1 response (TLR 3, 4, 5, 7, 8, 9 and 11) and may induce cross-presentation of antigens facilitating a cytotoxic T cell response under certain conditions (Manicassamy and Pulendran 2009). It should be mentioned that ligand binding to TLRs 3 and 4, 7 and 9 may also induce type I IFN responses via interferon regulating factors. Within this group of signal 2 facilitators, we have also included aluminium salts, as it has been recently discovered that these adjuvants directly interact with dendritic cells in a similar way to that of danger signals (Flach et al. 2011).

Aluminium-Containing Adjuvants

The adjuvant property of aluminium salts was discovered in 1926 (Glenny et al. 1926). Aluminium compounds (collectively termed as "alum"), especially aluminium phosphate and aluminium hydroxide, are some of the few adjuvants that have been allowed and considered safe to use in human vaccines. Aluminium adjuvants have been shown to induce Th2 responses almost exclusively (Jiao et al. 2010a), thus they have been used as adjuvants with great success, being particularly effective at promoting protective humoral immunity. However, alum is not optimally effective for diseases where cell-mediated immunity is required for protection. It was believed that alum activates NLRP3 inflammasome and induces necrotic cell deaths that release the danger signal "uric acid" (Coffman et al. 2010). However, it has been discovered very recently that being in a more crystalline form, alum binds

dendritic cell plasma membrane lipids with substantial force, independent of inflammasome and membrane proteins (Flach et al. 2011). The subsequent lipid sorting activates an abortive phagocytic response that leads to antigen uptake. Such activated dendritic cells, without further association with alum, show high affinity and stable binding with $CD4^+$ T cells via the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen-1 (LFA-1). Despite its potential, only a few studies have used aluminium adjuvants in the optimisation of fish vaccines (see below).

A vaccine against *A. salmonicida* mixed with potassium aluminium sulphate (alum) as an adjuvant was tested in Atlantic salmon more than 15 years ago (Mulvey et al. 1995). Alum appeared to enhance the protection against challenge, but not significantly. In another study, an *Escherichia coli* mutant was used for vaccination against *Edwardsiella ictaluri*-induced enteric septicaemia of catfish (*Ictalurus punctatus*). Killed *E. coli* bacteria with or without alum were administered i.p to catfish, and the fish were challenged with virulent *E. ictaluri* bacteria (Tyler and Klesius 1994). Fish given *E. coli* in alum showed an enhanced survival (92 %) compared with the fish in which *E. coli* was administered alone (54 %) or fish given saline (56 %).

Recently, an aluminium hydroxide-adjuvanted *E. tarda* vaccine was prepared and injected i.p in Japanese flounder. After an experimental challenge, the RPS obtained was 69 % (Jiao et al. 2010a), higher than when the antigen alone was used (RPS = 34), but lower than that obtained with the FIA-coupled vaccine (RPS = 81).

Another experiment has been recently carried out by Fan et al. (2012), in which formalin-inactivated reddish body iridovirus (TRBIV) was mixed with alum and either injected or bath administered (prime-boost) in turbot. The resulting RPS calculated was 83.3 % and 90.5 %, respectively.

ß-Glucans

ß-glucans are known to stimulate the nonspecific (innate) immune response of both mammals and fish through the action of dectin-1 (Dalmo and Bogwald 2008; Robertsen 1999). A high number of different ß-glucans with varying molecular assembly (e.g. linear, branching by single residues, and/or ß1,3-ß1,6-branching networks) and thus molecular weight exist – often dependent on their source; but it is acknowledged that the ß-glucans possessing the ß1,3-D conformation are the biological active ones (Dalmo and Bogwald 2008).

DeBaulney and coworkers prepared an oral vaccine against vibriosis for use in turbot, and after feeding the vaccine for 5 days, the fish were challenged 28 days thereafter. Fish given the vaccine alone resulted in a RPS of 52 %, while a combination of the vaccine and ß-glucan gave a RPS on 61 %, higher protection levels, but not statistically different from the vaccine alone (DeBaulney et al. 1996). In 1998, an attempt to establish immunisation protocols to obtain the highest immune response against *V. damsela* was performed in Spain (Figueras et al. 1998). These

authors i.p. injected the O-antigen of *V. damsela* in combination with ß-glucan. As a correlate to vaccine efficacy, the phagocytic index of head kidney macrophages was evaluated. There was an enhancement of the phagocytic index in fish injected with ß-glucan at the same time or after the antigen injection when compared with fish injected with ß-glucan before the antigen. Similar results were obtained with regard to antibody titres (Figueras et al. 1998).

Yeast glucan (mainly a ß-1,3-D glucan) was included in a furunculosis vaccine based in a formalin-killed culture of *A. salmonicida* and *V. salmonicida* (Rørstad et al. 1993). The vaccine, either with or without ß-glucan, was injected i.p. and salmon challenged 3–46 weeks after vaccination. Vaccines supplemented with ß-glucan induced significantly higher protection against furunculosis than vaccines without this adjuvant (Rørstad et al. 1993), but ß-glucan alone did not result in protection. In another study, ß-glucan-adjuvanted vaccines against furunculosis seemed to give protection at an early time point after vaccination (6 weeks), but no protection was seen after 3 and 6 months (Midtlyng et al. 1996). As a side effect, the average weight of the ß-glucan-adjuvanted group was significantly lower compared to the controls, but this weight loss was even higher in fish given oil adjuvant (Midtlyng and Lillehaug 1998). In a further study performed in coho salmon, Nikl et al. evaluated the potentiating effect of seven substances in combination with a formalin-treated *A. salmonicida* bacterin (Nikl et al. 1991). Statistically significant improvement in survival over the group receiving bacterin alone was noted in fish groups that received ß-glucans like VitaStim-Taito and lentinan (Nikl et al. 1991).

Catla is one of the major Indian carp species often affected with *A. hydrophila*; thus a formalin-inactivated *A. hydrophila* vaccine was developed, and protection was studied in the absence and presence of ß-glucan (Kamilya et al. 2006). A reduction in mortality was found in the presence of ß-glucan compared to the vaccine itself, although the differences were not statistically significant (RPS of 67.7 % and 58.0 % with and without the adjuvant, respectively). In carp, a vaccine against *A. hydrophila* showed a higher antibody titre when ß-glucan was i.p. injected prior to vaccination, while bath and oral administration of ß-glucan before vaccination did not result in enhanced antibody response (Selvaraj et al. 2005). In a further study by Selvaraj and coworkers, carp were vaccinated against *A. hydrophila* with LPS from a virulent strain of the bacterium in the presence of different concentrations of ß-glucan and administered through various routes such as i.p, oral or bath (Selvaraj et al. 2006). The RPS was significantly higher in i.p.-injected groups even at the lowest concentration of ß-glucan, and fish given a mixture of LPS and ß-glucan orally obtained a higher RPS compared to controls. The administration of the LPS-glucan by bath did not result in increased survival, and antibodies were never detected in fish vaccinated either orally or by bath. However, no possible analysis of the contribution of β-glucan in the vaccine efficacy could be established because an obvious control group in this study was missing, namely, the protective effect of LPS without adjuvant (Selvaraj et al. 2006).

In another study, the i.p. injection of ß-glucan on days 1 and 3 followed by two i.p. immunisations of *E. ictaluri* on days 7 and 14 performed in channel catfish resulted in higher serum antibody levels relative to catfish receiving PBS instead of

ß-glucan before administration of *E. ictaluri* (Chen and Ainsworth 1992). Serum antibody levels were determined on day 7 (day 21) after the last immunisation, reaching antibody titres twofold higher in fish that had been treated with ß-glucan.

In order to investigate possible treatments against *A. hydrophila* in blue gourami, laminaran, a ß-1,3-D glucan, was injected i.p. in the absence and presence of formalin-killed *A. hydrophila* bacteria (Samuel et al. 1996). A single i.p. injection of 20 mg kg^{-1} laminaran alone was sufficient to protect the fish against infection by a virulent strain of *A. hydrophila* up until 29 days after injection in correlation with an increased phagocytic activity of head kidney phagocytes. Despite this, the addition of 20 mg kg^{-1} laminaran to a formalin-killed *A. hydrophila* did not significantly improve the protection (Samuel et al. 1996).

Recently, the potential immunostimulatory effect of orally administered β-glucan was investigated in combination with immersion vaccination against *Yersinia ruckeri* in rainbow trout (Skov et al. 2012). Although the β-glucan had no effect on survival in either unvaccinated or vaccinated fish, some immune effects due to β-glucan were observed in vaccinated fish. These effects included differences in plasma lysozyme activity, bacterial clearance and immune gene transcription in fish that were fed the β-glucan and unfed fish.

Saponins

Saponins are naturally occurring glycosides of squalenes or triterpenes that have been widely explored as adjuvants in different mammalian systems due to their capacity to stimulate both Th1 and Th2 responses (Sun et al. 2009). The most widely studied saponins are Quil A (saponin extracted from the cortex of the South American tree *Quillaja saponaria* Molina consisting on a mixture of more than 25 different saponin molecules and one out of three components of ISCOMs) and their derivatives; however, due to their high cytotoxicity and instability in aqueous phase, the use of different kinds of saponins is being explored.

In Japanese flounder, formalin-killed *E. tarda* cells were administered to fish by feeding in the absence or presence of curdlan (a ß1,3 glucan) or curdlan together with Quil A saponin. Although the incorporation of curdlan gave higher survival rates, only the group in which the vaccine was administered with both curdlan and Quil A showed a statistically significant increased survival (Ashida et al. 1999).

Poly I:C

Polyinosinic:polycytidylic acid (Poly I:C) is a double-stranded polyribonucleotide that mimics a viral infection and therefore has been widely used to induce type I IFN in many species including fish (Eaton 1990; Jensen et al. 2002; Plant et al. 2005). The number of residues of Poly I:C normally spans from 200 to 8 kb, but unfortunately, in most instances, the molecular weight of the Poly I:C used is not

listed in the various reports. This makes comparisons difficult – as the number of Poly I:C molecules added to the biological system differs from one study to another. This may be crucial when receptor-mediated and biological responses are addressed. IFNs are cytokines with a major role in the early defence against viral infections, and Poly I:C induces indeed a nonspecific antiviral response after its binding to TLR3 and the subsequent activation of intracellular signalling events inducing, e.g. type I IFNs. This nonspecific antiviral activity of Poly I:C has been tested in rainbow trout infected with infectious haematopoietic necrosis virus (IHNV) (Kim et al. 2009). Fish pre-injected with Poly I:C were protected against IHNV challenge 2 days later, and IHNV-specific antibodies were detected in survivors. The survivors showed a 100 % survival rate following re-challenge with IHNV both 21 and 49 days after the primary IHNV challenge (Kim et al. 2009), demonstrating the fact that fish were at an antiviral state during the initial infection by a virus, gave them an important advantage for posterior specific antibody production. A similar study was performed in the sevenband grouper *Epinephelus septemfasciatus* in which fish were immunised against the nodavirus red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al. 2009). Fish injected with Poly I:C intramuscularly (i.m.) and challenged i.m. with RGNNV 2 days post-injection showed more than 90 % survival rate. When surviving fish were re-challenged with RGNNV 3 weeks after the primary challenge, no mortalities were detected in the group that had been previously exposed to Poly I:C; probably because upon RGNNV challenge, the antibodies against the virus were higher in these fish. All survivors that were re-challenged with RGNNV showed even higher levels of specific antibodies. In addition, the RGNNV titres in brain tissues of the survivors in the Poly I:C-RGNNV-RGNNV group were all under the detection limit (Nishizawa et al. 2009). Following up this work, this research group conducted a field trial exploring the vaccine efficacy of a RGNNV vaccine followed by Poly I:C injection. The Poly I:C-adjuvanted vaccine showed a relatively high efficacy, but a one-shot Poly I:C injection in sevenband grouper 20 days after a natural RGNNV outbreak also induced a high survival rate (93.7 %) compared to non-treated fish (9.8 %) (Oh et al. 2012).

A prophylactic strategy using Poly I:C was also used by Takami and coworkers in Japanese flounder experimentally infected with viral haemorrhagic septicaemia virus (VHSV) (Takami et al. 2010). The survival rate in Japanese flounder pre-injected with Poly I:C before a VHSV challenge was 100 %, while all untreated fish died within 9 days. Survival rates of the fish given a secondary challenge with VHSV were 100 % in the Poly I:C-VHSV group (Poly I:C-VHSV-VHSV group), while non-immunised fish showed a 0 % survival.

Lipopeptides

Lipoproteins and lipopeptides have been found in a large number of microorganisms, the most prominent being mycobacteria and mycoplasms. These molecules have been found to exhibit both a strong inflammatory response and a long-lasting

adaptive immune response in mammals; however, very few studies have been performed on lipopeptides in fish. The adjuvant effect of polar glycopeptidolipids in experimental vaccines against *A. salmonicida* was investigated (Hoel and Lillehaug 1997), using polar glycopeptidolipids (pGPL-*Mc*) from *Mycobacterium chelonae*, one of three mycobacteria species that are fish-pathogenic. Twelve weeks after vaccination, the antibody response of fish given 0.25 mg kg^{-1} pGPL-*Mc* in combination with an *A. salmonicida* bacterin was significantly higher than that induced by a non-adjuvanted bacterin. Increased doses of pGPL-*Mc* suppressed the antibody response, while no significant side effects were observed in the peritoneal cavity after the use of this adjuvant (Hoel and Lillehaug 1997).

Flagellins

The structural protein of Gram-negative flagella is called flagellin. Flagella are composed of several monomeric flagellins assembled to a core region where the filaments then possess helical shape. Flagellin is a potent activator of a broad range of cell types within the innate and adaptive immune system, promoting cytokine production (Mizel and Bates 2010). Flagellin is known to induce immune responses via the TLR5 signalling resulting in a mixed Th1 and Th2 response, although it has also been reported that inflammasomes containing NLRC4/IPAF may bind cytosolically located flagellin (Coffman et al. 2010). During the last decade, the adjuvant effect of flagellin has widely been studied in vertebrates and, during the last couple of years, also in fish (Jiao et al. 2009, 2010b; Wilhelm et al. 2006).

Piscirickettsiosis is a severe disease reported in salmonids that has caused especially great problems for the Chilean aquaculture industry. In 1989, the bacterium *Piscirickettsia salmonis* was isolated from a moribund coho salmon and was found to be the etiological agent of this disease. The pathogen is a Gram-negative obligate intracellular bacterium. The disease has also been reported to affect Atlantic salmon, rainbow trout and other farmed salmonid species (Wilhelm et al. 2006). A recombinant subunit vaccine was developed in order to control the disease due to poor responses to antibiotic treatment. Three experimental formulations were prepared containing two or three recombinant proteins of the bacterium, and the formulations were emulsified with one volume of FIA (Wilhelm et al. 2006). The highest protective response was obtained with a vaccine formulation containing the subunit of the flagellum and chaperonins Hsp60 and Hsp70 of *P. salmonis*, suggesting that the use of more than one recombinant protein antigen is needed to obtain a good protective effect against this infectious bacterium.

Jiao and coworkers have been studying different vaccine concepts against *E. tarda* in the Japanese flounder to obtain effective protective formulations, based on both recombinant proteins and DNA vaccine constructs (Jiao et al. 2009, 2010b). The most promising vaccine concept was the one consisting in a chimaeric DNA vaccine coding for the *E. tarda* proteins Eta6 fused in-frame to FliC, the flagellin for *E. tarda*. Although they found that *E. tarda* FliC induced low protective immunity

by itself, it could function as a molecular adjuvant and potentiate the specific immune response induced by the *E. tarda* antigen Eta6. Fish immunised with pEta6 and FliC produced specific serum antibodies and exhibited enhanced expression of genes that are involved in both innate and adaptive immune responses (IL-1ß, IFN, Mx, CD8α, MHC-Iα, MHC-IIα, IgM) (Jiao et al. 2009, 2010b). Such upregulation following immunisation with flagellin has also been described by Hynes et al. (2011), where TNF-α, IL-6, IL-8 and IL-1ß were significantly upregulated compared to non-adjuvanted controls. In this study, however, there was no induction of specific antibody response against flagellin or the model antigen *Limulus polyphemus* hemolymph (LPH) in the Atlantic salmon.

Synthetic Oligodeoxynucleotides

Bacterial DNA and synthetic oligodeoxynucleotides (ODNs) expressing unmethylated CpG motifs trigger an immunostimulatory cascade that culminates in the maturation, differentiation and proliferation of multiple immune cells, including B and T lymphocytes, NK cells, monocytes, macrophages and dendritic cells. CpG motifs are approximately 20 times less common in mammalian than microbial DNA and therefore are recognised as a danger signal by cells that express TLR9. In mammals, it has been widely demonstrated that CpG ODNs function as adjuvants when co-administered with vaccines, being able to both accelerate and magnify the immune response (Bode et al. 2011). In fish, although many studies have been carried out on the immunomodulatory effects of CpGs (Carrington and Secombes 2007; Liu et al. 2010a, b; Rhodes et al. 2004), only a few studies have focused on the adjuvant effect of these molecules.

Chinook salmon (*O. tshawytscha*) reared in the Pacific Northwest of the United States often suffers from infection with *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD). A study in which whole cell vaccines with or without CpG adjuvants were used revealed that both the vaccine alone or that with CpG provided protection against i.p. challenge with *R. salmoninarum* (Rhodes et al. 2004). However, a combination of a commercial *R. salmoninarum* vaccine (Renogen) with a CpG adjuvant significantly reduced the level of bacterial antigens in the kidney of naturally infected fish (Rhodes et al. 2004).

In rainbow trout, four groups were i.m. injected with a commercially available, a non-adjuvanted aqueous vaccine against furunculosis containing inactivated cultures of *A. salmonicida* (AquaVac Furovac 5) alone, or together with CpG ODN 1982, CpG ODNs 2133 or ODN2143. The fish were challenged by i.p. injection using a pathogenic *A. salmonicida* strain 7 weeks after injection. The only group that showed a significantly lower mortality compared to those injected with Furovac alone (mortality of 52 %) was the group injected with Furovac and the CpG ODN 2143 in which only 21 % of the fish died (Carrington and Secombes 2007).

The protective effect of CpG motifs was also studied by Liu and coworkers in turbot and Japanese flounder (Liu et al. 2010a, b). Sixteen different CpG ODNs

were synthesised and examined for the ability to inhibit bacterial dissemination in Japanese flounder blood. Four ODNs with the strongest inhibitory effects were selected, and a plasmid pCN6 was constructed containing the sequences of the four selected ODNs. Japanese flounder were injected i.m. with plasmids pCN6 and pCN3 (control) and PBS. Four weeks post-vaccination, the fish were challenged with *A. hydrophila* and mortality was monitored over a period of 20 days. Accumulated mortalities were 30, 66.7 and 63.3 % in pCN6-, pCN3-, and PBS-immunised flounder, respectively (Liu et al. 2010b). Fish were also vaccinated as above and challenged with *E. tarda* 4 weeks after vaccination, and the mortalities were 53.3, 90 and 93.3 %, respectively. Therefore, the pCN6 plasmid provided a nonspecific protection against both *A. hydrophila* and *E. tarda* infections. These nonspecific protective effects have also been observed in fish parasitic infections, since certain CpGs (e.g. CpG ODN 1668 and CpG ODN 2359) have also proved to be protective against *Miamiensis avidus* (Kang and Kim 2012). Following on, a salmonid alphavirus (SAV) vaccine containing antigen plus CpG and Poly I:C as adjuvants induced a significant production of neutralising antibodies and conferred some level of protection – as evaluated by percentage of SAV-positive fish compared to controls (Thim et al. 2012). The authors reported that the adjuvanted vaccines induced a prominent type I IFN expression, that is, a key factor in providing antiviral response.

To analyse the adjuvant effect of CpGs in turbot, fish were vaccinated with a *Vibrio harveyi* recombinant subunit vaccine, DegQ, in combination with a CpG that had previously been shown to provide anti-infectious effects in the host species after injection. Fish were vaccinated by i.p. injection including all the appropriate controls, and 28 days after vaccination, the fish were challenged with a virulent strain of *V. harveyi*, and accumulated mortalities were recorded (Liu et al. 2010a). The only vaccine formulation that induced a significant protection was DegQ in combination with this pCN5 CpG. The duration of the adjuvant effect was found to be at least 50 days.

One of the unique features of DNA vaccines is the ability to stimulate both cellular and humoral immune responses through the administration of a bacterial plasmid coding for a protective antigen (Weiner and Kennedy 1999). Plasmid DNA vaccines possess intrinsic immunostimulatory capacity due to the presence of CpG motifs in the bacterial plasmid backbone. Therefore, the inclusion of additional CpG motifs in the vaccine plasmid would provide higher intrinsic adjuvant activity, compared with the control plasmid, being this an easy method to increase the immunogenicity of DNA vaccines. Following this, Martinez-Alonso et al. (2011) explored the possibility of increasing the immunogenicity of a VHSV DNA vaccine though the introduction of several copies (either two or four) of a fragment containing multiple CpG sequences of known immunostimulatory effects into the DNA vaccine. The addition of these CpG motifs significantly increased the titre of neutralising antibodies in serum and increased the levels of transcription of several immune genes such as Mx or MHC-I, demonstrating for the first time that additional CpG motifs may be introduced in the plasmid to increase the immunogenicity of these DNA vaccines.

Cytokines

In the past years, a great number of cytokine genes have been identified in many fish species; however, despite the fact that the use of cytokines as adjuvants has been widely explored in mammals, not many studies have focused on the possible use of cytokine genes as vaccine adjuvants in fish (Wang and Secombes 2013). This may be due to the fact that for the majority of these molecules, many details concerning their immunological role are still lacking, and until we know what immune processes they are regulating, their use would be a mere trial and error process. In any case, some attempts to explore their potential have been made in some fish species.

Interferon regulatory factors (IRFs) form a large family of transcription factors. IRF-1 has been shown to have a role in cytokine signalling and host defence against pathogens. For example, IRF-1 is upregulated in response to virus infection in fish cells, inducing an antiviral state (Caipang et al. 2005). The potential use of IRF-1 as a vaccine adjuvant was thus investigated in Japanese flounder. The co-injection of IRF-1 plasmid with a DNA vaccine encoding the major capsid protein (MCP) gene of red sea bream iridovirus (RSIV) resulted in elevated serum neutralisation antibodies but was not significantly different from those in fish vaccinated with the DNA vaccine alone (Caipang et al. 2009). Despite the moderate effect in protection, IRF-1 was responsible for the upregulation of antiviral substances like nitric oxide (NO), IFN ß and IFN-inducible genes such as Mx.

IL-8 is a CXC chemokine produced by many cell types in mammals like macrophages, monocytes, epithelial cells, neutrophils and fibroblasts upon infection, or stimulated by cytokines like IL-1ß and TNF-α. In mammals, chemokines have been widely used as adjuvants in vaccines against viral infections, since not only they attract more cells to the site of inflammation but also regulate the immune functions of the recruited cells. In fish, IL-8 has been characterised in rainbow trout among other species, and its chemoattractant property is established (Harun et al. 2008). In this species, a vaccine plasmid coding for the glycoprotein gene of VHSV was co-injected with a plasmid coding for rainbow trout IL-8 to explore its potential adjuvant effect (Jimenez et al. 2006; Sanchez et al. 2007). When the plasmid coding of IL-8 (pIL-8+) was administered together with the VHSV vaccine, an increase of IL-1ß in the spleen was found together with a greater cellular infiltration at the site of inoculation. Furthermore, fish injected with pIL-8+ alone showed a significantly higher expression of TNF-α, IL-11, TGF-ß and IL-18 in the spleen (Jimenez et al. 2006). In a further study, the transcription of different inducible CC chemokines were studied in rainbow trout in response to both the VHSV DNA vaccine and/or pIL8+, demonstrating that when IL-8 is used as an adjuvant, the expression of other chemokines such as CK5A, CK6, CK7 and CK5B is also modulated (Sanchez et al. 2007). All these results showed that IL-8 was able to modulate the early immune response and could be a potential adjuvant in fish.

Although the administration of IL-1ß-derived peptides to rainbow trout by i.p. injection reduced the mortality of fish when exposed to VHSV 2 days after injection and induced leukocyte migration to the peritoneal cavity (Peddie et al. 2003), the

possible use of these peptides as adjuvants was not further explored. The role of IL-1ß as an adjuvant was investigated in carp after i.p. injection of killed *A. hydrophila* in the absence and presence of recombinant C-terminal peptide of carp IL-1ß. The agglutinating antibody titre obtained was significantly higher in the fish injected with killed bacteria plus recombinant IL-1ß peptide compared with killed bacteria alone 3 weeks after vaccination (Yin and Kwang 2000).

Immersion Delivery of Fish Vaccines

Immersion vaccination is the simplest method for vaccine delivery; however, it is not suitable for all antigens or for all farming situations. It can be performed using hyperosmotic infiltration (HI), direct immersion (DI) or spray. Vaccination by HI involves immersing the fish in solutions such as urea or sodium chloride for a short period of time followed by immersion in the vaccine. For vaccination by DI, fish are transferred to the vaccine for a certain period of time and then moved back to the holding tank (Plant and Lapatra 2011). This last method has been proven less stressful and equivalently effective; thus, HI is not commonly used. Although DI or spray of bacterins can provide significant levels of protection (Villumsen and Raida 2013), not many successful strategies to vaccinate against viruses through immersion have been reported. In 2008, Kai et al. performed a 20 min immersion of grouper with inactivated betanodavirus and obtained high protection levels when BEI was used to inactivate the virus (RPS > 75), but not when formalin was used (RPS = 39–43) (Kai and Chi 2008). Furthermore, the efficacy of formalin-inactivated vaccine could be significantly improved by nano-encapsulation (RPS = 85).

Some authors have used ultrasound to increase the uptake of vaccine antigens through immersion. Ultrasounds should open routes in the skin, thus facilitating the transdermal delivery of vaccines that will improve the effectiveness of vaccination by immersion (Navot et al. 2005). Delivering a *V. alginolyticus* bacterin with ultrasound resulted in similar protection levels that those obtained by injection vaccination, once the parameters for the application of ultrasound were optimised (Zhou et al. 2002). The delivery of a VHSV DNA vaccine in rainbow trout by immersion with short pulses of low-intensity ultrasound also provided some protection, although the levels were lower than those obtained after intramuscular injection (Fernandez-Alonso et al. 2001).

Novel Strategies for Oral Vaccination

From the practical point of view, oral vaccination is the most suitable strategy, because the vaccine would be delivered together with the feed to large groups of fish at the same time without stress. However, the main limitation is that not all animals eat the same amount of feed, thus the vaccine dose varies from fish to fish.

Traditionally, a lot of emphasis has been made on the fact that the antigen has to reach intact the second or third segment in order for the vaccine to be effective; however, although this might be true in most cases, a recent study has demonstrated that responsive immune cells are present all along the digestive tract in rainbow trout (Ballesteros et al. 2013). Furthermore, there is still a great lack of knowledge on how immune recognition and adaptive immune mechanisms are orchestrated in the digestive tract of different fish species that would most probably respond differently among species and according to the nature of the antigen delivered (Rombout et al. 2011). All these limitations have led sometimes to poor and inconsistent results when different strategies to orally deliver antigens to fish have been addressed.

Microparticles/Nanoparticles

Microparticles or nanoparticles offer a promising option to oil emulsions, and their beneficial use as carriers for vaccine delivery has been widely discussed (Sinyakov et al. 2006). An association of antigen(s) with microparticles can be achieved by covalent linkage or physical entrapment. Compared to the latter technique, where the antigen is non-covalently and physically incorporated in the interior of the microparticle, covalent coupling offers distinct advantages: lower amount of antigen is required; processing and presentation by antigen-presenting cells may be more efficient; a higher stability during storage is obtained and any excess of material can easily be regained. With the use of microparticles, even a very low dose of antigen can give rise to a robust humoral response. The structure and the properties of microparticles may change markedly with slight alterations in production conditions, but nanoparticles can be prepared in a physicochemically reproducible manner within narrow size limits. In addition to being vehicles for oral delivery of vaccines, the particles have also been suggested as potent adjuvants in mammalian systems (Cui and Mumper 2003). Therefore, all these encapsulation techniques could be catalogued as delivery methods, as well as signal 1 facilitator adjuvants.

PLGA Particles

Encapsulation of vaccines in biocompatible and biodegradable Poly(lactide-co-glycolide) (PLGA) polymers has been studied for over 20 years. Antigen is released from the microspheres by diffusion through matrix pores and by matrix degradation. Biodegradation rates can be regulated by alterations in polymer composition and molecular weights.

So far, a few studies have been carried out on fish with regard to uptake and degradation of PLGA particles and the immune response obtained. For the most part, these studies have focused on oral administration and have been performed in species such as Japanese flounder (Tian et al. 2008a; Tian and Yu 2011) or salmonids like rainbow trout (Adomako et al. 2012; Altun et al. 2010; Lavelle et al. 1997) or

Atlantic salmon (O'Donnell et al. 1996). In the case of Japanese flounder, a plasmid encoding the major capsid protein of lymphocystis disease virus (LCDV) was constructed and encapsulated in PLGA. Controls were naked plasmid vaccine and blank PLGA particles (Tian and Yu 2011). The fish were orally intubated, and 28 days post vaccination, the fish were challenged by intramuscular injection with LCDV. Vaccine effects were evaluated by observing the presence of lymphocystis nodules. The cumulative percentage of Japanese flounder with nodules after challenge was greatly reduced in the group receiving the plasmid coding for the LCDV protein in PLGA particles in the period of 15–120 days post-immunisation (Tian and Yu 2011). In addition, the levels of antibody in sera of fish vaccinated with PLGA microcapsules increased for up to 9 weeks; although from this point, it started to decrease (Tian et al. 2008a).

In rainbow trout, oral vaccination (as a feed additive) against lactococcosis was attempted with antigens encapsulated in PLGA particles (Altun et al. 2010). RPS of the PLGA vaccine amounted to 63 %, and booster vaccination with oral administration of the PLGA vaccine gave a RPS of more than 60 % 120 days after the first vaccination. Also in rainbow trout, HGG was microencapsulated in PLGA (Lavelle et al. 1997). Specific antibodies were detected in the intestinal mucus of fish fed the microencapsulated antigen after boosting with soluble HGG, but not in fish that were primed with the soluble antigen. The fate of orally administered HGG in Atlantic salmon was determined, demonstrating that 15 min after administration, the HGG-PLGA was found in the intestine as was the free HGG (O'Donnell et al. 1996). The results from this study indicate that orally delivered HGG-PLGA had higher levels and greater persistence of HGG systemically than free HGG.

A recent article appeared on parenteral immunisation of Indian major carp, rohu (*Labeo rohita*) with PLGA-encapsulated antigen (Behera et al. 2010). Outer membrane proteins (OMP) of *A. hydrophila* were encapsulated in PLGA microparticles and mixed with FIA in an emulsion or administered alone by i.p. injection in rohu. Twenty-one and 42 days after immunisation, the antibody titres were significantly higher in the PLGA-encapsulated antigen group containing FIA (Behera et al. 2010). A dose-dependent transient increase of antibody response following i.p injection of PLGA particles containing human gamma globulin (HGG) has been shown by Fredriksen and Grip (2012), where it was shown that microparticle carriers were superior compared to nanoparticles to induce antibody response. Furthermore, when the formulation of PLGA-entrapped HGG was performed with ß-glucan or oil, it resulted in a continuous increase of antibodies over time (up to day 120). Finally, feeding of rainbow trout with feed containing plasmid DNA encoding IHNV G protein induced slightly higher amount of neutralising antibodies against IHNV but no increased survival after experimental challenge with IHNV (Adomako et al. 2012).

ISCOMs

Immune-stimulating complexes (ISCOMs) were conceived to co-formulate antigen and adjuvant in a particle (Morein and Bengtsson 1999). ISCOMs represent an interesting approach to stimulate both the humoral and cell-mediated immune

response towards amphipathic antigens. A stable and non-covalently bound complex of Quil A with amphipathic antigens (approx. 40 nm diameter) in a molar ratio of approximately 1:1:1. ISCOMs produced through the patented Matrix™ technology by Isconova have been widely studied in combination with different veterinary vaccines and are currently incorporated in a number of commercialised animal vaccines. At this moment, Pharmaq is studying the introduction of these adjuvants in commercialised fish vaccines.

Alginate

Alginate is a copolymer of β-D-mannuronic acid and α-L-guluronic acid found in the cell wall of brown algae. It has been widely used to encapsulate antigens because it is cheap, has low toxicity and is adhesive to the mucosa (Wee and Gombotz 1998). Furthermore, the adjuvant effects of alginate have also been demonstrated in fish, since it has been shown to have effects on fish weight, innate immunity and disease resistance (Cheng et al. 2008; Yeh et al. 2008).

Concerning its use for encapsulating bacterial antigens, alginate microparticles with or without a *Vibrio anguillarum* bacterin were administered with feed to both carp and trout (Joosten et al. 1997). Although optimal responses were obtained in the different species with different alginate microspheres, mucus-IgM and mucosal plasma IgM cells were detected in both cases. On the other hand, the administration of *A. salmonicida* recombinant A-layer proteins in alginate beads delivered orally to carp did induce serum antibodies (Maurice et al. 2004). However, the encapsulation of *Flavobacterium columnare* bacterin did not induce the production of serum antibodies nor was able of conferring protection in Nile tilapia (Leal et al. 2010). Better results were obtained when the oral administration of bacterins in alginates is used as an oral booster after i.p. immunisation (Romalde et al. 2004). In this case, the oral vaccination alone provided some protection (RPS = 50), but when administered as a booster, the protection was significantly increased (RPS = 87), and longer protection periods were achieved in comparison to i.p. immunisation alone.

Concerning viral antigens, most studies have focused on the use of alginate to deliver DNA vaccines orally. Thus, a DNA vaccine against lymphocystis disease virus (LCDV) was delivered orally to Japanese flounder (Tian et al. 2008c). In this study, the antigen was detected in different tissues from day 10 to 90 post-vaccination, and serum antibodies were detected up to week 16. In a similar study, de las Heras et al. (2010) encapsulated a DNA vaccine against IPNV in alginate and also detected antigen expression and serum antibody production. In this case, IFN was also upregulated, and 80 % relative survival rates were obtained when fish were challenged 15 and 30 days after vaccine delivery.

Aeromonas salmonicida subsp. *salmonicida* bacterin was encapsulated in liposome-alginate particles (Eggset et al. 1995). Atlantic salmon were vaccinated by oral intubation into the stomach. As control, the fish were given liposome-alginate particles without antigen and non-encapsulated *A. salmonicida* bacterin by intubation. The fish were orally intubated each day for 2 days. Three weeks after, the fish were revaccinated by intubations on two successive days, and 7 weeks after the last

intubations, the fish were challenged by cohabitant salmon intraperitoneally injected with virulent *A. salmonicida* bacteria. Fish vaccinated by oral intubation with *A. salmonicida* bacterin in liposome-alginate showed moderately increased survival, and also increased anti-*A. salmonicida* antibody responses.

Chitosan

Chitosan is a mucopolysaccharide obtained from marine crustaceans with great potential for oral delivery of antigens (Rao and Sharma 1997). It has been used to deliver a DNA plasmid coding for a reporter gene (β-galactosidase) together with the feed (Ramos et al. 2005). β-Galactosidase expression could be observed in the stomach, spleen and gills, demonstrating the potential of this encapsulation method. Similar results were obtained in two other studies. Tian et al. (2008b) vaccinated with a DNA plasmid containing the major capsid protein (MCP) gene of lymphocystis disease virus (LCDV) encapsulated in chitosan and observed antigen expression in tissues up to day 90 post-vaccination and serum antibodies for up to 16 weeks post-vaccination. A DNA vaccine against the porin gene of *V. anguillarum* was also delivered after chitosan encapsulation to sea bass (Rajesh Kumar et al. 2008). In this case, although the antigen was detected in different tissues, only a moderate protection against experimental *V. anguillarum* infection was obtained.

Alternative Methods for Oral Delivery

Some other alternative methods to microencapsulation have been briefly explored by some authors for oral vaccine delivery in fish. For example, feeding young fish with brine shrimp (*Artemia* nauplii) used to bioaccumulate *Vibrio anguillarum* bacterin was studied as an oral vaccination method (Joosten et al. 1995). Although immunosuppression was encountered in younger fish, sea bream orally vaccinated showed significantly higher secondary responses compared with the control at days 57 or 69 post-immunisation. In a more recent experiment, formalin-killed *E. coli* expressing the *P. aeruginosa* exotoxin was fed to *Artemia* that were subsequently fed to zebrafish (*Danio rerio*). The fish were protected from *P. aeruginosa* challenge with 81 % of vaccinated fish surviving compared to 31 % of the controls (Lin et al. 2005). Furthermore, it is possible to genetically modify plants to express protective proteins that can be delivered directly in food. Although this method has not been widely explored in fish, it seems as an interesting area of research. When a fusion protein consisting in a gut adhesion molecule and a viral peptide was expressed from potato tubers and fed to carp, the adhesion molecule mediated the binding to and uptake from the gut, whereas the viral peptide induced a humoral immune response (Companjen et al. 2005). Alternatively, microalgae can also be

used to produce the antigen. For example, the *Renibacterium salmoninarum* protein 57 (p57) was expressed in the microalgae *Chlamydomonas reinhardtii* (Siripornadulsil et al. 2007). The delivery of the transformed algae either by immersion or in feed induced a specific antibody response. Whether these novel methods based on plant or microalgae are capable of conferring, real protection has still to be demonstrated.

Conclusive Remarks and Perspectives

The development of effective vaccines should be approached by combining the search for protective antigens together with the application of specific, and targeting, adjuvants that maximise the immunogenicity with a desired immune response. At the same time, the route chosen for immunisation has to be taken into account, because despite the fact that many details of immune regulation are still unknown in fish, it is clear that the site where the antigen is presented will strongly condition the immune response that is mounted. These vaccine-specific adjuvants should be able to trigger specific immunological processes, without producing a generalised response with strong side effects.

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Chapter 5
Fish Vaccines: The Regulatory Process and Requirements from the Laboratory Bench to a Final Commercial Product, Including Field Trials

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Abstract Vaccines are recognised as important tools for the prevention and control of diseases in fish. The regulatory requirements for registering veterinary vaccines have grown considerably over the last 50 years; nevertheless, they have contributed to a steady increase in the availability of vaccines of high quality with good safety profiles and proven efficacy against many diseases. In the EU, there are stringent requirements for vaccine manufacturers to comply with good manufacturing practice (GMP); consequently, the cost of vaccine production is high. Compared with vaccines for other animal species, the market for fish vaccines is limited in size; however, the cost in meeting the regulatory requirements is similar and the cost of development is equally expensive. Fortunately vaccines for use in small markets may take advantage of the Minor Use Minor Species Limited Market (MUMS) and limited market process for which the regulatory requirements are reduced where a successful application can be made for inclusion in the MUMS listings. Also, the field of animal ethics is constantly changing, leading to some reductions in the regulatory requirements for animal studies performed to generate safety, quality and efficacy data. The pharmaceutical industry needs to keep abreast of such changes and amend product development plans accordingly to remain competitive in the market.

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Introduction

This chapter will explain the EU regulatory requirements to bring a fish vaccine through the basic development stages to the final product, including the registration process leading to the granting of the authorisation to market the product.

Although the registration process itself will be the same for any type of veterinary vaccine, for simplicity, this chapter will focus on the regulatory requirements for a monovalent inactivated bacterial vaccine for fish.

Where appropriate, the text will indicate the locations in the EU registration dossier where the data should be included and will provide references to EU guidelines which explain how to generate data suitable for inclusion in the dossier.

The regulatory requirements for fish vaccines differ slightly for each type of vaccine; however, guidelines (General requirements for the production and control of live mammalian bacterial and viral vaccines for veterinary use WC50004652 Vol 7BIm1a; Requirements for the production and control of inactivated mammalian bacterial and viral vaccines for veterinary use in 1992) are available to assist applicants intending to apply for marketing authorisations for all types of immunological veterinary medicinal products (IVMPs), whilst another guideline is available specifically for fish vaccines (Guideline on the design of studies to evaluate the safety and efficacy of fish vaccines & EMA/CVMP/IWP/314550/2010). The first two of these three guidelines have been superceded by a new, simplified guideline (Guideline on requirements for the production and control of immunological veterinary medicinal products EMA/CVMP/IWP/206555/2010) on the requirements for the production and control of immunological products, which clarifies some aspects of the regulations that applicants had previously been found to be ambiguous.

Vaccine Types

There are several categories of vaccines for use in humans and animals, and vaccines for fish fall within the same categories, namely, viral, bacterial, fungal or parasitic. Within these categories, vaccines can be live, attenuated or inactivated. To stimulate a protective immunity against a disease, the antigenic component of the vaccine may be prepared from whole cells, cell supernatant or parts of an immunising agent, e.g. subunit or vector vaccines.

Antigen Development

Once a market for a vaccine for immunisation against a specific disease has been identified and evaluated, a suitable source for antigen preparation must be found.

Generally, the strain of bacteria (or virus) selected for antigen production will have been isolated from a diseased fish. The strain will have been identified by

genus and species and allocated a strain designation. The strain will be tested to ensure that it is pure rather than a mixture of different bacteria. Its origin, date of isolation, passage history and storage conditions are recorded for presentation in the registration dossier (Part 2. C). If the strain has come into contact with any material of bovine origin during the development process or even in the finished product, a declaration to this effect must be included in the dossier under the section on "Minimising the Risk of Transmissible Spongiform Encephalopathies".

- Master Seed
  Once purity and identity are confirmed, a master seed is prepared. Several vials of the master seed will be produced. Normally these will be lyophilised (freeze-dried) by adding a stabiliser such as lactose, sucrose or bovine serum albumin to the culture. The master seed is a critical part of vaccine development and production. Its stability under the chosen storage conditions is essential to enable a continued supply of batches of the vaccine. The testing required for viral master seeds is far more extensive than for bacterial seeds since the absence of several potential extraneous agents (Table of Extraneous Agents to be Tested for in relation to the General and Species Specific Guidelines on Production and Control of Mammalian Veterinary Vaccines Vol 7BI10a) that could contaminate the seed must be shown. The master cell seeds for viral vaccines also require testing.
- Working Seed
  From the master seed, subcultures are prepared that will be used for development testing and for production. The generation or passage level to be used for production is known as the working seed. Preparation and storage of the master seed and working seed are known as a seed lot system. For bacterial vaccines, the number of passages between the master seed and the working seed is not limited but must be specified. The same passage level must be used for production of all subsequent batches of final product as is used in the batches with which target animal safety and efficacy are demonstrated. In contrast, the passage level for virus vaccine production is limited to five passages from the master seed, as specified in the European Pharmacopeia (Ph Eur) monograph 0062.

Vaccine Development Process

The next step in vaccine development is to determine what form of the antigen is to be selected to provide the best protection whilst remaining safe to the target species and stable in the final vaccine under the proposed storage conditions. This may already be known from previous experience or from the literature. If not, a series of experiments will be required to determine the optimum antigen preparation. Once the most suitable preparation is known, it is necessary to test its efficacy in a proof-of-concept experiment. There are no specific protocols to be followed for such testing, and indeed the results of these tests are not required to be included in the registration dossier. The design of all the tests carried out during this development

phase is the responsibility of the scientists working on the product. Experiments such as those described below may need to be performed several times, with slight modifications each time until a satisfactory outcome has been reached. The types of testing recommended and the parameters to be measured in such laboratory experiments include the following:

- Route of administration – this will depend on the age and species of fish to be vaccinated. For this example, testing for a vaccine to be administered by immersion will be used.
- Optimising the challenge dose – this involves the use of a heterologous strain of the same bacterium species as the vaccine strain and selecting the dose capable of killing 80–100 % of unvaccinated fish. The optimum dose will be measured in terms of colony-forming units (CFU).
- Efficacy – a preliminary efficacy test is performed by setting up, for example, three tanks containing an identical number of young fish (fingerlings) in each one. One tank of fish will remain as untreated controls. To one of the other two tanks, experimental vaccine is added at a high concentration and to the other one experimental vaccine at a low concentration. After a suitable time interval to allow immunity to develop, the optimised challenge dose is administered to each of the three tanks. The number of fish that die in each tank is counted to determine the protective index of the vaccine. This preliminary efficacy test is not a legal requirement. Eventually a controlled challenge test will be performed which is reproducible and supportive of the claim for efficacy.
- Dose – optimisation of the vaccine dose will not only confirm that the vaccine is efficacious but will avoid the unnecessary wasteful cost of using excess antigen for vaccinating fish. Dose optimisation is normally achieved by performing a dose titration study followed by a challenge infection to identify the minimum immunising concentration.
- Safety – unlike other animal species in which vaccines are mostly administered by subcutaneous or intramuscular injection or by intraocular administration, the assessment of safety at the injection site cannot be evaluated if vaccine is to be administered by immersion nor by oral administration with feed. The only practical way to assess safety in this case is to look for and record morbidity and mortality in vaccinated fish in the efficacy test prior to administration of the challenge dose. These tests are part of product development. There are no regulations describing what must be done. The manufacturer decides what to do at this stage and then, based on the results, decides whether or not to proceed with registration studies and apply for a marketing authorisation.

Although none of the results of these proof of concept studies are required to be included in the registration dossier, sufficient supportive information about them should be presented in the quality section of the dossier, “Part 2.4 Product Development”, which requires the applicant to provide an explanation regarding the composition, components and containers proposed for the commercial vaccine, supported by scientific data on product development.

In-Process Testing

During the studies to establish that the antigen is safe and effective, it may become necessary to introduce certain purification steps prior to formulating the vaccine. When this occurs, it will be necessary to introduce additional testing at different stages to ensure the identity, purity and safety of the antigen.

For inactivated vaccines, either before or after any early purification steps, it will be necessary to subject the bacterial culture to inactivation. Agents such as formaldehyde or betapropriolactone (BPL) are commonly used. During the development stages of vaccine production, the kinetics of any inactivation process must be validated. The Ph Eur requires that it be shown that the time required for inactivation shall be not more than 67 % of the duration of the inactivation process, thus allowing a 33 % margin of safety for inactivation during routine production of the vaccine.

Assay Development and Validation

Depending on the type of vaccine under development, one or more assays will be required to test the antigen quality during the in-process stages and in the final product. If the tests are not established ones, such as those included in the relevant pharmacopoeia monographs, it will be necessary to fully validate them. The following aspects of the test method should be demonstrated – specificity, precision, linearity, sensitivity (includes limit), repeatability, reproducibility and robustness.

Ideally, once a vaccine formulation has been developed and batches are being manufactured routinely, the batch release tests will not involve the use of live fish. For all veterinary vaccines, it is a normal practice, once efficacy has been demonstrated by challenge studies in the target species, to develop in vitro assays for batch testing, especially for batch potency testing. If an in vivo potency assay for a fish vaccine can be replaced by an in vitro assay, it is necessary to validate (VICH GL1 Guideline on validation of analytical procedures definition and terminology; VICH GL 2 Validation of analytical procedures methodology) the test and report such validation and its correlation with the in vivo assay in the registration dossier or by means of a post-licensing variation application. All inactivated vaccine potency tests must be validated to show that they are capable of detecting a subpotent batch.

Formulation/Blending of Bulk Vaccine

Some vaccines, particularly live attenuated virus vaccines, are presented in their final containers as lyophilised powder for reconstitution with a diluent. To ensure their stability following lyophilisation, they are usually blended with a stabiliser prior to filling into containers and being freeze-dried.

In the present example of a liquid monovalent inactivated bacterial vaccine, it is normal practice to include one or more adjuvants in the formulation when administered by injection. The advantage of an adjuvant in an inactivated vaccine is that it stimulates the slow release of the vaccine antigen, thereby improving the duration of exposure of antigen to the animal's immune system to improve the stimulation of antibody production. However, for inactivated vaccines administered by immersion, adjuvants are not normally included in the formulation.

Selection of Final Containers

Selection of the vaccine container is important. It must be sufficiently robust to ensure the stability of the vaccine at least until the end of the supported shelf life. It must also be suitable for the user to broach and use under field conditions. Consideration of the volume of the container is important and it is often necessary to select different container sizes to meet the needs of different customers. Details of the containers and closure, together with data to demonstrate the integrity of the closure system, are required in the registration dossier (normally Part 2. A.2). The marketing authorisation (MA) lists the volumes or sizes of all the containers approved for the product on the summary of product characteristics (SPC). Any additional containers required following the granting of the MA must be added by means of a variation to the MA before they may be commercialised.

Development Testing/Preregistration Testing

In parallel with developing the vaccine formulation and validating the assays, the development tests required by EU legislation (Commission Directive 2009/9/EC) can be started. These tests only need to be performed once. They are not the same as the batch tests performed on each batch of vaccine prior to its release.

The timing of these tests is important in the EU since they must be conducted using vaccine produced exactly according to the method that will be described in the registration dossier (Part 2. B.1 and 2. B.2). If any changes are subsequently made to the production process or the formulation of the finished product, the safety and efficacy tests will need to be repeated, prior to submission, using the revised formulation. It is therefore critically important that these studies are not performed too early in the development process.

An important and useful specific EU guideline (Guideline on the design of studies to evaluate the safety and efficacy of fish vaccines & EMA/CVMP/IWP/314550/2010) for testing the safety and efficacy of fish vaccines has been published as a result of the recognition by EU regulatory authorities of the problems faced when conducting safety and efficacy studies in fish compared with other species. This guideline allows for a reduction in the normal requirements for veterinary vaccines under certain cir-

cumstances. For example, for some fish vaccines, it may be appropriate to apply for classification under minor use–minor species/limited markets (MUMS). Once MUMS classification has been obtained, it is possible to register such vaccines in the EU with considerably reduced fees and in some cases with no fees applicable at all.

For inactivated vaccines, there is generally a requirement that the target species safety test is performed using a batch produced at maximum potency, whilst efficacy testing is performed using vaccine formulated at minimum potency. In practice, this means that the minimum potency allowed for batch release is established based on the potency of the batch used to demonstrate efficacy under controlled laboratory conditions. Likewise the batch release limit for safety is established by the antigen content/potency of the batch used for the development safety studies.

- Safety

Details of the target animal safety tests are described in VICH guideline GL44 (VICH GL44 Guideline on target animal safety for veterinary live and inactivated vaccines EMEA/CVMP/VICH/359665/2005). These tests must be conducted according to good laboratory practice (OECD principles on good laboratory practice) (GLP). They are divided into laboratory tests and field trials as follows:

– *Laboratory tests*, in which vaccinated fish are observed with each morbidity/mortality being recorded daily for at least 14 days after vaccination:

  (i) Single dose
  (ii) *Overdose* – (this is no longer required in the EU for inactivated vaccines)
  (iii) *Repeated dose* – if the recommended vaccination schedule requires more than a single dose

– *Reproductive safety,* only if relevant, i.e. if the fish to be vaccinated are to be used for breeding purposes

Omitted from this guideline, but available in its own separate guideline, is the test to be carried out with live attenuated vaccines – VICH GL41 – "Target Animal Safety: Examination of Live Veterinary Vaccines in Target Animals for Absence of Reversion to Virulence".

– *Field safety* – this is achieved by monitoring safety, e.g. weight gains and local reactions, in the efficacy field trials.

- Efficacy

As with safety, the data to be generated to demonstrate the protection afforded by the vaccine, as described on the label, falls into the two categories of laboratory tests and field trials.

– *Laboratory Tests* – these are normally challenge tests in the target species using the recommended route of administration and the proposed vaccination schedule. They must be performed in each category of species for which claims are made. The batches used must be manufactured according to the method described in Part 2 B.1 and 2. B.2 of the dossier, formulated to reach minimum potency.

It is normally required to demonstrate both onset and duration of immunity by challenge infection unless an alternative method is available which correlates with the protection afforded against challenge, e.g. serology. The duration of immunity is used to recommend the interval between the primary vaccination course and any subsequent booster dose if a booster dose is recommended. If duration of immunity is studied in field trials, these should be large-scale research facilities where fish can be taken from holding tanks at different intervals and subjected to challenge infection or specific antibody response if this has been shown to correlate with protection. The conditions in the holding tanks, e.g. water temperature, quality, etc., should be similar to the conditions under which the vaccine will be used naturally in the field.

It should be noted that in the fish vaccine guideline (Guideline on the design of studies to evaluate the safety and efficacy of fish vaccines and EMA/CVMP/IWP/314550/2010), it has been recognised that for some disease situations in fish, no or only poor challenge models exist. The guideline states that in such situations, with appropriate justification, more emphasis may be placed on field studies conducted under conditions which reflect the disease situation in the field. Ideally, duration of immunity (Note for guidance: duration of protection achieved by veterinary vaccines) should be based on the results found in both laboratory studies and field trials; however, it is important to monitor the fish at regular intervals in field trials to detect the occurrence of disease outbreak, so that the recommended time interval between the primary course and booster vaccination can be justified.

– *Field Trials* – as with all veterinary medicinal products, field trials must be carried out in accordance with good clinical practice (GCP) (VICH GL9). The fish vaccine guidelines (Guideline on the design of studies to evaluate the safety and efficacy of fish vaccines & EMA/CVMP/IWP/314550/2010) provide useful information about the design of the trials explaining that they should be carried out in established commercial fish farms; the field studies are to be performed in established commercial farms where the relevant disease is anticipated and should preferably include unvaccinated controls. Allocation of groups should be done randomly, and the prevalence of disease, daily mortality, clinical symptoms and other relevant parameters should be monitored in both vaccinates and controls for comparison. The treatment of the controls, i.e. mock vaccinated, vaccinated with a comparator vaccine or non-vaccinated, should be justified. Studies should be conducted at the time of year when the disease normally occurs.

Field trial permits must be applied for before trials can begin. The method for applying for field trials is not the same in every member state. Advice should be sought from the relevant authority. Informed consent must be obtained from the owner of the fish farm, and consent is usually required from the relevant environmental protection agency.

A GCP (Setting up GCP trials in fish; Note for guidance) protocol of the planned trial must be prepared. Some flexibility in the details of the protocol will be necessary for trials carried out offshore since weather conditions may affect the planned start and end dates of the trial.

At the end of the trials, the results must be collected for the GCP report. The results must be analysed statistically in accordance with EU guidelines.

Reports of the GLP safety studies and GCP efficacy studies are included in Parts 3 and 4, respectively, of the registration dossier.

Final Product Testing

Part 2. E of the registration dossier is where the tests performed for batch release are described, together with the limits of acceptance for each test. The test methods themselves are also included in the dossier, normally in the Annex to Part 2, as standard operating procedures (SOPs). In addition to the descriptive summary of the tests including the stages at which they are performed, it is useful to prepare a tabulated summary of the tests and their acceptance criteria since this serves as the summary of the finished product specification and can be used as a guide to preparing batch certificates.

Samples for these tests are normally taken from filled containers; however, some tests may be performed on samples of bulk vaccine prior to filling. Guidance on this is available in Ph. Eur. (European Pharmacopoeia). Typical tests for an inactivated bacterial vaccine would include:

Test	Limit of acceptance (examples)
Appearance	*For example, pale white suspension*
Purity	Pure
Sterility	Sterile
Identity and assay of active ingredient	*For example, Yersinia ruckeri* 10^6–10^7*/ml*
Identity and assay of adjuvant	*For example, 5 % aluminium hydroxide gel*
Tests on excipients	*As relevant*
Identity and assay of preservative	*For example, thiomersal*
Safety	*Since January 2013* (Guideline on requirements for the production and control of immunological veterinary medicinal products EMA/CVMP/IWP/206555/2010), *this test is no longer mandatory for inactivated vaccines*
Inactivation	Completely inactivated

Batch Consistency

In Part 2. F of the dossier, it is necessary to include the results of tests on three consecutive batches of the vaccine. These will normally be pilot-scale batches that will also be used for stability testing. The purpose of this section is to demonstrate that

the quality of the product is consistent from batch to batch and to demonstrate conformity with the specification. Full batch protocols of these batches should be included in the Annex to Part 2.

Stability

Stability studies need to be set up as soon as possible so that some stability results can be included in the dossier. Samples of each container size should be stored at the recommended temperature. There should be sufficient samples so that testing at intervals of, say, every 6 months can be performed to support the claimed shelf life for the vaccine. Data must be provided for 3 months longer than the proposed shelf life. It is not necessary to carry out the entire list of tests described under Part 2. E controls on the finished product. It is only necessary to perform stability-indicating tests such as potency and any other important parameter that would highlight degradation or a reduction in stability over time. A minimum of 6 months stability data should be included in Part 2. G together with a commitment to provide further data, when available, to support extensions to the shelf life approved initially.

If the vaccine contains a preservative, this must be shown to be efficacious according to the requirements in the Ph. Eur. preservative efficacy test (Ph Eur 01/2005:50103). The preservative must be shown to retain its effectiveness up to the end of the shelf life.

Compiling the Registration Dossier

The regulatory authorities of all EU member states accept the same standard registration dossier, in English (Commission Directive 2009/EC). Depending on the nature of the vaccine, data intended for inclusion in different parts of the registration dossier are usually being generated simultaneously; therefore, it is normal practice to work on each section as the information becomes available rather than progress through the dossier sequentially.

The dossier is divided into five parts:

Part 1 – This is the *administrative* section containing the application form (1A), the SPC and draft packaging (1B) and the expert reports (1C).

Part 2 Manufacture and Quality – There are eight sections in this part ending with 2. H "Other Information", which should include any quality information not already included in sections 2. A to 2. G. Supportive documents such as specifications, certificates of analysis and SOPs are normally included in an Annex to Part 2.

Part 3 Safety – Reports of all the GLP safety reports go into this part together with reports of the reports on field safety. The headings to be addressed are listed in the legislation (Commission Directive 2009/9/EC) and must be referred to in the dossier, justifying those which are not applicable.

Part 4 Efficacy – All the reports of challenge studies and GCP field trials are included in this part.

Part 5 Bibliographical References – Any supportive literature is included in this section.

The wording for the SPC and packaging materials should be completed using the QRD templates. A guideline (Revised position paper on indications for veterinary vaccines) is available to explain how claims may be worded depending on the outcome of the efficacy studies. Each recommendation on the SPC and labelling must have been supported with data generated with the product itself. This is why it is so important to follow the intended dose, route of administration and vaccination schedule in the safety and efficacy studies intended to be reported in the registration dossier.

Expert reports are still expected in the EU, although they are now called "Detailed and Critical Summaries".

The Application for a Marketing Authorisation

Guidance on how to apply for a MA in the EU can be found on the European Commission's website under "EURALEX". The relevant volume is 6A, Notice to Applicants. Guidance on the submission of the dossier and the application form are provided in this volume.

Manufacturing Standards

All stages of vaccine production, including the production of antigen, must be carried out to good manufacturing practice (GMP) standards. Most parts of the world accept the quality of manufacturing standards of facilities inspected and approved as EU-GMP compliant. In contrast, vaccines and antigens produced in some other parts of the world, including the USA, are not acceptable for importation into the EU unless the facility has been issued with a GMP certificate following a EU-GMP inspection.

The standard of GMP demanded in the EU is extremely high, having increased steadily over the last 35 years. Nevertheless, the rewards for compliance with such standards include the reduction and even elimination of some vaccine testing compared with the requirements of 10–15 years ago.

Routes to Obtaining a Marketing Authorisation in the EU

(a) Centralised Procedure

Since the implementation of Council Directive 92/18/EEC, Title II, harmonised requirements have been publicly available concerning the studies required to obtain an MA for immunological veterinary medicinal products in the EU. Initially marketing authorisations were only issued on a national basis, until Council Directive 90/676/EEC introduced the centralised procedure (CP) with effect from 1 January 1992. However, most veterinary vaccines were not eligible for registration through the centralised procedure, and although some changes in the criteria for eligibility have been introduced, the use of the CP basically remains limited to vaccines derived from biotechnology (mandatory) and vaccines having something about them that is deemed novel (voluntary).

The advantage of using the centralised procedure is that a positive opinion followed by a positive Commission Decision results in MAs being granted in all member state countries plus Norway, Iceland and Lichtenstein.

The great disadvantage for manufacturers of fish vaccines that are eligible on a voluntary basis or even obligated to use the centralised procedure is the fees. Also, the requirements for translation of the SPC and packaging into 23 languages are a burden when the vaccine may only have markets in two or three EU member states.

More information about the centralised procedure is available on the EMA website. http://www.ema.europa.eu/under Veterinary Regulatory/Application Guidance.

For fish vaccines that are not derived from biotechnological methods, three other registration routes are available:

(b) National Procedure

It is still possible to obtain a national MA by submitting the application dossier to just one regulatory authority. This may be desirable for fish vaccines with a specific, limited market.

(c) Mutual Recognition Procedure

If an applicant has obtained one national MA and then subsequently wishes to market the vaccine in another one or more member states, they must use the mutual recognition procedure in which the first MA is mutually recognised by the other selected member states. The process is similar in parts to the decentralised procedure described below.

(d) Decentralised Procedure

The third route is the one that is most appropriate for a new inactivated bacterial vaccine for fish. The decentralised procedure (Best practice guide for veterinary decentralised procedure (DCP) CMDv/BPG/002) is used when more than one EU member state has been identified as a suitable market for a new product.

The first step for the applicant is to select the reference member state (RMS). This is the EU regulatory authority that will assess the dossier and prepare an assessment report.

The next step is to select the Concerned Member States (CMS). The applicant informs both the RMS and the CMSs of their intention to apply for MAs through the decentralised procedure. The entire procedure runs to a strict timeline and normally takes 210 days to complete, after which the RMS and CMS have 30 days to issue identical national marketing authorisations for the vaccine.

Preparing for the Launch

Preparations for the launch of the product will begin during the vaccine development stage. Obviously if the research and development team developed a vaccine that required vaccination of fish every 3 weeks for 6 months to provide protection against the target disease, this would not be a marketable proposition. R&D and marketing need to work together during the development phase to ensure that the SPC and label claims that are eventually authorised are practical and useful for the market.

The initial drafts of the packaging materials that were included in Part 1 of the registration dossier may have been revised by the regulatory authorities during the DCP. Thus the draft packaging will need to be amended accordingly prior to printing.

Although the MAs that have been issued by the individual member states must be identical, there are certain items which have not been completely harmonised in the EU and are left to each country to apply according to their national rules. These include legal category/distribution category – this affects whether the product is to be sold as a prescription-only medicine (POM) or under some other nationally allowed category.

Launch batches will have been prepared in anticipation of the marketing authorisations. The batch tests on these will need to be completed and batch protocols forwarded to the relevant authorities requesting batch release approval. Samples of these batches, in their final packaging, should be provided to the regulatory authorities on request.

Post Launch

The marketing authorisation holder has certain responsibilities after MAs have been granted. These include pharmacovigilance reporting and applying for batch release for subsequent batches of the vaccine. Any changes in the licensed production process or test methods must be subject to variations to the marketing authorisation. Fortunately, by using the decentralised procedure, the approval of variations is a harmonised process in which the RMS and CMSs issue approval to introduce the requested change simultaneously.

Finally, EU legislation and guidelines for veterinary vaccines have undergone a series of changes and improvements over the years, with new guidance being issued and published all the time. It is important that applicants check the EMA website and their national regulatory authorities' websites for relevant updates.

References

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European Pharmacopoeia (Ph Eur) monograph 0062: Vaccines for Veterinary Use
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Guideline on data requirements for IVMPs intended for minor use or minor species/limited markets. EMA/CVMP/IWP/123243/200
Guideline on requirements for the production and control of immunological veterinary medicinal products EMA/CVMP/IWP/206555/2010, effective 1 January 2013
Guideline on the design of studies to evaluate the safety and efficacy of fish vaccines, EMA/CVMP/IWP/314550/2010, effective 1 May 2012
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Revised position paper on indications for veterinary vaccines, EMA/CVMP/042/97-Rev.1-FINAL
Setting up GCP trials in fish, by Janet Stone and Alastair McLean, Ch 5, veterinary clinical trials from concept to completion edited by Nigel Dent and Ramzan Visanji ISBN 1-57491-121-X
Table of Extraneous Agents to be Tested for in relation to the General and Species Specific Guidelines on Production and Control of Mammalian Veterinary Vaccines Vol 7BI10a, (A draft replacement, for this guideline, EMA/CVMP/IWP/105112/2011, is currently under consultation)
VICH GL 2: Validation of analytical procedures: methodology
VICH GL1: Guideline on validation of analytical procedures: definition and terminology
VICH GL41 Target animal safety: examination of live veterinary vaccines in target animals for absence of reversion to virulence
VICH GL44 Guideline on target animal safety for veterinary live and inactivated vaccines EMEA/CVMP/VICH/359665/2005, effective July 2007
VICH GL9, (GCP) Guideline on Good Clinical Practices (CVMP/VICH/595/98-FINAL)

Chapter 6
Methods for Measuring Efficacy, Safety and Potency of Fish Vaccines

Paul J. Midtlyng

Abstract This chapter summarises the common methods for demonstrating safety, potency and efficacy of fish vaccines, allowing them to be licensed and marketed as veterinary medicinal products. It comprises a brief overview of clinical method development since the beginning of fish vaccinology and discusses the current guidelines, requirements and recommendations for choice of study methods. Historically, the same methods have been used for routine product quality testing (batch safety and potency) and for generating scientific documentation of the vaccines' clinical safety and efficacy that forms the basis for granting marketing authorisations. With the development and publication of new experimental disease models and methods, evaluation of fish vaccines is in the process of becoming more sophisticated, and further efforts to increase the diversity of clinical methods are being advocated. The essential role of field trials for confirmation of experimental efficacy results and for the assessment of long-term side effects of fish vaccines is acknowledged. In addition, recent work to reduce and refine the use of live fish in batch quality testing of fish vaccines is reviewed, representing a clear welfare benefit for the fish being used during vaccine manufacture. For future improvement of fish vaccine safety and efficacy assessment, emphasis is placed on the development of alternatives to current challenge assays for batch potency and for a broader characterisation of immune mechanisms, including the use of quantitative (real-time) PCR assays to demonstrate up- or downregulation of relevant cytokines and immunorelevant genes.

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Efficacy, Safety and Potency: What Does It Mean?

This chapter will attempt to summarise how the essential biomedical effects of immunological preparations for fish are being scientifically demonstrated, allowing them to be termed "vaccines", to be licensed by regulatory authorities and to be marketed as veterinary medicinal products (VMPs). For this purpose, methods allowing the quantification of their biological effects or features are required. However, scientifically sound, quantitative methods are equally important for veterinarians and animal owners in their benefit–cost evaluation and when planning the use of vaccines for fish health management and disease control. Although many of the study setups described in this chapter have rightfully attained the status of "standard methods" that enjoy wide acceptance by regulators as well as by the scientific community, real life entails great diversity and variability, always dictating a critical assessment of their applicability and relevance to the specific situation or question at hand.

Because fish vaccines are (veterinary) medicinal products, the definition of the terms used in the title of this chapter is determined by the corresponding regulations and guidance documents. The term "efficacy" means the disease therapeutic (pharmaceuticals) or preventative (immunologicals) value that a medicinal product offers and is by default a measure of its performance in real patients – its *clinical effect*. Any fish vaccine must therefore be "immunogenic", i.e. able to induce clinical immunity to virulent challenge with the infectious organism it is claimed to protect against. However, in animals as in humans, there is no *per se* requirement that vaccine protection raised against illness must be complete nor that vaccination must provide sterile immunity (inability of the challenge organism to infect the immunised individual). This is logical, given a variety of diseases that prove notoriously difficult to prevent against and also given the emergence of new infections.

The documentation of vaccine efficacy thus normally includes scientific studies to demonstrate that the clinical benefits vs. adverse effect balance is better or equal compared to existing vaccines, and evidence that at least part of the immunity elicited by the vaccine formulation is *antigen specific*.

The term "safety" encompasses the absence of clinical effects that are unacceptably disadvantageous to the patient group itself (*clinical safety*, *reproductive safety*), but medicinal legislation also requires VMPs be documented with regard to *environmental safety*, safety to the personnel that may come in contact with the product during manufacture or administration (*operator safety*) and, in products intended for food-producing animals, also safety of the foods to be derived from treated animals (*consumer safety*). This chapter will mainly focus on methods for determining clinical safety, while the other aspects of safety assessment and documentation will not be discussed.

In a regulatory context, the term *potency* is normally being used to describe the "strength" (amount of active substance per dose unit) of a medicinal product including vaccines. *Batch potency tests* form part of routine quality control during manufacture of medicinal products, normally being performed as final product quality tests immediately prior to release of each batch. In pharmaceuticals, the chemical structure of the active ingredient is well defined, and quantitative chemical methods are therefore dominant in batch potency testing. In vaccines, however, the antigen(s)

constitutes the active ingredient, and often the detailed antigenic structures giving rise to the protective immune responses are relatively unknown or comprise a complex mixture of epitopes and molecules that induces immune responses and protection together and in concert. Titration using culture methods is therefore the most common antigen quantification method for vaccines, but does of course not work after inactivation of the antigen.

Historically, this has rendered indirect assays, most common simplistic animal protection tests (demonstrating clinical protection upon challenge in vaccinated vs. unvaccinated animals) the standard for assuring a consistent batch-to-batch vaccine potency. This is likely also a reason why the terms potency and efficacy are sometimes being employed interchangeably in regulatory, scientific or technical literature.

A successful potency test is considered proof that the vaccine has been produced according to the licensed manufacturing outline and that the amount and quality of the antigen does not fall below the licensed specifications. For fish vaccines like for many classical animal vaccines, the establishment of *in vitro* or antibody-based methods for batch potency testing that satisfy the methodological quality doctrines of medicinal product manufacture has proven very challenging. But irrespective of *in vivo* or *in vitro*, any potency test should be able to reveal a vaccine batch of inferior quality, i.e. falling below the final product specification irrespective if expressed as antigen amount, the ability to induce of antibody responses, or as induction of clinical protection against virulent challenge.

Controlled Clinical Trials with Fish Vaccines

In his famous first paper on oral vaccination against furunculosis, Duff (1942) reported seven small-scale infection experiments during which orally immunised cutthroat trout (*Salmo clarki*; now *Oncorhynchus clarkii*) were exposed to virulent *Bacterium* (now *Aeromonas) salmonicida* by addition to the rearing water, by introduction of clinically diseased fish or by intramuscular inoculation with an approximately threefold lethal dose. The survival in vaccinated fish was variable but in part significantly higher than in the controls in the bath exposure experiments, while only faint and non-significant protection was seen after challenge by inoculation.

The second and well-controlled live animal study series that more convincingly demonstrated the disease-protective capacity of a fish vaccine was reported from North America by Krantz et al. (1964), who immunised brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta*) with a formalin-killed culture of *A. salmonicida* formulated with a mineral oil adjuvant. In several small-scale experiments in which immunised and control fish were inoculated intraperitoneally (i.p.) with an estimated LD50 dose of viable bacteria ca 3 months post immunisation, the fish that had received the adjuvanted bacterin showed full protection. In two of the experiments, fish were included that had been vaccinated with bacterin alone, but were not protected.

In a third early vaccine evaluation paper, Spence et al. (1965) were able to prove that transfer of serum derived from adult rainbow trout previously immunised with a formalin-killed *A. salmonicida* bacterin via repeated i.p. injections would protect

juvenile coho salmon (*Oncorhynchus kisutch*) against furunculosis challenge. These authors performed experimental challenge by swabbing a scarified skin area along the lateral line and subsequently adding the *A. salmonicida* broth culture to the water. Orally immunised coho juveniles were subjected to daily addition of broth cultures to the rearing water and also to natural furunculosis outbreak, predicted from the hatchery's historic records.

Quite interestingly, these pioneering papers report nearly all of the principal clinical study models that have later proven invaluable for the research, development and licensing of fish vaccines, including the use of so-called "mark-and-mix" design that will be discussed towards the end of this paper. Unfortunately, this methodological diversity was only to a limited degree employed for demonstration of fish vaccine potency and efficacy for the subsequent 30 years.

Clinical Method Development for Licensing and Batch Release

In the following decade, research and development work on fish vaccines initiated by further academic groups in North America, Europe and Japan (see Evelyn 1997; Gudding and Goodrich 2014 for summaries) resulted in a *Yersinia ruckeri* bacterin to protect against enteric redmouth disease in rainbow trout in the USA in May 1976, being the world's first licensed fish vaccine. A review of the experimental methods and field data to demonstrate this vaccine's efficacy and potency was published by Tebbit et al. (1981). Representing the commercial startup companies that had embarked upon fish vaccine licensing during the mid-1970s, Antipa and Croy (1979) summarised the procedures required by the US Department of Agriculture (USDA) for veterinary vaccines that had to be satisfied prior to licensure:

> After initial laboratory testing has established the methods of choice for producing and delivering the vaccines to fish, the company submits a production outline to the USDA. The production outline specifies the exact means of producing the vaccine, as well as the methods for delivering it to fish and evaluation vaccine efficacy, potency, and safety. Approval of the production outline by the USDA authorizes the first commercial production of three serial lots of vaccine….
>
> Vaccine Testing: Each of the three serial lots of vaccine must then be tested individually with appropriate fish species for safety, efficacy and potency at two levels: one as normal field dose and a more diluted concentration than a field dose (often one-half of on-field dose) that could possibly occur in field usage. If these tests prove satisfactory, a field testing permit is issued by the USDA.
>
> Before field vaccination tests begin, further experimentation is needed to provide additional information, such as the safety of a given delivery system under field conditions. Field tests are conducted on large number of fish to assure adequate statistical analysis. The logistics and methods of managing large numbers of fish must be carefully planned…..
>
> Each of the three serial lots of vaccine must be tested with separate lots of fish under field conditions. Control groups must be handled in the same way as experimental groups, except for exposure to the vaccine…….
>
> Because of varying challenge conditions in the field, laboratory challenges are used more frequently than natural exposure to pathogens in the field to assess vaccine efficacy…… Methods for laboratory challenges are specified in the production outline. Field challenges should be designed to correspond as closely to production conditions as possible …

Apparently, this led to the practice of demonstrating satisfactory potency outcome of three pre-licensing batches (so-called consistency batches) to form the main basis for efficacy documentation, irrespective of the caveats expressed towards the clinical relevance of inoculation challenge methods in the early vaccine evaluation papers.

Being perhaps the most cited scientific paper in fish vaccinology literature, Donald F. Amend (1981) published a more detailed methodology for potency testing of fish vaccines using infection experiments. The article is most frequently cited with reference to the algorithm for calculating relative percentage survival (RPS), based on the author's amendment of the formula recommended by the USDA for expressing relative potency (RP).

The formula $\mathrm{RPS} = 1 - \left[\frac{\%\ \text{vaccinate mortality}}{\%\ \text{control mortality}} \right]$ gives a figure that expresses the relative ratio of survival in the vaccinates over the controls, with a maximum of 100 when no mortality at all occurs in the vaccinated fish and zero when there is no difference in mortality.

What appears less known is that the same article contains a comprehensive discussion about a variety of further aspects of vaccine potency and efficacy testing methods in fish and proposes detailed recommendation of test setup, challenge conditions and outcome acceptance criteria for controlled trials:

- Duplicate setup (2 × 25 fish) of vaccinate and control group
- Exposure by bath challenge in two concentrations
- Maximum 10 % non-specific mortality and 20 % within-group variation after challenge
- Control mortality ≥60 %, vaccinate mortality ≤24 %

Following the author's recommendations, the proposed acceptance criteria for potency equate to a standardised RPS of 60 % or above.

Further discussion of the experiences made in the first period of testing fish vaccines was provided by Cardella and Eimers (1990), who detailed the method development carried out by the National Veterinary Services Laboratories in Ames, Iowa, when establishing their protocols for independent testing of batch purity, safety and potency in samples from federally licensed fish bacterins. The outcomes of 46 fish vaccine serials (each 21 ERM and *Vibrio anguillarum*/*ordalii*, plus 4 furunculosis vaccine lots) showed that all passed the proposed batch safety test protocol and criteria (single-dose vaccination of 30 5-gramme rainbow trout fingerlings, observation for 7–14 days, survival ≥95 %). This is apparently the only scientific report providing data from routine batch quality testing of fish vaccines. For potency, testing protocols involving challenges by i.p. injection and by immersion were evaluated in parallel, with tight pass criteria. While 18 out of 20 ERM batches and all of the tested vibriosis and furunculosis vaccine batches satisfied the RPS criteria, three i.p. challenges with *Vibrio* species failed to reach the minimum control mortality. In the discussion, the authors concluded that:

> The intraperitoneal challenge system is reproducible, dependable and provides a high degree of reliability in a bacterin testing system; however its use must be closely scrutinized

because it is not a natural route of exposure and may represent a too severe a challenge in that a large number of organisms are directed internally and not exposed to the natural resistance factors located on the surface of the fish.

The authors also endorsed the reproducibility of the immersion challenge methods. The work reported in this paper resulted in standard operating for the potency testing of ERM and vibriosis bacterins by the US licensing agency being implemented from the mid-1980s, while *further development studies are required to establish a standard test system for the injectable Aeromonas salmonicida bacterins* (Cardella and Eimers 1990). Quite interestingly, the same report includes original data confirming clear susceptibility differences to the tested challenge organisms between salmonid species and a discussion of the relevance of this finding to vaccine testing.

Evolution of (European) Scientific Guidelines for Licensing Fish Vaccines

It appears that the work carried out and reported above by the early North American fish vaccine companies and the US licensing agency became strongly normative for establishing the methods for evaluation of fish vaccines worldwide. Following the rapid development of industrialised salmon aquaculture and in particular the breakthrough of mass immunisation programmes in Norwegian salmon farming in the early 1990s (reviewed in Midtlyng et al. 2011a), several regulatory agencies issued specific scientific guidance for development and licensing of fish vaccines. In Europe, this first led to a standardised European Pharmacopoeia monograph for inactivated, oil adjuvanted furunculosis vaccine (Ph. Eur. Monograph 1521) followed by monograph 1580 (inactivated cold-water vibriosis vaccine) and 1581 (inactivated vibriosis vaccine), all concerning salmonid vaccines. In these monographs that came into effect in 1994, a specific experimental design for documentation of immunogenicity was outlined (see Table 6.1). This methodology was also recommended for final product potency testing prior to batch release. A very similar vaccine monograph covering yersiniosis vaccine for salmonids (Ph. Eur. 04/2013: 1950) has been adopted later. It appears that when adopting these first standardised methodologies, the main emphasis was placed on reproducibility, likely in order to avoid inconclusiveness and/or erroneous rejections of manufactured batches due to method variability of routine potency tests. This also becomes evident in the first EU guidance on fish vaccines where i.m. or i.p. inoculation challenge of 50 fish per vaccine or control group was accepted as a "generic" method for immunogenicity and batch potency testing (CVMP/III/3590/92-EN).

Reflecting the experience made during evaluation of a large number of developmental furunculosis vaccine formulations both experimentally and in the field (Midtlyng et al. 1996a, b; Midtlyng 1996), a specific session of the first International

Table 6.1 Scientific documents and guidelines on fish vaccine potency, safety and efficacy issued by or implemented by European regulatory agencies, cited in this paper

VICH GL 44	Target animal safety for veterinary live and inactivated vaccines
VICH GL 50	Biologicals: harmonisation of criteria to waive target animal batch safety testing for inactivated vaccines for veterinary use
Ph. Eur 04/2013: 50206	Evaluation of efficacy of veterinary vaccines and immunosera
Ph. Eur 04/2013: 50207	Evaluation of safety of veterinary vaccines and immunosera
Ph. Eur. 01/2015: 0062	Vaccines for veterinary use
Ph. Eur. 01/2015: 1521	Furunculosis vaccine (inactivated, oil adjuvanted, injectable) for salmonids
Ph. Eur. 04/2013: 1580	Vibriosis (cold-water) vaccine (inactivated) for salmonids
Ph. Eur. 04/2013: 1581	Vibriosis vaccine (inactivated) for salmonids
Ph. Eur. 04/2013: 1950	Yersiniosis vaccine (inactivated) for salmonids
EMA/CVMP/IWP/582970/2009	Reflection paper on the control of the active substance in the finished product for immunological veterinary medicinal products
EMA/CVMP/IWP/206555/2010	Guideline on the requirements for the production and control of immunological veterinary products
EMA/CVMP/IWP/314550/2010	Guideline on the design of studies to evaluate the safety and efficacy of fish vaccines

Symposium on Fish Vaccinology in Oslo in 1996 was devoted to discussion of methodology aspects of vaccine evaluation in fish. Nordmo (1997) discussed at length the strength and weaknesses of various routes of challenge exposure (i.p., i.m., bath, cohabitation) and emphasised the relative nature of RPS as a quantitative measure:

> …However, RPS numbers are often presented by vaccine manufacturers without reference to the test system employed. Unless these details are presented and scrutinised, the results obtained in testing facilities may be irrelevant to field conditions. Comparison of RPS between different test systems is also irrelevant, as RPS for identical products will vary between test systems. RPS is a dynamic parameter that will change throughout the post challenge period….
>
> Although standardized test models have been described for several infectious diseases, the question still remains whether competitive products can be compared using these models on different sites. If the test results from different laboratories were to be compared, one would have to establish standardised test systems with respect to environment, pathogen and host. Such standardization is unlikely to take place….
>
> As some vaccine product have now been introduced and shown to have very good efficacy under field conditions, they could be introduced as reference standards when testing new candidates for marketing authorisation….

The status of regulatory standards and tests was critically revisited again by Midtlyng (2005), who presented several examples of bacterial infections where

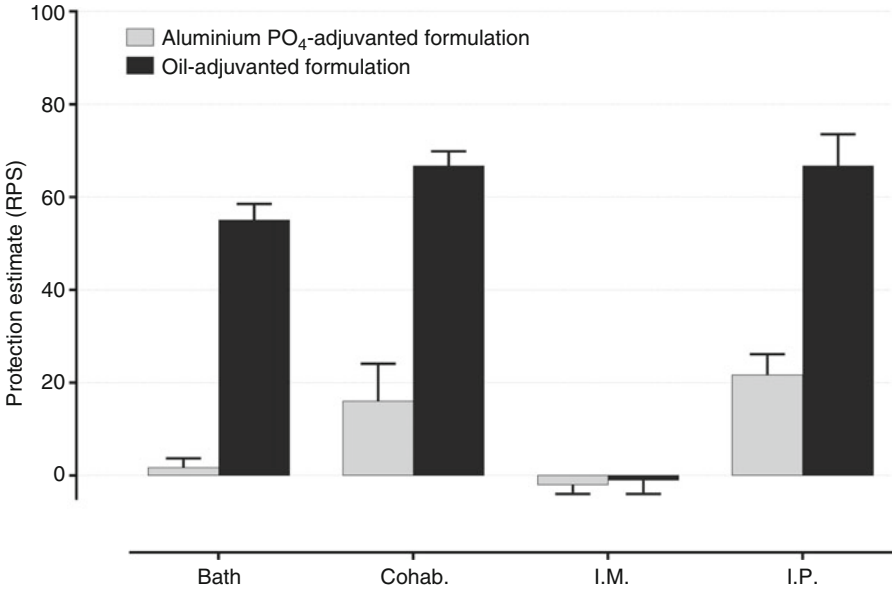

Fig. 6.1 Two anti-furunculosis vaccine formulations in Atlantic salmon (*Salmo salar*) parr, assessed by four different challenge methods 12 weeks post immunisation. As opposed to the waterborne challenge routes or intraperitoneal (i.p.) inoculation, intramuscular (i.m.) challenge failed to demonstrate any protective effect of the vaccines. Based upon data from Nordmo and Ramstad (1997) (Reproduced from Midtlyng 2005 with permission from Karger AG Medical and Scientific Publishers, Switzerland)

vaccine protection had been found to differ largely, solely depending on the route of experimental exposure (Fig. 6.1). Pointing to the activation of peritoneal macrophages as a potentially strong interference factor when using i.p. injection for experimental exposure, the author suggested that *intraperitoneal challenge is inappropriate to evaluate specific immune parameters in fish, or at least there is a high risk for assay interference caused by non-specific mechanisms…… Careful validation is therefore required to ensure that intraperitoneal challenge tests are indeed adequate to ensure batch-to-batch vaccine potency, let alone that they may on their own suffice to document the efficacy of emulsion-type vaccines for intraperitoneal administration*. Also the low number of fish required to document acute and subacute target animal safety was questioned on grounds of poor statistical power.

In summary, the standardisation of methodologies through regulatory monographs and guidelines has undoubtedly both simplified the development and licensing procedures and ensured a more "level playing field" between competing pharmaceutical companies with regard to licensing. In this author's opinion, this standardisation has, however, historically also reduced the exploration of method diversity and slowed down further sophistication of methods to predict fish vaccine performance under real-life conditions.

Evolution and International Harmonisation of Methodologies

In the above-mentioned symposium, the status of regulatory requirements and guidelines on the licensing of fish vaccines as of the mid-1990s were summarised by representatives of the European (Lee 1997), US (Birnbaum 1997) and Canadian (Sethi et al. 1997) authorities. On part of the pharmaceutical industry, Goodrich (1997) summarised that despite efforts to honour differences in immunisation practices between species,

> …historical regulations today are based on injectable vaccines for warm-blooded animals. Administration of vaccines products to cold-blooded aquatic species by immersion, bath and oral routes, as well as by injection, creates interpretive problems for manufacturers and regulators alike. Industry encourages and awaits regulatory standardization, harmonization and equivalence….

To some degree, these aspects have since been fulfilled, not least through the so-called International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (abbreviated VICH). Status and perspectives for international harmonisation of methods and requirements were reviewed by Castle (2005), pointing to batch safety and potency testing, duration of immunity and field documentation being on top of the priority list. A major step towards harmonisation of methods and requirements within Europe was the EMEA recommendation of compliance of veterinary vaccines in the community with the relevant monographs of the European Pharmacopoeia in 1998. An in-depth review of how the European regulatory requirements for pharmaceuticals and vaccines in aquaculture have evolved is provided by Alderman (2009). Since then, however, a number of regulatory guidelines have been revised and/or new guidelines have been issued, being adequate responses to the criticism expressed by experts in academia and industry and representing significant improvement of scientific guidance (Table 6.1).

The Difference Between Batch Quality Testing and Studies to Scientifically Demonstrate Vaccine Safety and Efficacy

Even though the final product quality of vaccine batches is being verified through specific tests, it is important to realise that batch safety or potency tests are *not* primarily intended nor designed for scientific documentation of the vaccine's clinical safety and efficacy. The batch tests are routine quality control procedures to ensure that the antigen amount per dose remains within the approved specifications despite batch-to-batch variation. This purpose calls for highly standardised, reproducible, technically simplistic and preferably rapid methods that lend themselves well for routine implementation. Methods that do not involve steps where biological variation can occur are therefore preferred. In contrast, the scientific documentation of a vaccine's safety and efficacy calls for study designs that are as representative as

possible of its intended use, of the target animal population and of natural exposure to various environmental factors including any relevant diversity of field strains. Safety and efficacy studies must therefore be designed to avoid bias and in summary be comprehensive enough to capture all major conditions of use and all relevant outcome parameters.

As mentioned in the first paragraph of this paper, vaccine batch testing predominantly confirms the integrity and amount of the active ingredient *indirectly*, by confirming that the degree of immunity or immune response the serial is able to elicit in live animals under well-controlled and highly standardised experimental conditions remains as expected. The pass criteria are being set in accordance with published vaccine monographs, or where no monographs exist, to the batches that were used for demonstrating the clinical safety and efficacy of the product. Despite the fact that recommended vaccine batch test protocols are live animal vaccination or vaccination and challenge experiments, the batch test method will always differ from a realistic clinical setting.

Methods for Batch Safety Testing of Fish Vaccines

Through the adoption of VICH Guideline 44, the European, US and Japanese veterinary medicinal agencies agreed to reduce routine safety testing of veterinary vaccine batches, but until April 1, 2013, batch safety tests were generally required for release of veterinary vaccine batches in Europe (Ph. Eur chapter 5.2.6; monograph 0062). In countries where the requirement still prevails, batch safety tests test for inactivated fish vaccines typically involve the administration of the vaccine to between 10 and 50 individuals of the target species, with subsequent observation of the animals for signs of general toxicity for at least 3 weeks, completed by euthanasia and evaluation of injection-site reactions. Detailed pass criteria are set to assure that no abnormal systemic or local reactions are induced. Overdose testing is commonly required for batch release of live vaccines only.

The relevance of the routine batch safety tests for veterinary vaccines has been frequently questioned due to their lack of sensitivity to detect moderate variation in antigen content (VICH GL 50). The introduction of good manufacturing practice (GMP) or similar quality systems in vaccine manufacturing has increased the batch-to-batch consistency and hence reduced the risk for quality variation beyond the licensing specifications. Last but not least, the introduction of “real-time” monitoring and reporting of suspected adverse effects from the field (so-called pharmacovigilance routines) created new and more sensitive means of signal detection, should a licensed product display deviation from the accepted safety profile. This led to the adoption of an international recommendation to harmonise the criteria to waive the target animal batch safety testing for inactivated veterinary vaccines (VICH GL 50). For existing fish vaccines manufactured in Europe, USA and Japan, the batch safety tests can therefore be waived based on satisfactory historic information. Supportive data would include the number of batches manufactured (typically 10), the number

of years the product has been on the market, the number of doses sold and the frequency and seriousness of any adverse reactions in the target species and any investigations into the likely causes of these events.

Recently, the mandatory batch safety testing for veterinary vaccines has been removed in European vaccine standards, thus allowing veterinary vaccines to obtain marketing authorisation or to be manufactured without the performance of a routine batch safety test. Consequently, the quality of the pre-licensing evidence for consistency of production and clinical safety to target species will have to be assessed thoroughly by the licensing authority. If considered incomplete, the licensing authority may request specific commitments from the marketing authorisation holder to supplement the documentation or to request batch safety testing until further evidence has been presented. Batch safety testing can also be requested on an *ad hoc* basis upon changes or irregularities in manufacturing or upon indications from the field.

Methods for Batch Potency Testing of Fish Vaccines

Probably unknown to the majority of readers, one of the most renowned early fish immunologists, Douglas P. Anderson contributed a very early paper suggesting potency and efficacy evaluation of fish vaccines by antibody responses (Anderson et al. 1980). In the absence of suitable premises for conducting challenge experiments, the authors documented the kinetics of the antibody response of rainbow trout to two *Yersinia ruckeri* and one *Aeromonas salmonicida* bacterin administered by injection, hyperosmotic immersion or plain antigen baths in dose–response experiments. A so-called passive haemolytic plaque assay was employed to titrate the agglutinating antibodies in the serum of immunised fish. The results demonstrated a dose–response relationship between antigen dose and antibody response, leading the authors to propose the method for both potency and efficacy determination. In hindsight it seems that their conclusion was pioneering when it comes to batch potency testing but somewhat optimistic when it comes to efficacy, as only a part of the evidence for immunogenicity can be generated by antibody assays.

According to current regulatory standards, batch potency testing of veterinary vaccines should be performed on final product after packaging in tamper-proof containers, and the generally recommended methodology for live vaccines is titration (EMA/CVMP/IWP/206555/2010) using viral or bacterial culture. Also PCR techniques have now been developed for potency estimation of live human (Schalk et al. 2005) or veterinary (Prabhu et al. 2012; Susanj et al. 2012) vaccines. For potency testing of inactivated viral or recombinant protein (subunit) vaccines, quantitation of immunorelevant epitopes may also lend itself for validation, like reported for Newcastle disease in poultry (Claassen 2011). But because antigen quantitation represents a major challenge with inactivated and in particular with adjuvanted formulations, regulators are still recommending indirect methods (experimental challenge or antibody response methods) for potency testing of today's most common fish vaccines (Table 6.2).

Table 6.2 European Pharmacopoeia recommended methods for immunogenicity documentation and batch potency testing of fish vaccines as of January 2015

	Experimental challenge assay		Antibody-based batch potency assay	
Monograph ID	# of fish	Control mortality	# of fish	Specific conditions
Ph. Eur 01/2015: 1521 furunculosis vaccine (inactivated, oil adjuvanted, injectable) for salmonids **Ph. Eur. 04/2013: 1580** Vibriosis (cold-water) vaccine (inactivated) for salmonids **Ph. Eur. 04/2013: 1581** Vibriosis vaccine (inactivated) for salmonids **Ph. Eur. 04/2013: 1950** Yersiniosis vaccine (inactivated) for salmonids	≥30 vaccinated ≥30 unvaccinated Observe until the end of mortality (no death over 2 consecutive days)	≥60 % within 21 days after onset	≥25 vaccinated ≥10 unvaccinated	Water temp ≥12 °C Immunisation period ≥500 degree days No specific requirements for immunisation conditions

The main aspects of when developing indirect batch potency assays are:

- The measured outcome must be an indicator of efficacy, preferably a relevant aspect of immunogenicity in the target species.
- The test must be sufficiently sensitive to distinguish between potent and subpotent batches.
- During development, the assay conditions giving the most discriminating and the most reproducible dose–response relationship must be explored, and dose–response and assay precision must be satisfactorily validated.
- The use of positive and negative control formulations or reagents or of minimum-potency reference batches may be considered.

In their paper discussing various humoral and cellular immune response assays for vaccine evaluation, Reitan and Secombes (1997) recommended the enzyme-linked immunosorbent assay (ELISA) for vaccine evaluation and pointed out that for several bacterial infections of salmonids, a good correlation between humoral antibody levels and clinical protection has been proven. The antibody responses in fish are, however, water temperature dependent and generally more variable between individuals than seen in mammals (Avtalion and Clem 1981), a situation that adds to the challenges of developing antibody-based potency tests. Generally the first batch potency assay for a new inactivated vaccine antigen is therefore developed using the principal experimental challenge design already accepted in existing monographs.

Refinement of Animal Use During Routine Batch Potency Testing

Batch potency testing is part of manufacturing and the method must be subject to validation; this remains a major cost and risk factor in development of alternatives to challenge assays. Together with the inherent fish-to-fish variability in antibody responses, this has undoubtedly delayed the development and acceptance of alternative potency test methods. However, the use of live animal challenge tests as for quality control should ideally be of temporary nature and limited to the research and development phase for alternative methods that can be carried out without notable animal welfare issues. The need to move away from welfare compromising animal testing in vaccine batch control has therefore been repeatedly advocated (Hendricksen et al. 2008; Romberg et al. 2012).

In the outcome of a specific workshop conducted at the European Centre for the Validation of Alternative Methods (ECVAM) in Ispra, Italy, numerous elements for the reduction of animal welfare-relevant impacts during licensing and control of fish vaccines were proposed (Midtlyng et al. 2011b). Simultaneously, a project aiming to develop an antibody-based assay for furunculosis batch potency was initiated in Norway. When administering a multivalent oil-adjuvanted vaccine and subsequently keeping salmon pre-smolts at three different temperatures, a good correlation between anti-furunculosis antibodies and protection was found after ca 500 day-degrees of immunisation. An immunisation period of 6–9 weeks at a water temperature of 12 °C was therefore proposed for an Ab-based batch potency test (Romstad et al. 2012). In a follow-up trial, the correlation between *A. salmonicida* antigen dose, antibody levels as determined by ELISA and clinical protection to virulent challenge was demonstrated (Romstad et al. 2013), the antibody-based potency assay seemingly being superior in identifying subpotent formulations that were included. Finally, the antibody ELISA proved suitable for testing both multivalent and monovalent oil-adjuvanted vaccine formulations from two fish vaccine manufacturers (Romstad et al. 2014). These results have led to the recent revision of the European recommendation for the antibody-based batch potency test for oil-adjuvanted, injectable furunculosis salmon vaccines (Ph. Eur 01/2015: 1521).

In the interest of animal welfare, further work along these lines should be carried out to cover a wider range of vaccine antigens and fish species, first and foremost the bacterial antigens included in the vaccines that are most widely used (*Yersinia ruckeri*, *Vibrio* spp., *Aliivibrio* spp., *Moritella viscosa* and *Photobacterium damselae* subsp. *piscicida*). Correlation between antigen dose, humoral immune response and protection to waterborne challenge has recently been reported for inactivated infectious pancreatic necrosis virus (IPNV) antigen (Munang'andu et al. 2013a, b), indicating that an IPNV potency assay based on antigen or antibody quantification may be within reach. Promising work to develop an antibody-based potency assay for vaccines covering a non-salmonid target species was also recently been published from Japan (Hirano et al. 2014).

Methods for Documentation of Clinical Safety

Well-controlled laboratory studies constitute the backbone for documentation of clinical safety of veterinary vaccines, focussing predominantly on the absence of acute or subacute toxicity after administration of a single dose to the presumably most sensitive stage of the target species (Ph. Eur. 5.2.6). Safety of repeated administration is only indicated when revaccination is foreseen, and overdose toxicity studies are only required for live vaccines. For fish vaccines, reproductive performance trials are mostly omitted, because of the limited proportion of broodfish amongst the target population. Overdose testing (10×) and specific safety aspects (dissemination of the vaccine strain in target animals, spread to unvaccinated animals and reversion to virulence studies) are needed for all live (replicating) veterinary vaccine formulations including for fish.

The minimum number of animals to be included in laboratory safety trials is 50 fish per vaccinate or control group. For immersion vaccines, the fish should be treated for twice the recommended time and in twice the recommended concentration of vaccine; otherwise, single-dose administration is recommended. The minimum observation period is generally set at 14 days, but 21 days is recommended minimum in the European fish vaccine monographs adopted hitherto. The European regulatory guideline for studies on safety and efficacy of fish vaccines (EMA/CVMP/IWP/314550) contains further methodology details, amongst others the optimum water temperature for safety trials in the most common farmed species, on recording of adverse reaction outcomes and of post-mortem examinations for injection-site lesions upon completion of safety trials. It is also emphasised that for adjuvanted vaccines administered parenterally, adverse effects develop slowly and may have protracted effects manifesting well into the rearing cycle. Studies should therefore be conducted in the field in order to evaluate the safety profile over the life span of the target species and should include assessments of weight gain and of injection-site reactions at the time of harvest.

The mortality rate and the length of the inappetence period after vaccine administration are typical outcomes recorded in short-duration laboratory safety studies of any fish vaccine. The recording of behavioural signs of adverse vaccine effects (Midtlyng 1997) is also relevant in well-controlled safety studies and should be encouraged. For documentation of adjuvanted, injectable formulations, the duration of laboratory trials may be extended up to 6 months in order to allow a meaningful evaluation of injection-site pathology (Midtlyng et al. 1996a; Mutoloki et al. 2004, 2006) and may include å preliminary assessment of growth effects. Well-controlled laboratory studies are generally acceptable to document the absence of acute or subacute toxicity, but less suited to assess injection-site reactions that may result in granuloma formation. Investigations of prolonged inflammatory tissue reactions should therefore also be performed in field studies, together with the assessment of any longer-term and more subtle growth effects (Midtlyng and Lillehaug 1998; Aunsmo et al. 2008a, b).

For the evaluation of abdominal side effects post i.p. vaccination of salmonids with multivalent, oil-adjuvanted vaccines, an ordinal scoring scale from 0 to 6 based on macroscopic pathology findings ("the Speilberg scale"; Midtlyng et al. 1996a; annexed in EMA/CVMP/IWP/314550/2010) has gained wide acceptance. At water temperatures of 10–12° C, the progression of injection-site reactions in Atlantic salmon may take 6–12 months, and peak averages remaining ≤2.5 can be considered acceptable. Intraperitoneal vaccination of salmon with emulsion vaccines causes only slight reduction in growth rate when injection-site lesions are moderate but may increase beyond 10% amongst fishes showing Speilberg scores of 3 or higher (Midtlyng and Lillehaug 1998).

Methods for Efficacy Documentation

Demonstration of vaccine efficacy requires well-controlled clinical trials showing that the vaccine is able to provide the intended or claimed protection against clinical illness and its consequences, morbidity and/or mortality (Ph. Eur. 5.2.7; EMA/CVMP/IWP/314550/2010). For licensing, the methods used and the outcomes to be measured in efficacy studies are therefore highly dependent on which protection claims shall be proven. If a fish vaccine is claimed to protect against infection or to suppress replication of the agent, this must be proven using re-isolation techniques; a fish vaccine example was provided by Frost and Ness (1997). Claims for protection against clinical illness or mortality must be substantiated by data showing effects on corresponding outcomes. While protection against challenge-specific mortality is the most common outcome in efficacy studies of fish vaccines, evaluation of clinical (i.e. lethargy) or pathological (i.e. target tissue inflammation or necrosis) signs may be acceptable outcomes for determination of vaccine efficacy. Specific studies are required to substantiate duration of protection claims, and this represents a particular challenge especially in species normally being reared for 18–24 months before harvest, like most marine cultured finfish (both salmonids and others). Where protection is predominantly antibody mediated, such studies can, however, be elegantly performed by the use of passive immunisation techniques (plasma transfer assays) designed in a classical way (Ellis et al. 1988; LaFrentz et al. 2003).

To provide evidence for clinical protection, the experimental challenge conditions must as far as possible mimic the natural conditions of infection and use a heterologous strain whose relevance to clinical disease should be justified. While maintaining the recommendation to use sufficient fish to ensure statistically significant results, current European guidelines do recommend imbalanced study designs (reduced number of unvaccinated and consequently unprotected controls) and humane (non-lethal) end points that allow euthanasia once the outcome for that fish has been determined (EMA/CVMP/IWP/206555/2010).

Many authors have successfully employed natural (waterborne) experimental challenge models for the common diseases of salmonids (Adams et al. 1987; Ferguson et al. 1991; Bricknell 1995; Nordmo et al. 1997; Madsen and Dalsgaard

1999; Caipang et al. 2006; Løvoll et al. 2009), and this has eventually reduced the use of inoculation methods for efficacy documentation of fish vaccines. Midtlyng (2005) provided examples how protection estimates may vary depending on challenge route (based on data reported by Nordmo and Ramstad 1997) or depending on the cumulative control mortality cutoff (Fig. 6.2). Upcoming research to reveal the more detailed pathogenesis and entry mechanisms for fish pathogens like *V. salmonicida* (Bjelland et al. 2012; Kashulin and Sørum 2014) will serve to improve choice of experimental exposure methods for determining clinical vaccine efficacy in controlled trials.

It is nevertheless notable that certain diseases of Atlantic salmon post-smolts (e.g. IPN) have been notoriously difficult to reproduce experimentally by the use of injection challenge (Rimstad et al. 1991), while success was achieved via waterborne exposure (Stangeland et al. 1996; Bowden et al. 2002). In the case of IPN, it was eventually revealed that the outcome of vaccination and challenge studies was strongly influenced by genetically determined innate resistance amongst the test fish and that the use of an IPN-susceptible recruitment population was able to provide a more predictable and reproducible outcome (Ramstad et al. 2007; Ramstad and Midtlyng 2008). Waterborne challenge studies in susceptible Atlantic salmon were

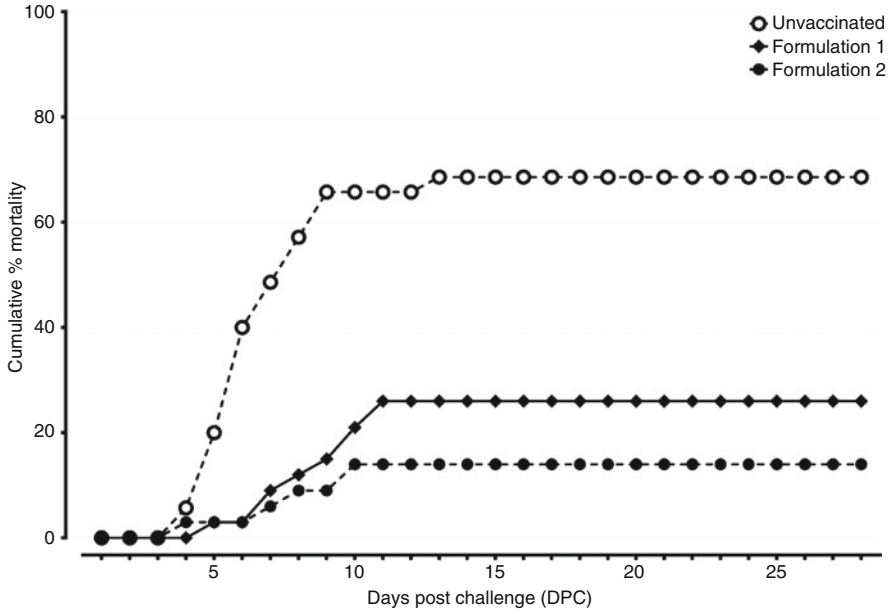

Fig. 6.2 Development of mortality in groups of Atlantic salmon (*Salmo salar* L.) parr immunised against winter ulcer disease using two injectable vaccine formulations and challenged per intramuscular injection with 4000 colony-forming units of *Moritella viscosa*. An important discrepancy was found between relative vaccine performance assessed at 60% control mortality (RPS_{60}) and after cessation of the experimental epizootic ($RPS_{endpoint}$) (Reproduced from Midtlyng 2005 with permission from Karger AG Medical and Scientific Publishers, Switzerland)

instrumental when identifying the genetic determinants of IPNV virulence (Santi et al. 2004) and were recently employed to compare the efficacy of different antigen delivery systems for IPNV antigens (Munang'andu et al. 2012).

Quantification of protective vaccine effect is commonly expressed as relative percentage survival (RPS) or relative percentage protection (RPP), and the warnings as to the interpretation of these figures quoted in the beginning of this chapter (Nordmo 1997) should be re-emphasised. Jarp and Tverdal (1997) pointed to survival analysis and comparison of hazard rates as an alternative technique for comparing the outcomes of experimental or field vaccine trials, where not only the final outcome but also the time course of disease events (time until illness or death) is being taken into account, potentially providing a more sensitive means of statistical outcome analysis.

Part of vaccine efficacy documentation for licensing purposes are state-of-the-art studies to describe relevant immune mechanisms evoked by the vaccine. Typically, these are investigations describing functionally protective antibody responses (ideally), but also non-protective responses (Håvarstein et al. 1990; Frost et al. 1998), or the absence of antibody responses is relevant. Cellular immune response investigations may comprise demonstration of increased spontaneous proliferation, increased responsiveness of leucocytes for immunised fish to antigen stimulation *in vitro or* increased respiratory burst activity of macrophages (Tatner 1990; Marsden et al. 1994, 1996; Midtlyng et al. 1996b). Also the expression of immune genes is highly relevant to describe the immune responses to vaccination in greater detail (Bridle et al. 2013; Munang'andu et al. 2013b) to support – but not to replace – the clinical evidence of vaccine protection.

Field Trial Methodology

The purpose of field trials with veterinary vaccines is to ensure that the evidence for safety and efficacy obtained in controlled laboratory settings can be confirmed under the conditions prevailing where target species populations are being held commercially. This is particularly important for fish vaccines for at least two reasons: firstly because both immunity development and disease occurrence in fish can be heavily influenced by site-specific water temperature and water chemistry profiles and secondly because physical rearing conditions may vary strongly depending on rearing technology (tank or ponds vs. in marine net pen farming). In Europe, a "satisfactory number of sites with conditions representative for the normal in-use conditions" is recommended for field trials (EMA/CVMP/IWP/314550/2010). For efficacy, field trials are particularly valuable to document protection towards a representative variety of field strains and to support duration of immunity (provided natural challenge occurs late in the rearing period). For safety, field trials are of particular importance to assess if there are long-term growth effects under relevant industrial conditions or if there are persistent injection-site reactions that may cause impaired quality of the flesh at harvest. For diseases where no challenge model

exists, the efficacy data from the field may be justified as an alternative. Conversely, where infections are well controlled by existing vaccines (e.g. salmonid furunculosis), sites using a new vaccine under development may unlikely experience any field challenge that can generate efficacy relevant data. In such cases, comprehensive laboratory studies using waterborne challenge methods can be justified for documentation of fish vaccine efficacy, and this situation is acknowledged by regulatory authorities (EMA/CVMP/IWP/314550/2010).

For successful conduct of vaccine field trials, avoiding breakdown of study setup and conduct, farmer and/or investigator compliance with the protocol and accurate recording of the outcomes are important, as are unbiased environmental and rearing history (Mitchell 1997, Nordmo 1999). Blinding is traditionally considered important; however, a favourably performing vaccine will soon reveal itself, and complete blinding may not be ethically or practicably feasible in a full-blown commercial setting. However, the dominant challenge when conducting field trials is in this author's opinion to secure unbiased allocation of test vaccine or reference vaccine/non-vaccinated groups. Wherever groups of fish (tank or pen populations) remain the unit of concern, paired allocation of test vaccine and reference (comparator) vaccine is recommended. This allows the data per group to be stratified for the statistical analysis, with subsequent aggregation of the site results for interpretation of the data. An example of a large-scale multicentre vaccine field trial carried out following this design can be found in Midtlyng (1996).

In their discussion of statistical aspects of fish vaccine trials, Jarp and Tverdal (1997) pointed at the inherent risks of clustering or herd effects (between-site or between-pen variation) that needs to be taken into account in both design and statistical analysis of the outcome data from fish vaccine trials. With an unbalanced design with a dominant proportion of vaccinated and presumably protected fish, herd immunity may completely prevent manifestation of challenge even in the control group and vice versa; a vast majority of unprotected fish may result in a vigorous challenge of the vaccine group and breakdown of vaccine protection. Perhaps the most effective way to avoid clustering bias is the "mark-and-mix" study design where the recruitment population is being randomly allocated to one of the vaccination groups, marked physically to allow easy recognition of group or individual identity, and the returned to the same pen where rearing is continued in a random mixing situation. If the study comprises several mark-and-mix pens, stratified analysis of the results should be performed. In trials using this design, marking can be performed under anaesthesia concurrently with administration of the vaccine, avoiding handling bias and pain during the marking procedures. Recent results showing wound closure within few hours after adipose fin clip suggest that the mark-and-mix design using this group mark should be considered a minimally invasive procedure and appears unlikely to give negative animal welfare effects (Andrews et al. 2014).

Certain safety relevant outcomes (e.g. persistent injection-site lesions or growth) need to be studied over the entire rearing period from vaccination through harvest. After the use of multivalent salmon vaccines, Midtlyng and Lillehaug (1998) found higher abdominal side effects and 23 % reduced growth rate in one of the parallel sites included, while differences in the remaining two

sites were small and non-significant. In another study where vaccine-induced abdominal lesions were initially associated with the development of spinal deformities in Atlantic salmon (Aunsmo et al. 2008a), the weight loss at harvest attributable to vaccination was estimated approximately 10 % (−508 g) and clearly smaller than the effects attributed to gender (+1211 g in males) and presence of spinal deformity (−1177 g).

Outlook

As of 2015, the current regulatory requirements and guidelines for licensing of fish vaccines have – at large – taken the scientific criticism expressed during the IABS Fish Vaccinology Symposia in 1996 and 2003 and by Alderman (2009) into account. Experimental infection models are being developed for both established and emerging diseases of aquaculture species, allowing fish vaccine development and licensing to move forward. In this author's opinion, the development and validation of new standardised antibody response (Romstad et al. 2012, 2013, 2014) and/or antigen quantification methods for batch potency of inactivated vaccines remain, however, a major task. Research on how quantitative (real-time) PCR can be utilised for quantification of fish vaccine antigens and potentially used for batch potency testing should be welcomed.

For efficacy assessment and documentation, there are good arguments to place stronger emphasis than hitherto on morbidity (behavioural, infection and/or tissue pathology) outcomes as the end point of controlled fish vaccine trials. Methods for characterisation of the humoral, cellular and cytokine immune responses (Bridle et al. 2013; Munang'andu et al. 2013b) should form a more comprehensive part of efficacy documentation in the future.

The improvement potential of imbalanced groups' design for studies to evaluate and document fish vaccine safety and efficacy (Midtlyng et al. 2011b) still remains to be realised. In this author's opinion, there is a need to revise current Ph. Eur. monographs accordingly, so that comprehensive safety and efficacy documentation of fish vaccines does not fall victim to animal welfare motivated considerations.

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Chapter 7
Potential of DIVA Vaccines for Fish

Sean J. Monaghan, Kim D. Thompson, Patrick D. Smith, and Alexandra Adams

Abstract The expanding aquaculture industry continues to encounter major challenges from highly contagious viruses. Control and eradication measures for lethal and economically damaging notifiable viral diseases involve 'stamping out' policies and surveillance strategies. Mass-culling of stock and restricted movement of fish and fish products, used to control the spread of notifiable diseases, has considerable impacts on the trade of fish products. Although effective, these measures are expensive and ethically complex and could possibly be reduced by emulating innovative vaccination strategies used by the terrestrial livestock industry. DIVA (*differentiating infected from vaccinated animal*) strategies provide a basis to vaccinate and contain disease outbreaks without compromising 'disease-free' status, as antibodies induced during infection can be used to distinguish from those induced by vaccination. The potential and feasibility of DIVA vaccination in aquaculture is explored here with reference to DIVA strategies applied in higher vertebrates. Three economically important notifiable viruses, causing major problems in three different cultured fish industries, are considered. The increased availability and application of sophisticated biotechnology tools has enabled improved prophylaxis and serological diagnosis for control of viral haemorrhagic septicaemia in rainbow trout, infectious salmon anaemia in Atlantic salmon and koi herpesvirus disease in

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carp. Improving the specificity of serological diagnostics in aquaculture in conjunction with suitable vaccines could enable the application of DIVA strategies, but the immunological variation between different fish species and contrasting pathobiological characteristics of different viruses determines the feasibility and potential of such DIVA approaches for aquaculture industries.

Introduction: Disease Management in Aquaculture Using Vaccination and Serology

Vaccination and serology are effectively used in combination in disease management programmes for terrestrial animals, as serology is regarded as a primary epizootiological and diagnostic tool (Uttenthal et al. 2010); however, serological procedures for monitoring viral diseases in fish are not routinely used in aquaculture (La Patra 1996; Office International des epizooties, OIE 2012). This is despite establishing, more than 40 years ago, that serology can be used to indicate if fish have been exposed to a viral pathogen, e.g. infectious haematopoietic necrosis virus (IHNV) in rainbow trout (*Oncorhynchus mykiss*) (Jorgensen 1974, Amend and Smith 1974 both cited in La Patra 1996) and channel catfish virus (CCV) in channel catfish (*Ictalurus punctatus*) (Plumb 1973 cited in La Patra 1996). Long-lasting, antiviral antibody responses have been detected in fish during certain infections (reviewed in La Patra 1996), including *O. mykiss* infected with IHNV or viral haemorrhagic septicaemia virus (VHSV) (Lorenzen and La Patra 1999; Fregeneda-Grandes and Olesen 2007), Atlantic salmon (*Salmo salar*) infected with infectious salmon anaemia virus (ISAV) (Kibenge et al. 2002; Cipriano 2009) and carp and koi (*Cyprinus carpio*) infected with koi herpesvirus (KHV) (Ronen et al. 2003; Adkison et al. 2005; Perelberg et al. 2008; St-Hilaire et al. 2009; Matras et al. 2012). Serology, therefore, has the potential to make a valuable contribution for diagnosing viral infections in fish (reviewed in Adams and Thompson 2011). Advancements in fish vaccinology for viral diseases have been made possible by the use of molecular biology, thus helping to improve vaccine protection, production and cost-effectiveness (Sommerset et al. 2005; Dhar and Allnutt 2011; Gomez-Casado et al. 2011), but there are currently no DIVA vaccines for fish. Such vaccines would be useful for controlling aquatic notifiable diseases. Application of vaccines for economically important notifiable diseases in other animals has been shown to take advantage of sero-compatible diagnostic tests that are based on antigens unique to the vaccine and/or the pathogen, when implementing vaccination regimes as part of disease surveillance programmes (Van Oirschot et al. 1996; Van Oirschot 1999; Clavijo et al. 2004; Henderson 2005; Suarez 2005, 2012; Vannie et al. 2007; Uttenthal et al. 2010). Determining the most suitable antigen(s) for inclusion and/or exclusion for both the vaccine and diagnostic test is key for developing a reliable system for differentiating between infected and vaccinated animals.

Control of Notifiable Diseases in Aquaculture

The rapid growth of the aquaculture industry since 1970, with a mean annual growth of 8.8%, has also seen an increase in viral disease outbreaks. Strict surveillance is carried out for a number of notifiable viral diseases caused by members of the *Rhabdoviridae*, *Orthomyxoviridae* and *Alloherpesviridae* that have proved both economically and ethically destructive to the aquaculture industry (Olesen 1998; Miller and Cipriano 2003; Haenen et al. 2004), and such programmes sometimes forbid the application of a vaccine, e.g. as a consequence of the designation of ISA as a 'list 1' disease in Norway and the European Union (EU) until 2006 (OIE 2012; Falk 2014).

Viral haemorrhagic septicaemia (VHS) is one of the most economically important viral diseases in salmonid aquaculture. The large number of susceptible host species to viral haemorrhagic septicaemia virus (VHSV) has made control of this rhabdovirus very difficult (Skall et al. 2005; Kim and Faisal 2011; OIE 2012; Biacchesi and Brémont 2014). In Denmark, the rainbow trout industry suffered massive losses due to VHS, with 400 rainbow trout farms infected, with associated costs in the region of £40 million per year (Olesen 1998). More recently the cost of rhabdoviral diseases to Canadian aquaculture has been estimated to be CAD$ 200 million (Salonius et al. 2007). Sanitation and stamping out programmes have proved very effective for reducing and controlling the number of cases throughout Europe (OIE 2012; Jensen et al. 2014), although outbreaks have since been reported in the UK and Norway (Stone et al. 2008; Dale et al. 2009). Nonetheless, implementation of strict control and eradication measures has led to the eradication of VHS in Denmark with no disease outbreaks recorded in the last 2 years (Biacchesi and Brémont 2014; Jensen et al. 2014).

Twenty years after the introduction of sanitation programmes to control VHS, ISA became a threat to the success and sustainability of the Atlantic salmon industry, both in the northern (Lyngstad et al. 2008; Kibenge et al. 2009a; Murray et al. 2010) and the southern (Godoy et al. 2008; Mardones et al. 2009, 2011) hemispheres. ISA is now regarded as one of the most economically important diseases for this industry, representing the only 'list 1' classified notifiable fish disease under the OIE disease classification system of 2009 (OIE 2009). Regulatory controls implemented by the Norwegian authorities included banning the use of non-disinfected sea water in hatcheries and movements of fish between sea sites (Lyngstad et al. 2008; Rimstad et al. 2011), the requirement of health certificates for operating fish farms and the implementation of disinfection measures for water effluent from processing plants and slaughter houses (Thorud and Håstein 2003). Since implementing these stringent sanitary measures, the number of ISA cases in Norway has reduced significantly, with between 1 and 20 cases reported annually (Lyngstad et al. 2008; Rimstad et al. 2011), although the economic impacts resulting from ISA still costs the Norwegian aquaculture industry an estimated US$ 11 million annually have been massive in Norway (Hastings et al. 1999). Globally, the losses to the Atlantic salmon industry resulting from ISA outbreaks has ranged from millions in the USA (Miller 2003), Canada (Hastings et al. 1999) and Scotland (Rodger et al. 1998; Hastings et al. 1999) to $2 billion in Chile (Kibenge et al. 2009a; Mardones et al. 2011).

Eradication programmes have been successfully implemented for the control of ISA (Bricknell et al. 1998; Rodger et al. 1998; Hastings et al. 1999; Stagg 2003; Murray et al. 2010); however, their success has been attributed to extensive culling, restricted movement of personnel and vehicles and fish and fish products. The implementation of control and surveillance zones with regular inspections during and following disinfection and fallowing, coupled with improved codes of practice for fish husbandry, has ensured no recurrences of ISA outbreaks (Royal Society of Edinburgh; RSE 2002). Epidemiological analysis of the recent ISA outbreaks in Chile suggests that control strategies in such highly populated farming areas should include control zones of at least 10 km rather than the 5 km currently recommended by the OIE (Mardones et al. 2011). Although this may be more effective, ultimately more animal slaughter and trade restrictions may occur as a result.

Another important aquaculture sector, the common carp and koi industry, has suffered major losses as a result of koi herpesvirus disease (KHVD), which is highly virulent and temperature-dependent. A lack of regulation of koi movements may represent the root cause for the worldwide spread of KHVD, because ornamental fish seem to have been less stringently screened than food fish (Haenen et al. 2004; Pearson 2004). The enormous impact of KHVD is now seen in carp fisheries (Peeler et al. 2009; Taylor et al. 2010), the ornamental koi and food carp industries (Perelberg et al. 2003; Antychowicz et al. 2005; Gomez et al. 2011; Azila et al. 2012), and also in wild carp populations throughout the world (Takashima et al. 2005; Grimmett et al. 2006; Uchii et al. 2009, 2011; Garver et al. 2010; Minamoto et al. 2011, 2012).

The first reported outbreaks of KHVD occurring in Israel in 1998 (Ariav et al. 1999), spread to 90 % of all carp farms in the country by the end of 2000, costing the Israeli carp industry $3 million per year (Perelberg et al. 2003). Disease outbreaks of KHVD also occurred in the USA and in Europe around the same time, which were initially reported at a koi show in New York and then later by koi dealerships (Bretzinger et al. 1999; Hedrick et al. 2000). The disease was possibly spread during the transportation of fish or when they were held together in tanks or ponds without quarantine (Haenen et al. 2004). The disease has since been reported in at least 28 countries (OIE 2012) throughout Europe (Haenen et al. 2004; Gotesman et al. 2013), Asia (Sano et al. 2004; Bondad-Reantaso et al. 2007; Ilouze et al. 2011; Rakus et al. 2013), Africa and North America (Haenen et al. 2004; Rakus et al. 2013).

High mortalities on carp farms in Indonesia (80–95 %) in 2003 resulted in losses worth ~ $15 million (Rukyani 2002 cited in Haenen et al. 2004). In Japan, 1200 tonnes (t) of carp died during a KHVD outbreak in Lake Kasumigaura, Ibaraki, in 2003, and by 2004, the virus was detected in 42/47 regions of Japan, with more than 100,000 mortalities reported (Iida and Sano 2005; Ishioka et al. 2005; Matsui et al. 2008; Yuasa and Sano 2009). The disease was a serious threat to the $75 million ornamental carp industry, and consequently, all nishikigoi shows were cancelled in November 2003 (Haenen et al. 2004). Although KHVD was initially prevented from being listed as a notifiable disease, because the absence of disease could not be ascertained and diseased fish could not be confidently identified, which limited the usefulness of OIE legislation (Haenen et al. 2004), vaccination was believed to hold great potential for controlling the disease in the future.

Definition of Marker and DIVA Vaccines

The term *differentiating infected from vaccinated individuals* was first proposed by Jan Van Oirschot and co-workers to replace the previously defined concept of 'marker vaccines' (Van Oirschot et al. 1986, 1996; Van Oirschot 1999). They defined marker vaccines as 'a vaccine (inactivated or live) based on deletion mutants or isolated microbial proteins that allow the differentiation between vaccinated and infected individuals based on the respective antibody responses which are elicited. Hence, a marker vaccine is used in conjunction with a test that detects antibodies against a protein that is lacking in the vaccine strain' (Van Oirschot et al. 1996). Although the 'marker vaccine' was, in general, initially based on deletion mutants of the wild-type microbe in conjunction with a differentiating diagnostic test (Pasick 2004), the term DIVA has now been extended to include subunit and inactivated whole vaccines (Pasick 2004; Uttenthal et al. 2010) or other vaccines that lack immunogenic proteins of the wild-type strain, e.g. DNA and recombinant vaccines. The accompanying serological diagnostic test is just as important as the vaccine itself, as detection of marker-specific antibodies is necessary in order to be able to distinguish between the host's antibody response to the vaccine and to the wild-type virus (Clavijo et al. 2004; Uttenthal et al. 2010). In the majority of cases, the primary concern is to determine whether or not an animal has been infected rather than vaccinated, thus *differentiating infection in vaccinated animals* (DIVA) (Uttenthal et al. 2010).

Application of DIVA Vaccines in Terrestrial Animals

The application of marker vaccines, in combination with additional management measures, such as reduced contacts between herds, can increase the possibility of disease eradication whilst providing a means to identify uninfected vaccinated animals. The first successful DIVA strategy to be implemented was for the control and subsequent eradication of pseudorabies virus (PrV), the causative agent of Aujeszky's diseases (AD), using a glycoprotein E (gE)-negative vaccine and a gE-specific serological test to differentiate between vaccinated and infected pigs (Van Oirschot et al. 1996; Stegeman 1995; Van Oirschot 1999; Vannie et al. 2007). The control of AD-infected farms involves emergency vaccination with a DIVA vaccine (i.e. ring vaccination) and containment of the virus within a 10 km infected zone implemented by movement restrictions. This avoids culling apparently healthy animals exposed to the virus, thus preventing the need for mass culling.

Although the majority of DIVA-compatible vaccines developed for animals have been based on the four most economically important trans-boundary diseases in Europe, i.e. AD, classical swine fever (CSF), foot and mouth disease (FMD) and avian influenza (AI) (Van Oirschot et al. 1996; Babiuk 1999; Van Oirschot 1999; Clavijo et al. 2004; Pasick 2004; Bouma 2005; Suarez 2005,

2012; Beer et al. 2007; Vannie et al. 2007; Uttenthal et al. 2010), the development of many other DIVA vaccines, also for other animal viral diseases, have been reported. Recent advances in various biological disciplines (i.e. immunology, microbiology, molecular biology, proteomics, genetics, genomics and microbial pathogenesis) have led to their application in the development of these various DIVA vaccines. The approach for developing a DIVA vaccine requires either (1) construction of vaccines that exhibit different immunogenic properties to the wild-type strain or (2) exploitation of the immunogenic variations that exist between vaccine and wild-type strain virus structural and nonstructural proteins, including vaccines engineered with gene deletions and additions, live vectored vaccines, chimeric vaccines and peptide and subunit vaccines (Babiuk 1999; Henderson 2005; Meeusen et al. 2007). Regardless of the approach taken to design the vaccine, it is necessary to specifically detect antibodies of the vaccinated/infected animals to the marker antigen(s) with a sensitive 'marker assay' (Beer et al. 2007), i.e. host response to those proteins present/absent in the vaccine.

A prerequisite for DIVA vaccination is that all field strains express the marker antigen and that infected animals always elicit antibodies to that protein after infection (Van Oirschot et al. 1996; Van Drunen Little-van den Hurk 2006). A number of requirements for the DIVA diagnostic test in *mammals* were proposed by Van Oirschot et al. (1996):

1. Antibodies must be detectable within 3 weeks after infection.
2. Antibodies must persist for a long period after infection.
3. Vaccinated and subsequently infected animals must elicit antibodies against the wild-type virus replicating within the host.
4. Repeatedly vaccinated animals must score negative to markers for infection.
5. The test must display high sensitivity, specificity and reproducibility.

The detection of a specific antibody response to a 'foreign' exogenous marker (positive marker) or absent endogenous marker (negative marker) is achieved through serological testing using an ELISA. Only animals inoculated with the vaccine containing the marker antigen will produce a detectable antibody response against the marker, whereas animals responding to epitopes associated with the pathogen indicate that they are infected or have been vaccinated with an alternative vaccine (James et al. 2008) (Fig. 7.1a, b). Although this approach does not enable a DIVA strategy, it can be applied for DIVA approaches if accompanied with a vaccine that differs sufficiently to the infectious agent to distinguish animals that had been vaccinated prior to becoming infected, which otherwise cannot be serologically identified by negative markers alone. The negative marker approach is achieved by detection of antibodies to an antigen absent from the vaccine to indicate infection, but antibodies to alternative antigens of the pathogen indicate vaccination (Fig. 7.1c, d).

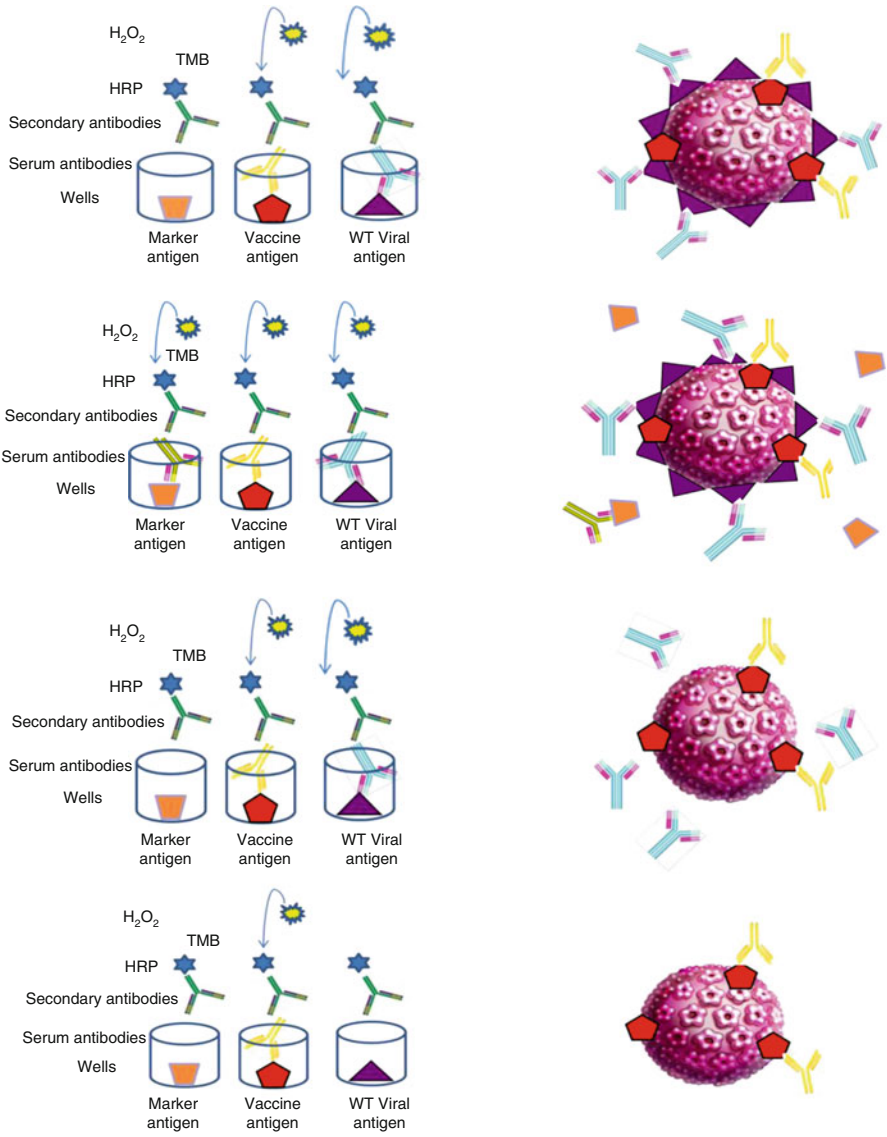

Fig. 7.1 Schematic diagram of antigen specific enzyme-linked immunosorbent assay (ELISA) and antibody response to exogenous 'positive' and endogenous 'negative' marker vaccine antigens. *A* conventional vaccine and ELISA, *B* 'positive exogenous' marker vaccine and ELISA, *C* and *D* 'negative endogenous' marker vaccines and ELISAs. (**a**) Antibody response to all vaccine antigens. Note no differential response to WT and vaccine; (**b**) antibody response induced to vaccine and marker antigens indicating vaccination; (**c**) positive signal induced to two viral antigens including antigen absent in the vaccine indicating infection; (**d**) positive signal only to vaccine antigen present in the vaccine indicating vaccinated but uninfected. Different coloured immunoglobulins represent specific antibody responses to *orange* = marker, *blue* = virus and *yellow* = vaccine. HRP and TMB are enzyme and substrate, respectively, involved in the reaction resulting in a chromogenic signal. *WT* wild-type antigen. Shapes represent epitopes of the marker , vaccine and virus antigens Figure virus particles modified from http://www.genesium.ro/proceduri/depistare-si-tipizare-hpv.html

Potential of DIVA Vaccines for Disease Control in Aquaculture

Despite extensive research into the development of a VHS vaccine, using killed, live attenuated or recombinant viral antigens (Lorenzen and La Patra 2005), no VHSV vaccine for mass delivery is yet available (Lorenzen et al. 1998; Lorenzen and La Patra 2005; Sommerset et al. 2005; Gomez-Casado et al. 2011; Biacchesi and Brémont 2014). Several candidate vaccines, based on inactivated virus, recombinant G protein and attenuated virus, have effectively protected fish against challenge (Biacchesi and Brémont 2014), but the most promising development for aquatic rhabdoviral diseases has been the use of DNA vaccines (Anderson et al. Anderson et al. 1996a, b; Lorenzen and La Patra 2005; Gomez-Casado et al. 2011), of which have recently been licensed for use in Atlantic salmon in Canada for IHNV (Biacchesi and Brémont 2014). Nonetheless, vaccination would not be permitted in VHS-free zones according to EU regulations (OIE 2012), whilst killed or non-replicating vaccines have been approved for use on infected farms (Olesen 1998), i.e. to limit viral spread, although survivors can also become long-term carriers, meaning that vaccinated animals could continue to spread disease. Surveillance is difficult by virus isolation, as this may not be able to identify subclinically infected fish (Skall et al. 2005); thus, detection of VHSV antigen using cross serotype reactive monoclonal antibodies (MAbs) (Lorenzen et al. 1988) or nucleic acid by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Garver et al. 2011; Jonstrup et al. 2013; Warg et al. 2014a, b) is undertaken to confirm a VHS-free status. Maintaining a disease-free status is complicated when restriction of fish movements is the only method of control, as reservoir rainbow trout carrying marine genotypes (i.e. genotype III) have been associated with reported VHS outbreaks in Norway, despite this genotype previously being considered non-pathogenic to rainbow trout (Dale et al. 2009; OIE 2012). If an approach differentiating infected from vaccinated animals (DIVA) could be applied, vaccination could still be possible without compromising the disease-free status of the fish.

The criteria for detecting ISAV according to the OIE *Manual of Diagnostic Tests for Aquatic Animals* (OIE 2012) include the use of virus isolation in cell culture, but many isolates and strains of ISAV have limited cytopathogenicity and thus may not replicate within the cell line and/or lack cytopathic effect (CPE) (Dannevig et al. 1995; Rolland et al. 2003; Kibenge et al. 2006). It may take a number of weeks or months before ISA develops in neighbouring pens, so surveillance is currently based on virus detection using an indirect fluorescent antibody technique (IFAT) and qRT-PCR for confirmation, whilst antibody detection is only accepted as an additional method for virus detection (OIE 2012). Vaccination against ISA has been included in control programmes in Canada since 1999, the Faroe Islands since 2005 and Chile since 2010 (Falk 2014), but does not provide complete protection in Atlantic salmon (OIE 2012). The current vaccines used are mostly inactivated virus vaccines (Dhar and Allnutt 2011) that do not provide clearance of the virus in immunised fish, which can subsequently become carriers (Kibenge et al. 2004; OIE 2012). A number of other vaccines targeting the haemagglutinin esterase (HE)

protein have also been produced (Mikalsen et al. 2005; Dhar and Allnutt 2011; Wolf et al. 2013), but if a DIVA vaccine is to be used for ISA, together with an antibody diagnostic test, it would be necessary to ensure that antibodies elicited by the vaccine are not induced by the virus itself and that the fish screened are not carriers. Additional complications for ISA surveillance programmes include the fact that there are a number of other salmonid hosts acting as reservoirs for the virus (Falk 2014) and the presence of many low pathogenic or avirulent strains in the aquatic environment. These have been associated with genetic variability of the HE protein of segment 6, mostly situated in the small hyper-polymorphic region (HPR), through which the full-length HPR (HPR0) of ISAV is considered to be a precursor to virulent ISAV strains (Aamelfot et al. 2014). A lack of clinical disease signs is often associated with fish carrying the HPR0 strain (Christiansen et al. 2011), and despite detecting the presence of HPR0 regularly for 8 years in the Faroe Islands, very few outbreaks of ISA have occurred there (Aamelfot et al. 2014). Nonetheless, as the HPR0 strain has been identified in clinically healthy wild and farmed Atlantic salmon in Scotland, Canada, Norway, the Faroe Islands and Chile, the HPR0 strain of the virus should be monitored and transmission controlled to limit outbreaks associated with mutations (deletions) that result in enhanced virulence.

Since being listed as notifiable, vast numbers of studies have been carried out for KHVD to develop sensitive, specific, convenient and cost-effective diagnostic tests for KHV surveillance (reviewed in Haenen et al. 2004; Sano et al. 2005; Gotesman et al. 2013; Rakus et al. 2013). However, there are still no validated tests that are accepted for declaring fish free of KHV (OIE 2012). Vaccine development for KHV is a major area of research, which has included live attenuated (Ronen et al. 2003; Perelberg et al. 2008; Dishon et al. 2014; O'Connor et al. 2014; Weber III et al. 2014), inactivated oral (Yasumoto et al. 2006; Miyazaki et al. 2008), inactivated injectable (Monaghan 2013; Schmid et al. 2016), recombinant multi-deletion (Costes et al. 2008, 2012; Vanderplasschen 2013; Boutier et al. 2015) and oral and injectable DNA vaccines (Aoki and Hirono 2011; Zhou et al. 2014a, b; Cui et al. 2015; Kattlun et al. 2016). Controlling the spread of KHV is complicated by a number of potential vectors such as goldfish (Bergmann et al. 2010b; El-Matbouli and Soliman 2011; Fabian et al. 2013), sturgeon (Kempter et al. 2009) as well as molluscs and crustaceans (Kielpinski et al. 2010).

Vaccination is seen as an important tool for controlling KHVD (Dishon et al. 2014) as eradication and disinfection have not proved effective (Ronen et al. 2003; Perelberg et al. 2008; Ilouze et al. 2011). A live attenuated vaccine was developed in Israel to enable emergency vaccination during mass KHVD outbreaks that occurred between 1998 and 2000, which provided good levels of protection against the virus. This vaccine (KoVax, KV3) has now been commercialised and is used widely across Israel (O'Connor et al. 2014; Weber III et al. 2014; Dishon et al. 2014). However, the implications associated with this include the potential spread and transmission of wild-type virus from vaccinated carp exported from Israel that may act as carriers (Peeler et al. 2009), presenting a risk to naïve, unvaccinated carp stocks. Regardless of the vaccine, being able to identify fish as vaccinated and uninfected is important for introducing vaccinated fish into unvaccinated naïve populations.

Use of Marker Vaccines

The first marker vaccines were developed for economically important alphaherpesviruses, e.g. PrV and bovine herpesvirus 1 (BoHV-1), which were generated containing a deletion of a non-essential, but immunogenic envelope glycoprotein (gE) and have been effectively used as both live and inactivated marker vaccines (Van Oirschot et al. 1986, 1996; Kaashoek et al. 1996; Bosch et al. 1996; Van Drunen Little-van den Hurk 2006; Romera et al. 2014). These allow identification of infected animals by detecting specific antibodies to the viral gE protein absent in the vaccine, whilst vaccinated, uninfected animals have antibodies only to other glycoproteins of the virus still present in the vaccine.

Live marker vaccines are often further attenuated by deletions of the thymidine kinase (TK) gene (Quint et al. 1987; Kaashoek et al. 1996; Anziliero et al. 2011), as achieved for alloherpesviruses (Hanson et al. 2011) during the development of a live vaccine for channel catfish against CCV (Kancharla and Hanson 1996) or for carp against KHV (Costes et al. 2008; Vanderplasschen et al. 2013). Some of the structural proteins identified on KHV, for example (Michel et al. 2010; Yi et al. 2014), include a number of immunogenic membrane glycoproteins of ORF 25, 65, 81, 148 and 149 (Rosenkranz et al. 2008; Fuchs et al. 2014), which could potentially be used for DIVA strategies, as production of subunit or DNA vaccines containing some of these antigens could allow serological differentiation between vaccinated fish and infected fish (Fuchs et al. 2014). However, it is unknown whether these KHV proteins are antigenically identical between all KHV isolates, as some studies have reported genetic differences between geographical distinct KHV isolates with respect to some of these immunogenic membrane glycoprotein targets (e.g. ORF25 and 65) (Han et al. 2013; Xu et al. 2013), which could affect the reliability of the diagnostic test, and furthermore, these studies have only screened the targets with a limited number of carp antisera. It has proven difficult to establish the most immunogenic antigens of KHV because of variations in antibody responses of individual carp to different virus antigens following infection and/or exposure to KHV (Adkison et al. 2005; Monaghan et al. 2011; Wasa et al. 2014).

The high sensitivity and specificity of the serology test for gE has contributed immensely to the success of intensive marker vaccination sero-surveillance programmes for AD in pigs (Pensaert et al. 2004), using synthetic gE peptides (Jacobs and Kimman 1994) and baculovirus-expressed recombinant gE antigen (Gómez-Sebastián et al. 2008) to coat the ELISA plates. Immunogenic epitopes of the G protein of VHSV, a virus notably affecting rainbow trout, have been previously mapped (Fernandez-Alonso et al. 1998). This protein has the potential to be used in a similar DIVA approach to that used for AD, if certain epitopes are absent from the protein expressed as a recombinant vaccine. Another potential approach for VHSV could be to utilise recombinant viruses containing a deletion of the nonstructural, non-virion (NV) protein, which previously resulted in irreversible attenuation in rainbow trout (Thoulouze et al. 2004), as long as antibodies could be detected to the NV protein in vaccinated fish.

Chimeric viruses have proved an effective approach for use as mammalian marker vaccines, e.g. for classical swine fever virus (CSFV). By substituting immunogenic proteins with a homologous protein of a closely related virus, the vaccine can be utilised as a marker vaccine, e.g. the envelope proteins E2 or E^{RNS} of the flavivirus CSFV substituted with an analogous sequence from the flavivirus bovine viral diarrhoea virus (BVDV) (Van Gennip et al. 2002; Koenig et al. 2007). The envelope proteins, E2 and E^{RNS} of CSFV, are known to have high and low neutralising activity, respectively (Huang et al. 2006), making them ideal targets for this approach as one immunogenic protein is dispensable for the vaccine. However, the problems with using a negative marker (i.e. its removal from the vaccine), where the most protective antigen is also the most immunogenic, compromise either the diagnostic assay sensitivity or the vaccine efficacy (Eblé et al. 2013). Other approaches, which have proved successful for CSFV, have used recombinant vectors and replicon vaccines designed to express the CSFV E2 gene (Hahn et al. 2001) or carry CSFV-E2 and CSFV-E^{RNS} deletions (Van Gennip et al. 2002; Stettler et al. 2002), respectively. Replicon vaccines for fish, e.g. against ISAV, have been designed using salmon alphavirus (SAV) to carry insertions of ISAV HE protein, constituting antibody responses and protection against ISAV challenge (Wolf et al. 2013). This vaccine also holds the potential for differential serological diagnostics for ISA using SAV or other alternative ISAV proteins not expressed in the replicon vaccine. However, the virus vector for the vaccine antigen must be taken into consideration as the prevalence of the vector virus in the environment, e.g. SAV, would compromise the serological test. One of the other few reports relating to marker vaccines for fish viruses involves the insertion of the G-gene of VHSV and IHNV into pathogenic bacteria (*Aeromonas salmonicida*) as a vector (Enzmann et al. 1998). A differential antibody response was seen between the vaccine strain and the wild-type virus using sera from immunised rainbow trout in Western blot (Enzmann et al. 1998). Recombinant technology using bacterial artificial chromosomes (BAC) for vaccine development (Costes et al. 2008, 2012; Vanderplasschen 2013) could also provide a basis for deletion marker vaccines for KHV. Recently a multi-deletion vaccine based on deletions of the proteins encoded by ORF56, −57 and −134 resulted in a safe and efficacious live attenuated KHV vaccine (Boutier et al. 2015). Recombinant antigens to ORF56, −57 or −134 used in serological ELISA tests could potentiate DIVA compatibility for this vaccine.

Exogenous marker vaccines, on the other hand, are developed by either administering 'foreign' antigens (i.e. proteins not naturally encountered by the host), within the vaccine formulation (James et al. 2007, 2008) or through insertion of heterologous genes into the vaccine strain genome in order to induce a detectable differential antibody response (Castrucci et al. 1992; Walsh et al. 2000a, b; Mebatsion et al. 2002; Fang et al. 2008). Antibody responses to the 'foreign' protein, indicate that the animal is vaccinated, regardless of infection status. There has been some debate regarding the use of positive marker vaccines, because of the difficulty in differentiating infected carrier animals from vaccinated animals (Van Oirschot 1999). However, the incorporation of a positive marker can ensure that only authorised vaccines are used during regulated vaccination programmes, which is essential both for trade and surveillance purposes with regard to successful control of notifiable

diseases (Suarez 2005). Positively marked vaccinated animals could then be traded or moved between regions or countries with full awareness of the product used to vaccinate the animal.

The incorporation of a foreign gene into the genome of a pathogen as an endogenous marker can prove complicated as the protein expressed must be sufficiently immunogenic. Green fluorescent protein (GFP) is a well characterised, commonly used reporter gene in transfections, microbial pathogenesis and virus mutagenesis studies, including expression in ISAV and KHV (Mikalsen et al. 2005; Fichtner et al. 2007), and previous studies have measured specific antibody responses to GFP in carp (Companjen et al. 2006), suggesting a potential application of GFP as an exogenous marker vaccine antigen.

Other approaches have been applied for positive marker vaccination against aquatic RNA viruses, e.g. IPNV. Subviral particles (SVPs) are formed by structural virus proteins self-aggregating to form particles that do not mimic the native virus capsid (Dhar et al. 2010). These have been synthesised for IPNV VP2 protein (Allnutt et al. 2007). The recombinant VP2 (rVP2) particles that result are able to carry foreign protein insertions, which reduced IPNV shedding in immunised fish and which elicited specific antibodies to both the foreign antigen, *c-myc* (human oncogene), and VP2 protein (Dhar et al. 2010). If antibodies are also detected in other IPNV proteins, e.g. VP3, then such a vaccine could be used as a DIVA vaccine. The G protein of VHSV and HE protein of ISAV have been incorporated into the genome of IHNV (as a vaccine carrier), resulting in efficiently stable expression of the recombinant virus that provided protection in the majority of fish (Harmache et al. 2006). Similar mutations of the IHNV genome have led to attenuation and the induction of specific antibodies in challenged trout (Romero et al. 2005).

Another marker which has been added to vaccines is tetanus toxoid (TT). This has been added to an inactivated avian influenza (AI) vaccine for use in chickens and ducks, and birds responding to the TT were subsequently identified as 'vaccinated' (James et al. 2007, 2008). As TT is immunologically foreign to fish, it has the potential to be used as a marker antigen in aquaculture vaccines, especially since it is already registered for applications in food animal vaccines (James et al. 2007). However, specific anti-TT antibodies were not detected in fish vaccinated with an inactivated ISA vaccine to which TT had been added (Monaghan 2013). The immunogenicity of molecules in mammals does not always reflect the response obtained in fish (Alcorn and Pascho 2002), as reflected in the TT marker vaccine studies conducted in salmon (Monaghan 2013), possibly because of their lack of high titre, high affinity antibodies, e.g. compared to IgG and IgY in mammals and birds, respectively (Kaattari 1994; Cain et al. 2002). Thus, finding a suitably immunogenic foreign antigen to use as an exogenous marker antigen still poses a major obstacle for establishing a marker vaccine for fish

The use of synthetic peptides has been suggested as a means of inducing an alternative antibody response by administrating specifically designed immunogenic peptides (Root-Bernstein 2005). Lower immunogenicity and/or antigenicity of B-cell epitopes in fish may impede this approach for aquaculture, however (Lorenzen and La Patra 1999).

Diagnostics Tests to Accompany DIVA Vaccines

Mammalian alphaherpesvirus subunit vaccines based on glycoprotein gD, a highly protective and essential protein of BoHV-1, has provided a cost-effective and safe DIVA approach using an accompanying sensitive and specific ELISA to the gE protein, as described above, to detect infected animals (Van Drunen Little-van den Hurk et al. 1997; Babiuk 1999). The disadvantage of the subunit vaccines is the delayed antibody response elicited compared to live attenuated vaccines (Van Oirschot 1999; De Smit et al. 2001; Beer et al. 2007) providing a longer window of potential false-negative diagnosis.

Research into diagnostic test development, rather than the vaccine, has also enabled DIVA strategies to be developed for already available protective subunit or whole inactivated vaccines for AIV, FMDV and CSFV (Mackay et al. 1998; Clavijo et al. 2004; Perkins et al. 2007a, b; Uttenthal et al. 2010; Mohapatra et al. 2011). These DIVA diagnostic tests have focused on nonstructural proteins, e.g. NS1 of AIV and any of FMDV (Mackay et al. 1998; Clavijo et al. 2004; Kwon et al. 2009; Mohapatra et al. 2011) as well as structural proteins, e.g. the matrix protein, M2e for AIV (Suarez 2005, 2012; Lambrecht et al. 2007; Kwon et al. 2009; Hemmatzadeh et al. 2013). Characteristics that make such proteins ideal for use as the DIVA ELISA test antigen include being expressed in high abundance during virus replication, e.g. Me2 matrix protein of AIV, or their absence/low abundance in vaccinated animals when the vaccine strain is not replicating (Clavijo et al. 2004; Uttenthal et al. 2010). These proteins should also be highly conserved amongst different serotypes and subtypes of the virus, e.g. of AIV and FMDV (Doel 2003; Clavijo et al. 2004; Uttenthal et al. 2010; Shao et al. 2011), unlike the vaccines themselves, which are based on viral surface proteins, where many serotypes and subtypes exist, although in CSFV this actually prevents their application for DIVA diagnostics because of high conservation, i.e. of NS3, leading to problems of cross reacting antibodies in the field (Van Gennip et al. 2001; Beer et al. 2007).

DIVA approaches for VHS could benefit from a G protein subunit vaccine based on an antigenic epitope shared by all isolates to circumvent the issues imposed by the multiple genotypes, although this is complicated by the fact that serogrouping does not correlate with genotypes (OIE 2012) and expressing the VHSV G protein in an antigenic form able to provide protection has so far failed (Biacchesi and Brémont 2014). Recently, a more promising oral subunit vaccine was developed for ISAV based on the HE protein (Dhar and Allnutt 2011). This could potentially enable a DIVA approach by screening for antibodies against the nucleoprotein (NP) that is lacking in the vaccine, as only infected fish would respond to this antigen. Such DIVA strategies usually comprise the administration of subunit and recombinant vaccines lacking the internal, usually non-protective, viral proteins in conjunction with recombinant ELISAs. These DIVA assays employ plates coated with (1) the vaccine antigen and (2) the viral antigen absent in the vaccine.

Multiplex assays have been developed to circumvent problems with variation in immunoreactivity of FMDV nonstructural proteins (Perkins et al. 2007a, b) and

detection of mucosal IgA, which is only produced in high levels in infected animals and has been proposed as an alternative diagnostic to alleviate the issues encountered with serological sensitivity of IgG detection (Parida et al. 2006). The mucosal immunoglobulin of fish, IgT/IgZ, may be useful in this regard, especially for developing non-lethal DIVA diagnostics, e.g. from gill and/or skin swabs, although research on this antibody is still in its infancy and the tools for its detection are still lacking (Hansen et al. 2005; Zhang et al. 2010; Tadiso et al. 2011; Castro et al. 2013; Rasmussen et al. 2013).

Antibody-based DIVA approaches have recently been based on differential antibody responses to structural proteins of live infectious KHV and an inactivated whole virus vaccine (Henderson Morley Ltd., 2009). By using recombinant proteins of an internal tegument protein region (rORF 62) and transmembrane protein region (rORF 68) (Aoki et al. 2011) of the virus, it has been possible to differentiate between carp that had been vaccinated and those that had been infected (Monaghan et al. 2011; Monaghan 2013). Similar approaches using inactivated vaccines have been applied for DIVA strategies based on HA surface protein and nucleoproteins (NP) for orthomyxoviruses, such as equine influenza virus (Minke et al. 2004; Kirkland and Delbridge 2011), and have also been considered for avian influenza virus (AIV) (Suarez 2005). Vaccines developed so far against ISA in Atlantic salmon include a recombinant subunit protein vaccine (Dhar and Allnutt 2011), a DNA vaccine (Mikalsen et al. 2005) and a salmon alphavirus replicon vaccine (Wolf et al. 2013), which have all been developed specifically to also express a protective surface haemagglutinin esterase (HE) protein but not other ISAV proteins. Since internal structural proteins of ISAV, i.e. the NP, has been reported to be highly antigenic for anti-ISAV Atlantic salmon sera (Kim Thompson pers. observation; Falk pers. comm. cited in Wolf et al. 2013), this protein holds potential for use in a DIVA strategy. The application of ISAV NP and HE proteins in a companion serology test could enable differentiation between infection and vaccination if antibodies from vaccinated fish could be detected by a HE-specific ELISA and infected fish detected by an NP-specific ELISA.

DNA vaccination has not only proven a very promising approach to vaccination, it has broadened the potential for DIVA strategies, as noted for CSFV targeting E2 and E^{RNS} envelope proteins and the nonstructural protein, NS3 (Beer et al. 2007). DNA vaccines have also provided encouraging results in fish, e.g. for rhabdoviruses VHSV, IHNV and spring viraemia of carp virus (SVCV) (Anderson et al. 1996a, b; Lorenzen et al. 1998; Lorenzen and La Patra 2005; Sommerset et al. 2005; Emmenegger and Kurath 2008; Tonheim et al. 2008), which may enable differentiation of antibody responses if viral antigens present in the infectious virus strain, other than the G protein expressed by the vaccine, are coated onto ELISA plates.

A KHV capsid-associated protein encoded by ORF84 (Monaghan 2013; Monaghan et al. 2016) and nonstructural protein encoded by ORF12 (Kattlun et al. 2014) have also been reported as immunogenic to carp. Such antigens could be applied as a companion diagnostic test for DNA vaccines, which have also been developed for KHV (Aoki and Hirono 2011; Zhou et al. 2014a, b), as only protective glycoproteins of the virus are present in the DNA vaccine which lacks expression of internal capsid

proteins or nonstructural proteins. However, recent serological studies on KHV have revealed a vast variation in reactivity of individual carp to this virus, which complicates the application of independent antigens for detection of seropositive fish (Adkison et al. 2005; Monaghan et al. 2011; Wasa et al. 2014). Furthermore, recent studies have failed to detect antibodies to highly immunogenic KHV antigens, such as the expression product of ORF81, following DNA vaccination (Kattlun et al. 2016).

Different antibody responses between fish to the same viral protein, but to different fragments of that protein, have been demonstrated for VHSV in rainbow trout (Encinas et al. 2011b). This may be associated with disulphide-dependent conformational epitopes (Einer-Jensen et al. 1998). Thus, such antigenic epitopes of the protein may be masked by cross-linking during formalin inactivation of the virus when developing the inactivated vaccine, and the native form is subsequently not recognised by these immunised fish. However, altered conformation of virus epitopes or incorrect protein folding following expression through *E.coli* can also affect antibody detection on the diagnostic test. Proteins expressed as inclusion bodies are generally misfolded and often biologically inactive (Sørensen and Mortensen 2005). Although recombinant proteins produced through prokaryotic expression systems, i.e. in *E. coli* vectors, have been successfully applied for fish sero-diagnostics (Huang et al. 2001; Kim et al. 2007; Encinas et al. 2011a, b; Monaghan et al. 2011; Wasa et al. 2014), often the folding of the target protein of interest is important for specific antibody detection. The yeast *Pichia pastoris* has been utilised for the production of antigens for recombinant protein ELISAs for mammalian herpesviruses (Ao et al. 2003) as proteins can be expressed with correct folding, disulphide bond formation and post-translational modifications such as glycosylation (Macauley-Patrick et al. 2005). Other studies developing recombinant ELISAs for VHSV used *P. pastoris* for generating highly specific fragments of the major antigenic 'G' protein of the virus (Encinas et al. 2011a, b). Alternatively, a baculovirus expression system has been used in a number of studies for sensitive detection of notifiable mammalian viral diseases (Kimman et al. 1996; Pérez-Filgueira et al. 2006; Gómez-Sebastián et al. 2008) and may prove useful for fish serology and DIVA diagnostic test development. Recombinant baculovirus-expressed glycoprotein of KHV ORF149 has been successfully generated for potential application in serological diagnostic tests and DIVA strategies (Fuchs et al. 2014).

Genetic DIVA Strategy

Novel 'genetic DIVA' strategies have also been developed for direct differentiation of the wild-type strain and vaccine strain viral genome within mammalian hosts. For example, CSFV and BoHV-1 wild-type strain and attenuated live vaccine strain were differentiated based on nucleic acid sequences (Schynts et al. 1999; Beer et al. 2007; Blome et al. 2011).

A number of genetic DIVA strategies have also been developed for fish including KHVD, VHS and ISA. As part of the VHS marker vaccine study conducted by

Enzmann et al. (1998) as described above, a genetic DIVA system was developed using a variable region of the G-gene to develop a differentiating qRT-PCR for an attenuated vaccine, thereby allowing differentiation of the vaccine strain virus from wild-type virus (Enzmann et al. 1998).

A PCR was developed to specifically detect an altered nucleotide sequence in the KHV KoVax vaccine, which can used to differentiate the vaccine strain from wild-type virus. More recently, a live attenuated KHV vaccine was developed by Boutier et al. (2015) using recombinant KHV carrying specific deletions, including the deletion of most of ORF56 (Boutier et al. 2015), potentiating its compatibility for a genetic DIVA strategy by using a highly sensitive ORF56-specific PCR-based diagnostic test (Bergmann et al. 2010a; Monaghan et al. 2015). The genetic DIVA approach used for ISA takes advantage of the different genotypes of ISAV. As Chile, Scotland, Norway and the Faroe Islands only experience ISA from genotype I (European genotype), a vaccine based on genotype II (North American genotype) could enable differentiation of viral and vaccine strains in the tissue of these fish using molecular methods, namely, a TaqMan qPCR (Kibenge et al. 2009b). This detects Segment 6 (genotype-specific) in vaccinated fish and Segment 8 (genotype non-specific) in infected fish. However, DIVA approaches based on virus nucleic acid detection may lack sensitivity when trying to detect fish carrying the virus, which may result in false-negative screening by conventional PCR due to low viral copy numbers (Gilad et al. 2004; Bergmann et al. 2010a). This approach could, however, be useful during acute stages of viral infection when antibodies to the marker have not yet been produced, e.g. to VHSV and KHV (OIE 2012).

The Role of Fish Antibodies as Markers of Infection

The success of DIVA/marker vaccines depends on the adaptive immune response to these antigens. Differences in adaptive immune responses of fish compared to those of higher vertebrates need to be considered when exploring the possibility of using DIVA vaccination.

There are five immunoglobulin classes in mammals (IgM, IgD, IgE, IgA and IgG), of which IgG is produced after class switching with higher affinity binding sites. Fish lack isotype switching (Workenhe et al. 2010) and possess predominantly IgM and to a lesser extent IgD and the mucosal immunoglobulin IgT/IgZ (Hansen et al. 2005; Tian et al. 2009; Tadiso et al. 2011). The IgM molecule in mammals is a large pentameric polymer of 5 4-peptide subunits (Roitt 1997), whereas its form differs in fish; this has led to much debate regarding the specificity of fish antibodies. Some reports have subsequently challenged the effectiveness of fish diagnostic serology, a critical component of DIVA systems, due to the apparent high avidity but low affinity of IgM (Denzin and Staak 2000). An antibody response against an antigen normally requires T-cell participation (Bly and Clem 1992; Secombes et al. 1996) and depends on the biochemical properties of the antigen. Polysaccharide antigens tend to induce a B-cell antibody response, which is T-independent (TI antigens),

whereas proteins will usually induce a T-cell-dependent antibody response (TD) (Kaattari and Piganelli 1996). However, in fish T cells are typically sensitive to low temperatures (Bly and Clem 1992; Secombes et al. 1996; Le Morvan et al. 1998), which may be a pertinent point with regard to immunisation regimes for marker vaccination in fish as specific antibody responses to the marker antigen may be suppressed at low temperatures. Therefore, the timing of the vaccination may be crucial for the development of specific antibodies to marker antigens, not only with regard to water temperature but also to the physiology of the fish. Marker antigens administered to anadromous fish during smoltification, e.g. Atlantic salmon vaccinated with an ISA vaccine, may impact on salmon physiological and immunological responses (Specker and Schreck 1982; Maule et al. 1987; Zapata et al. 1992; Eggset et al. 1997) including antibody production (Melingen et al. 1995a, b), compromising serological testing and thus DIVA vaccination strategies for such fish.

For rainbow trout, Atlantic salmon and carp, the antibody responses reported to infection and vaccination against VHS, ISA and KHVD, respectively, have major implications for the application of DIVA vaccination for these notifiable fish diseases.

Non-neutralising antibodies were considered to be highly abundant and long-lived in fish surviving infection to rhabdoviruses, e.g. IHNV and VHSV (Olesen et al. 1991; Enzmann and Konrad 1993; Lorenzo et al. 1995), which could be exploited for DIVA serological diagnostics with suitable ELISA antigens that share epitopes amongst the various genotypes. Specific antibody responses to VHSV have also been reported >6 months post challenge (Lorenzen and La Patra 1999; Fregeneda-Grandes and Olesen 2007), and DNA vaccination, i.e. with the G protein gene, induces a specific rainbow trout antibody response (Lorenzen et al. 1998; Lorenzen and La Patra 2005), which is important for implementing DIVA strategies for this rhabdovirus. Nonetheless, fish were found to respond to only some viral antigens after immunisation with whole virus, and sera from trout surviving exposure to virus often show no reactivity on immunoblots (Lorenzen et al. 1993), emphasising the importance of choosing the suitably immunogenic antigens, possibly in addition to the G protein, for development of an appropriate DIVA test.

Often only low antibody titres have been reported in sera from ISA-infected or vaccinated fish (Kibenge et al. 2002; Mikalsen et al. 2005; Lauscher et al. 2011; Monaghan 2013). Although specific antibodies produced in Atlantic salmon to ISAV do constitute a protective effect, the majority of antibodies are thought to be directed to the nucleoprotein on ELISA (Falk and Dannevig 1995; Lauscher et al. 2011). A recent investigation highlighted the possibility that detection of specific anti-ISAV antibodies may be associated with the dose of antigenic challenge, implying that detection of specific antibodies may be difficult unless the salmon immune system encounters an intense viral load. Lauscher et al. (2011) found that fish vaccinated with a very high dose of inactivated ISAV produced very good antibody responses of titres >1/3200 6 weeks post-vaccination (wpv); however, it was noted that fish vaccinated with lower doses, 80 % less than the high vaccine dose, produced very poor antibody responses or did not respond at all after the same period of time. Reduced relative percent survival has also been observed in fish vaccinated

with low concentrations of inactivated ISAV following experimental challenge (Jones et al. 1999). This would perhaps represent one of the major hurdles for ISA marker vaccination as it is economically challenging to produce such high quantities of inactivated virus for vaccination on a cost-effective commercial scale. Recently, antibody data from sera collected from Atlantic salmon farms in Chile indicated that long-lasting specific anti-ISAV antibodies can be detected using ELISA (Tobar et al. 2015). Furthermore, the recombinant antigens of ISAV HE and NP proteins were used in the ELISA, which could be suitable for DIVA diagnostics. The importance of booster vaccination, i.e. by oral administration, was emphasised for inducing long-term detectable antibodies. Regardless of protection, a specific antibody response must also be detectable prior to infection for a DIVA strategy to be feasible, which may not be possible in the field for Atlantic salmon, at least with regard to ISA. This is particularly pertinent with regard to HPR0 ISAV strains, which may take many years to become virulent (Aamelfot et al. 2014).

Although a heat-inactivated KHV vaccine has been generally unsuccessful (Ilouze et al. 2011; Schmid et al. 2016), a formalin-inactivated KHV vaccine applied in a recent study proved highly antigenic, with strong anti-KHV titres produced (Monaghan 2013), and high levels of protection recorded when used in field trials (Ian Pardoe, Henderson Morley PLc., pers. comm., 2009). Other studies have also demonstrated increased protection when formalin-inactivated KHV was administered orally (Yasumoto et al. 2006; Miyazaki et al. 2008). However, cross-reactivity of antibodies with closely related cyprinivirus, e.g. carp pox (CyHV-1), has proved problematic for effective assay development for KHV (CyHV-3) (Adkison et al. 2005; St-Hilaire et al. 2009; Taylor et al. 2010; Monaghan et al. 2011; Wasa et al. 2014). Nonetheless high anti-KHV antibody titres with increased affinity and duration have been reported (Ronen et al. 2003; Adkison et al. 2005; Perelberg et al. 2008; St-Hilaire et al. 2009; Uchii et al. 2009; Taylor et al. 2010), which is promising for facilitating DIVA strategies for the control of this lethal virus, especially as infected carp become latent/persistent carriers (Eide et al. 2011; Xu et al. 2013). An additional problem is the presence of highly abundant natural antibodies in different strains of carp, leading to false-positive diagnosis in KHV serological tests (Dixon et al. 1994; Kachamakova et al. 2006; Sinyakov and Avtalion 2009). Cyprinids expressing high levels of innate, non-specific (cross reactive) natural antibody are thought to be less capable of expressing specific IgM following immunisation, whereas fish with low natural antibody levels tend to produce higher specific antibody titres (Sinyakov and Avtalion 2009). This presents another challenge for improving the sensitivity of KHV diagnostic serology and thus the reliability of DIVA vaccination.

Concluding Remarks

Dhar et al. (2010) pertinently stated that ‘methods to reduce viral diseases in aquaculture will improve both the quality of life for the animal and make the industry more sustainable’. This could be potentially achieved through DIVA vaccination.

However, marker/DIVA vaccine development against fish viruses is limited and has not yet been extensively assessed (Monaghan 2013). Differences in humoral immunity between higher and lower vertebrates must be taken into consideration, as well as the DIVA approach to particular viral pathogens, as these influence the feasibility of using DIVA strategies in aquaculture. Challenges facing DIVA vaccination against VHS result from the presence of many reservoir species, the multiple genotypes of the virus and poor antibody responses reported in vaccinated rainbow trout. A DIVA approach for ISA would be difficult considering the impacts of smoltification on the Atlantic salmon immune response, the poor antibody responses reported in vaccinated fish and the prevalence of the HPR0 strain. This is not to say that DIVA approaches for aquaculture are impossible, as the carp industry, for example, could benefit from a DIVA approach for controlling KHVD as differential antibody responses are obtained in fish vaccinated with an inactivated vaccine when selected recombinant antigens are used in the ELISA test, and carp produce long-lasting and high titre antibody responses to the virus following KHV vaccination and infection/exposure. Furthermore, serology could soon be included in the OIE manual for diagnostic testing of KHV (OIE 2012). However, the large number of carrier species for KHV and the issue of latency may make containment of outbreaks through ring vaccination difficult.

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