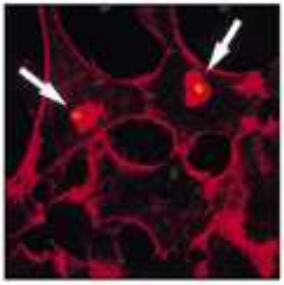
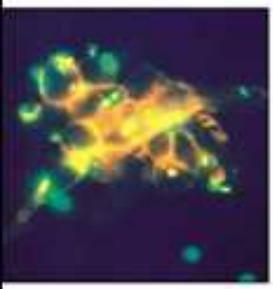
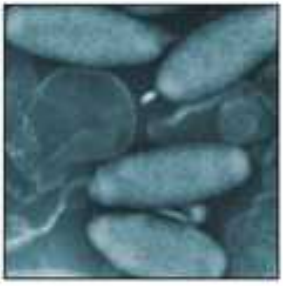
Molecular Aspects of Fish and Marine Biology – Vol. 3



Current Trends in the Study of Bacterial and Viral Fish and Shrimp Diseases





editor

LEUNG Ka Yin

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Current Trends in the Study of Bacterial and Viral Fish and Shrimp Diseases

editor

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Contents

Preface

This monograph summarizes the current trends and developments in the study of bacterial and viral fish and shrimp diseases. There are few books on these subjects and relevant review articles are mostly outdated. This volume will thus serve as a platform for scientists and aquaculturists to understand current limitations as well as new developments so that fish health and disease control can advance to new heights.

The first section (Chapters 1 to 3) provides readers with an overview of the bacterial and viral diseases and the current understanding of innate immunity and interactions with pathogens. Section II (Chapters 4 to 6) includes case studies, where three pathogens are presented, namely two bacteria (*Aeromonas hydrophila* and *Vibrio anguillarum*, the common causes of bacterial diseases in freshwater and marine aquaculture, respectively) and the white spot syndrome virus (a significant viral disease in shrimp). These case studies serve as models for the investigation of various bacterial and viral diseases. Section III (Chapters 7 to 10) presents new platform technologies that are widely used in the study of human pathogens. It aims to spur fish biologists to use modern and cutting edge technologies in their studies so that the study of fish diseases can move into the mainstream of microbiology studies and focus not only on applied research but also on basic research. The final section (Chapters 11 to 14) is on marine biotechnology, discussing biotechnology products (spin-offs from basic research,

including diagnostics, immunotherapy and vaccine development, and the use of probiotics) that are urgently needed for the aquaculture industry.

I wish to express my sincere thanks to all the authors for their contributions and valuable advice during the preparation of this monograph. It is my hope that this volume will bring a new dimension and inspiration to the study of fish health and disease control. Responsible and technology-driven aquaculture is urgently needed to aid food problems and should be a vital solution to improving diet all over the world. The study of pathogen-host interactions with up-to-date technologies will revolutionalize the future of fish and shellfish farming and produce high quality and safe food for our hungry world.

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Chapter 1

Bacterial Diseases of Fish — Where Do We Go from Here?

Recent advances in understanding the virulence mechanisms of fish pathogenic bacteria

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Introduction

Virulence mechanisms of pathogenic bacteria are important to understand because therein lies the nature of the host–pathogen interaction and knowledge of this allows progress to be made in developing control measures and especially vaccines. In this chapter, a critical overview of the available information regarding the virulence mechanisms of some of the major bacterial pathogens of farmed fish is presented.

Bacterial Diseases of Cold Water Fish Species

Renibacterium salmoninarum (*R. sal*)

This Gram-positive bacterium is the etiological agent of bacterial kidney disease (BKD) in salmonid fish. A key aspect of virulence of *R. sal* is its ability to enter, survive and multiply in host macrophages.[1,2] There have been quite a few studies on the interaction between the bacterium and macrophages *in vitro* but one of the problems with these data is the questionable status of the virulence of the bacteria used in the

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studies. While all isolates were able to infect and survive for some days in macrophage cultures, they were not all virulent *in vivo* but this has not frequently been tested. In one study, several strains were used for *in vitro* and *in vivo* experiments. Only one strain produced BKD with mortalities and only this strain of *R. sal* was resistant to killing by chemically generated (SNAP) nitric oxide (NO).[3,4] This correlated quite well with the *in vivo* production of NO (measured as serum nitrate) following infection of rainbow trout.[3] Infection with the avirulent strains resulted in slightly raised nitrate levels from days 4–12. No fish died in this group over a sampling period of 34 days. On the other hand, infection with the virulent strain resulted in nitrate levels increasing on day 8 and then progressively increasing to very high levels until day 21 when all the fish in this group had died from BKD. This suggests that the NO response may have cleared the avirulent strains while having no effect on the virulent strain, though the delay in the NO response to the virulent compared with the avirulent strains is interesting. None of the strains used in this study were killed by peroxynitrite.[4]

In most other studies, the virulence of the strains has not been determined at the time of the experiments. With this in mind, it has been shown that photochemically generated O_2^- reduced *R. sal* viability, even in the presence of superoxide dismutase (SOD), but not in the presence of catalase, suggesting that hydrogen peroxide (H_2O_2) was responsible for the killing of strains with undetermined virulence.[5] In the same study, this strain was not killed by normal macrophage cultures but it was killed by macrophage activating factor (MAF)-activated macrophages, even in the presence of an inducible nitric oxide synthase (iNOS) inhibitor, suggesting that reactive oxygen species (ROS) rather than reactive nitrogen intermediates (RNI) were responsible.

The bacterium activates the alternative complement system which allows adherence to macrophages within one hour of contact and by two hours the opsonized bacteria are phagocytozed.[6] Even non-opsonized *R. sal* (of known virulence) are phagocytozed rapidly and have escaped from phagolysosomes into the cytoplasm by 4.5 hours after infection of macrophage cultures.[2]

Following infection of macrophage cultures with *R. sal*, activation of the respiratory burst and production of O_2^- is rapidly detected (within

30 minutes). The production of O_2^- is enhanced if the bacteria have been previously exposed to normal rainbow trout serum (fresh or heat-inactivated).[7] Exposure to normal and especially immune serum containing antibodies to the p57 protein also enhance intracellular survival and growth of *R. sal* in macrophage cultures.[8] This may explain reports that induction of antibodies to *R. sal* can actually increase susceptibility to BKD.[9,10]

Thus, very soon after infection with *R. sal*, macrophages go into respiratory burst (RB). However, at one, three and six days after infection, macrophages are unable to produce ROS on stimulation with phorbol myristate acetate (PMA) indicating that the RB is either exhausted by the bacterium or that further production of O_2^- is blocked.[11] The same lack of RB induction by PMA occurs 18 hours after exposing macrophages to the major soluble antigen (MSA) also known as the p57 surface protein[12] but the ability of this protein to induce the RB at earlier times, resulting in exhaustion of the response at the time of testing was not investigated. While the same reduction in RB was observed in activated macrophages it was not to the same extent as in normal macrophages, and while three days after infection the RB of infected normal macrophages could no longer be elicited by PMA, the activated infected macrophages were still capable of O_2^- production.[5] Moreover, the activated macrophages were able to kill the bacteria, and while this was only partial at one day following infection, it was virtually complete after seven days. Furthermore, the killing was completely inhibited if catalase was added to the cultures suggesting that H_2O_2 was responsible. Over the same period the bacteria grew in the non-activated macrophages.[5] As mentioned above, the precise degree of virulence of the strain used in these experiments was not determined at the time so it is not known definitely if virulent strains of *R. sal* can be killed by activated macrophages because of their ability to produce higher levels of H_2O_2.

Nevertheless, the available information indicates that *R. sal* is susceptible to being killed by H_2O_2 and while its own catalase may be able to protect against this bactericidal mechanism in normal macrophages, it is not sufficiently protective in activated macrophages. In the normal host, it appears that *R. sal* becomes opsonized by serum

factors including alternative complement, and rapidly adheres to macrophages inducing the RB perhaps before phagocytosis has begun. The role of the MSA might be important here as it is released by the bacterium in large amounts *in vivo*[9] and may induce macrophages to go into RB at a distance so that the RB is completely exhausted before the bacterium can be phagocytozed. The phagocytic capacity of macrophages exposed to MSA was not inhibited.[12] The presence of opsonizing antibodies would be expected to increase the rate at which the macrophage RB is exhausted. Then the bacterium is taken up by the macrophage and the resilient cell wall components protect the bacterium from the hydrolytic enzymes in the phagolysosomes from where it rapidly escapes into the cytoplasm[2] and where it is presumably safe and can continue to multiply. But how does it achieve this escape process? Following infection *in vivo*, iNOS is expressed and NO is produced. While avirulent strains of *R. sal* can be killed by NO, virulent strains are resistant but the mechanism of this resistance is not understood.

Attempts to investigate the molecular events that occur following infection of macrophages *in vitro* have disclosed some interesting features.[13] There is a rapid abrogation of tumor necrosis factor (TNF)-α gene expression and concomitant stimulation of the expression of iNOS and other proinflammatory cytokine genes like interleukin (IL)-1b and cyclooxygenase (COX)-2 after two hours. This is somewhat paradoxical as expression of these cytokine genes is known to be up-regulated by TNF-α in rainbow trout.[14] Moreover, one day after infection, the expression of the TNF-α gene is markedly stimulated and the iNOS gene expression is switched off. Thus, *R. sal* appears to be modulating cytokine gene expression by the infected macrophages in a curious fashion, and more information of this nature should be helpful in understanding how this pathogen subverts the bactericidal mechanisms of the host phagocytes.

Vaccination with standard bacterins has not been very successful. Recently, vaccination with recombinant metalloprotease has induced good protection while recombinant p57 vaccines made the fish more susceptible.[10]

Aeromonas salmonicida subsp. *salmonicida* (*A. sal*)

A. sal causes furunculosis in salmonid fish and prior to 1990 caused devastating losses in Atlantic salmon farming but since then has been very effectively controlled by vaccination.

In salmonids, this pathogen is highly virulent and has a multitude of mechanisms for infecting and surviving in the host. While many of the pathogenic mechanisms are known concerning the acute disease, the bacterium can also cause an asymptomatic carrier state and very little is known concerning the host–pathogen relationship in this form of the infection.[15] The major defence factors of *A. sal* against the host can be summarized as follows. The major surface molecules are the A-protein together with lipopolysaccharide (LPS) which constitute the hydrophobic A-layer. This is involved with adhesion to the host, resistance to complement-mediated killing, resistance to phagocyte-killing mechanisms and iron (heme) uptake.[16] *A. sal* can very rapidly invade the Atlantic salmon (AS) epithelial-like cell line and also primary cultures of rainbow trout enterocytes within five minutes of incubation. Most of the host cells are lysed by a 30-minute incubation.[17] Upon contact with fish cells (RTG-2), *A. sal* produces an ADP-ribosylating toxin (AexT), probably after invasion of the cells, causing cell lysis within two hours.[18] Secretion of this toxin has been shown to be via a type III secretion system.[19]

In vivo, a polysaccharide capsule is produced which confers resistance to serum killing and, by covering the hydrophobic A-layer, renders the bacterium less able to auto-aggregate which may assist spreading within the host.[16] Outer membrane proteins (OMPS) include iron-regulated OMPS that serve as receptors for siderophore-iron complexes and constitute a high affinity iron-uptake system.[20] Besides siderophores, other substances are exported from the bacterial cell including toxic enzymes such as proteases and glycerophospholipid: cholesterol acyl transferase (GCAT) which digest host tissues and erythrocyte membranes liberating amino acids, fatty acids and heme for growth.[20] Under iron-restricted conditions and exposure to low levels of H_2O_2, the bacterium produces a periplasmic manganese superoxide dismutase (MnSOD), in addition to

the constitutive cytoplasmic iron SOD (FeSOD), as well as an inducible cytoplasmic catalase. These enzymes confer resistance to the bactericidal activity of ROS produced by phagocytes during the respiratory burst.[21,22]

Virulence mechanisms of *A. sal* are certainly multifactorial but it is still unclear which are essential ones. It was considered for a long time that both the secreted serine protease and the GCAT toxin were essential virulence factors but using knockout mutants this was shown not to be the case.[23] Certainly the A-layer structure, constituted of the A-protein and LPS, is an essential virulence factor[16] and maybe the AexT is also as a knockout mutant of this gene displayed no toxicity to RTG-2 cells.[18]

Piscirickettsia salmonis (*P. sal*)

P. sal is the etiological agent of salmonid rickettsial septicemia (SRS) in salmonid mariculture. It is a Gram-negative obligate intracellular bacterium and must be cultured in fish tissue culture cell lines.[24] The organism is believed to initially target blood monocytes followed by infection of endothelial cells in all organs.[25] No information is yet available concerning the interaction of this pathogen with the host defence mechanisms but the observation of infected monocytes in blood smears[25] would indicate that *P. sal* can survive within professional phagocytes in a similar manner to *R. sal.* The effect of serum factors including antibodies on the ability of *P. sal* to invade host cells has not yet been investigated.

A few vaccination trials have been conducted using whole cell bacterins but with variable results. Sometimes vaccinated fish were more susceptible to challenge,[26] reminiscent of some *R. sal* vaccine trials. Possibly antibodies to certain epitopes of the pathogen may enhance its ability to invade and survive within host phagocytes. Convalescent coho salmon sera recognizes a number of *P. sal* antigens in Western blots including a 17 kD putative outer surface protein OspA.[27] Vaccines prepared from recombinant OspA alone or fused with T-cell epitopes from tetanus toxin and measles fusion protein have shown good protection, up to 83% Relative Percent Survival (RPS) for the latter.[28] The function of the OspA is not known and the protection induced by these vaccines did not correlate with elevated antibody titers to the

OspA. Obviously much more work is needed to elucidate the mechanisms of virulence and interaction of this pathogen with host defences.

Flavobacterium psychrophilum (*F. psychrophilum*)

F. psychrophilum is a Gram-negative filamentous yellow-pigmented rod and the causative agent of rainbow trout fry syndrome (RTFS), a septicemic condition causing large mortalities in fry. Older fish appear to be resistant. The major surface structures and antigens have been characterized.[29,30] The bacterium is enveloped in a loosely attached slime layer comprised of low molecular mass (16 kDa) lipopolysaccharide (LPS) and several proteins. High molecular mass LPS is also present on whole cells and abundantly in culture supernatants. Slime layers are considered important virulence factors of some pathogenic bacteria, often associated with attachment to substrates, resistance to phagocytosis and maintaining degradative enzymes in close contact with substrates.

F. psychrophilum can survive in normal and immune serum where it depletes complement but curiously it does not grow.[31] The mechanism of the complement resistance is not known but the observation of a polysaccharide capsule by electron microscopy of bacteria *in vitro*[29] and *in vivo* in infected spleen tissue[32] could explain resistance.

The bacterium produces proteases which may account for its invasiveness and pathogenesis. A metalloprotease with 55 kDa, Fpp1, has been characterized.[33] This enzyme cleaved gelatin, laminin, fibronectin, fibrinogen, collagen type IV, actin and myosin — all basic elements of the fish muscular system. *In vitro,* the protease was produced in early exponential phase and was both calcium and temperature dependent being maximally produced at 12°C and in the presence of 10 mM $CaCl_2$. These parameters correspond to those in the natural host during outbreaks of RTFS.

The interaction of *F. psychrophilum* with host macrophages has received some attention recently. *In vivo,* following intraperitoneal injection of the bacterium into 1 g (ten weeks old) and 25 g (20 weeks old) rainbow trout fry, the bacteria were rapidly found in the spleen and later in the kidney only of the 1 g fish. In the latter, the percentage

of macrophages isolated from the spleen which contained intracellular bacteria increased from 12 hours (1.5%) up to three days (10%) and the number of bacteria per phagocytic cell similarly increased from four to ten bacteria, and at six days post-infection the number of bacteria/phagocyte had increased to over 70. Using epifluorescence microscopy, all these bacteria were determined to be viable.[34] Hence it would appear that *F. psychrophilum* is able to resist phagocyte killing mechanisms.

In vitro, *F. psychrophilum* rapidly associates with trout macrophages and this can be inhibited in the presence of *N*-acetylneuraminic acid (sialic acid) as well as modification of bacterial surface carbohydrates with Na-metaperiodate.[35] This indicates an opsonin-independent adhesion mechanism. No toxicity to the macrophages was detected over two hours of incubation but bactericidal effects were not measured.

The bacterium and its culture supernatants are able to rapidly induce the respiratory burst in trout phagocytes.[36] Using isolated kidney cells comprising 80% macrophages and 20% neutrophils [in a chemiluminescence (CL) assay using luminol to detect H_2O_2 production] both bacterial cells and culture supernatant induced a peak response in only three minutes and the response declined to zero in 40 minutes. Bacteria opsonized in normal rainbow trout serum induced two peaks, the first at three minutes and then a prolonged production over 40 minutes with a peak at 25 minutes. Strangely, when Zymosan A was added to the phagocytes two hours after the CL run, a second induction of H_2O_2 production was observed which was similar to that induced in uninfected phagocytes (peak at 12 minutes, duration two to 45 minutes). This is difficult to explain unless the bacteria induce a respiratory burst only in a subpopulation of the phagocytes, possibly only in the neutrophils as this peak was lower and faster than that induced by the Zymosan A. One might speculate that the bacterium can release surface material which is able to induce and exhaust the respiratory burst in neutrophils at remote sites while the cells can avoid inducing the burst in macrophages during phagocytosis. Obviously, more work is required to elucidate the nature of the interaction between this pathogen and host phagocyte responses. The bacterium is catalase-positive[29] which may confer resistance to H_2O_2 produced by phagocytes.

Injection vaccination using a formalin-killed bacterin in oil adjuvant is highly protective but immersion or injection vaccination without the adjuvant was not.[37] A vaccine based on a partially purified outer membrane fraction of the bacterium induced protection in rainbow trout by injection without the use of adjuvant but immersion delivery was not studied.[38] The nature of the protective antigens and protective mechanisms is not known. As the disease is mainly in fry which are difficult to inject, more research is required to produce a useful commercial vaccine.

Bacterial Diseases of Warm Water Fish Species

Gram-positive Pathogens

Diseases of fish caused by Gram-positive cocci have come to prominence over the last 15–20 years, but early isolations date as far back as the 1970s. They are now ubiquitous in aquaculture with outbreaks recorded in the UK,[39] France,[40] Italy,[41] Spain,[42] Israel,[43] South Africa,[44] Korea,[45] Japan,[46] Australia,[44] Taiwan[47] and USA.[48] Diseases have been caused by a number of different Gram-positive cocci, and the history of recording such outbreaks is characterized by confusion over identity of the pathogens, which to a large degree has now been resolved with the advent of sensitive and highly selective molecular techniques. Whilst there remains a great variety of species causing disease, the most frequently isolated, and the most important economically in warm water aquaculture are *Streptococcus iniae* and *Lactococcus garvieae*, and it is therefore these two species which will be discussed in this section.

Streptococcus iniae (*S. iniae*)

Background

Originally isolated from the Amazon freshwater dolphin (*Inia geoffrensis*),[49] *S. iniae* has been associated with outbreaks of disease in many species of farmed and wild freshwater and marine fish, including rainbow trout (*Oncorhynchus mykiss*), tilapia (*Oreochromis* spp.), hybrid

striped bass (*M. saxitilis x M. chrysops*), red drum (*Sciaenops ocellatus*), European sea bass (*Dicentrarchus labrax*), channel catfish (*Ictalurus punctatus*), barramundi (*Lates calcarifer*), Japanese flounder (*Paralichthys olivaceus*) and yellowtail (*Seriola quinqueradiata*). Distribution appears to be worldwide with serious outbreaks reported in Israel,[50,51] the United States,[48,52] Japan[53] and Australia.[54] *S. iniae* has also been reported to cause invasive infections in humans who have handled diseased fish,[55,56] which has provoked a great deal of research into epidemiology and mechanisms of pathogenicity.

Identification

The phenotypic and genotypic variability of *S. iniae* is a key characteristic which has been associated with its ability to cause invasive infection,[56] whether the isolate is from human or fish origin,[57] vaccine success or failure[58] and marine or freshwater origin.[51]

Shortly after the first isolation of *S. iniae* from freshwater dolphin,[49] the same group isolated a second type, also from freshwater dolphin, which differed in its ability to hydrolyze esculin, and was able to ferment lactose but not salicin.[59] Several phenotypic characteristics of *S. iniae* biotypes are similar to Enterococci, for example the wide permissive temperature range (10–45°C), and this has led to confusion in identifications in the past.[60,61] However, the coupling of molecular typing techniques with biochemical and phenotypic studies has clarified the taxonomic position. Generally, Gram-positive cocci, catalase-negative, ß-hemolytic, with inhibition of growth by 6.5% NaCl, pH 9.6 and 40% bile forms the basis of identification along with additional results from biochemical tests given in previous publications,[50,62] but some variability amongst other biochemical tests favors confirmation by molecular methods.[48,62,63] Amplification and sequencing of 16s rRNA from approximate nucleotide positions 50–300 including the variable regions V1 and V2 is useful for confirming identity.[48,63]

Epidemiology

Biochemical differences have been demonstrated between human and fish isolates of *S. iniae* based on pyrrolidonyl arylamidase, arginine

dihydrolase, ß-glucuronidase activity and fermentation of ribose and glycogen. In spite of the biochemical differences, the same isolates were identical by randomly amplified polymorphic DNA (RAPD) analysis and repetitive primer polymerase chain reaction (REP PCR).[57]

However, by refining the methodology, sensitive molecular techniques can be employed to distinguish the origin of particular isolates. In Israeli aquaculture, there is some debate over whether *S. iniae* has infected cage-reared fish from a reservoir in the wild population on the Mediterranean coast,[64] or whether the infection was introduced to wild marine populations by import of infected fish for culture into the northern Red Sea region.[51] The isolates from the Mediterranean could not be distinguished from the Red Sea isolates by comparison of 16s rRNA sequences, or by amplified fragment length polymorphism (AFLP) or restriction fragment length polymorphism (RFLP) methods[51] and the resolution to this poses some interesting questions on environmental impact of aquaculture.[65] Eldar *et al.*[66] demonstrated differences between US and Israeli tilapia and trout isolates by analysis of RFLP of ribosomal RNA (ribotyping), effectively ruling out the possibility of an epidemiological link between freshwater isolates in the US and Israel. It was also demonstrated that the type strain from *I. geoffrensis* belonged to a different ribotype compared with these fish isolates.[66] RFLP was also used effectively to demonstrate genotypic differences between two *S. iniae* serotypes isolated from farmed fish in Israel prior to and following vaccination.[58]

Specific genetic differences, determined by pulsed-field gel electrophoresis (PGFE),[55] were found in isolates that were able to cause invasive infections in both fish and humans compared to commensal isolates,[56] with virulent isolates falling into one PFGE clone, whilst isolates from non-diseased fish were genetically diverse. These differences were reflected phenotypically by the formation of a granular precipitate by commensal isolates when in suspension, whilst virulent isolates remained buoyant.[56] More recently, however, Lau *et al.*[67] reported that isolates from infected patients in China differed from those in Canada morphologically, and were genetically unrelated by PGFE profile. Thus it would appear that the virulence factors necessary for causing invasive infections are present on more than one PGFE clone.

Host–pathogen interactions, virulence and vaccination

Route of entry

Rather unusually, much work has been carried out on routes of infection of various species of fish by *S. iniae*. Perera *et al.* indicated that infection was possible by immersion and oral route[68] in tilapia. More specific studies have also been performed. Infection via the nares was shown to be effective in inducing disease at low dose rates in tilapia and hybrid striped bass, while infection via the eye did not result in pathology.[69] More recently, the same laboratory demonstrated infection of striped bass via the gill, although mortality was lower compared to infection with similar doses via the nares.[70] Tissue distribution was rapid when high doses were used, spreading to the olfactory system, brain, blood and kidneys within 12 hours of inoculation with 5×10^6 cfu, and 48 hours following inoculation with 2.6×10^6 cfu.[70] In barramundi, immersion and oral routes of infection have been demonstrated.[71] The oral route resulted in the chronic form of the disease, displaying symptoms identical to those seen in naturally infected fish. Infection by immersion resulted in more acute symptoms, and infectivity was not affected by salinity or skin trauma.[71] In contrast, in Japanese flounder, only very high doses induced mortality when administered orally, whilst low doses induced high mortality by immersion.[72] Thus, infection may be via gills, nares, or orally, but appears to be somewhat species dependent. Clearly immersion infection will infect via all these routes to a degree and this may account for the generally higher infectivity reported following immersion compared to the specific gill, oral or nares routes. It would appear that, at least in barramundi, cutaneous infection is not a significant cause of mortality as skin trauma prior to immersion challenge did not increase infectivity or morbidity.

Capsule

S. iniae is a capsulated bacterium with at least two serotypes.[58,73] Serotypic variation appears to result from capsular variation as protein antigens are conserved and cross-reactive between the serotypes. Both serotypes are resistant to killing by normal and immune trout serum[73] and both express a non-immune immunoglobulin binding protein when

grown in trout serum.[74] This protein is able to bind trout immunoglobulin by the Fc region,[74] and may be effective in reduction of opsonization by the immunoglobulin and subsequent phagocytosis, particularly bearing in mind the evidence for the presence of Fc receptors on fish macrophages.[75] In most Gram-positive coccal pathogens studied to date, increased opsonophagocytosis and killing by macrophages, mediated via anti-capsular polysaccharide antibodies appears to be the major means of defence,[76–78] thus reverse binding of immunoglobulin may indeed make an important contribution to the resistance of *S. iniae* to host defences.

Hemolysin

The hemolysin of *S. iniae* is a functional homologue of streptolysin S (SLS), demonstrated by complementation of an SLS negative group A streptococcus (GAS) mutant and inhibition by trypan blue. The hemolysin is regulated by nine genes with high homology to the GAS *sag* operon. Whilst hemolysin appears to be responsible for soft tissue damage and necrosis, it does not contribute to the establishment of septicemia or resistance to phagocytic clearance.[79] Indeed the relationship between *S. iniae* and host phagocytes and other immunocompetent cells is altogether more interesting and has been further studied.

Interaction with immunocompetent cells

Recent studies have shown that *S. iniae* is able to colonize salmonid macrophage-like cell lines and purified trout head-kidney phagocytes.[80] Indeed, in septicemic fish, approximately 70% of the bacterial load in the blood was found in the phagocytes.[80] However, merely colonizing and surviving within phagocytic cells appears to form only part of the strategy for host colonization and subsequent propagation. Virulent *S. iniae* is capable of inducing apoptotic death of infected macrophages.[80] Cells undergoing apoptosis lack immunocompetence, thus induction of programmed cell death is considered advantageous to the invading pathogen as apoptotic cells are phagocytozed without initiating an inflammatory response, with the consequent activation of antigen presenting cells and non-specific defences.[80,81] In tilapia, *S. iniae* is able to modulate apoptotic death of non-specific cytotoxic cells,[81] and the

authors speculated that those isolates that did not increase apoptotic death may not be virulent in fish.[81] Thus it appears that the ability of *S. iniae* to survive in the phagocytic cells, coupled with the induction of apoptosis, enables it to establish infection and even progress from bloodstream to infection of the central nervous system.[80]

Vaccination

Encouraging results following vaccination of trout with *S. iniae* inactivated bacterins has been reported.[82] The vaccines were autogenous, inactivated cultures without adjuvant and were delivered by intraperitoneal injection. Both laboratory and field trials indicated protection (RPS approx 90%) for up to four months following immunization, with antibody titers reaching 1:20–30 one month after immunization falling to 1:1 after six months.[82] Passive transfer of immunity suggested that the antibody response plays a key role in protection against this disease.[82] Under routine field use in Israeli trout farms, the vaccines reduced mortality from in excess of 50% to less than 5%, and increased weight gain in farmed trout by 20%.[83] However, whilst the success of these early vaccinations was encouraging, their dependence on an antibody response to capsular polysaccharide resulted in vaccine failures after two years of use, following the emergence of a different serotype.[58] The novel variant was readily identified by PCR, using RAPD analysis, and was negative for arginine dihydrolase using the API strep system.[58]

In tilapia, the efficacy of combined isolate and single isolate-containing vaccines were compared. Vaccines were prepared using inactivated cultures supplemented with concentrated extracellular products (ECPs).[84] The combined vaccine containing two isolates (ARS10 and ARS60) of *S. iniae* performed well, regardless of the challenge isolate used. When the vaccines containing the single isolate (ARS10) were used, protection was better against the heterologous challenge (ARS60) than the homologous challenge. In light of recent publications on the pathogenicity mechanisms of *S. iniae* in fish,[80,81] this unusual performance may reflect the nature of the strains used. If the ARS10 strain was able to induce apoptosis in host macrophages,[80,81] then increased opsonization and phagocytosis of the apoptotic factor

resulting from the specific antibody may actually reduce efficacy of the vaccine by depleting the resident macrophage population to a degree. In contrast, strain ARS60 may not possess the apoptotic factor and antibodies only against capsular polysaccharide may be protective. Indeed, this effect may have been exacerbated by the inclusion of concentrated ECPs (and therefore putative apoptotic factor) in the vaccine. Thus, whilst speculative, the relative abilities of these two isolates to induce apoptosis in tilapine macrophages requires further investigation in order to clarify the reason for the reduced efficacy of the ARS10 vaccine against homologous challenge. The positive efficacy recorded in these studies is encouraging, although further work is required to determine the extent of strain and serotypic variation in *S. iniae* before routine vaccination programs can be safely designed.

Lactococcus garvieae (*L. garvieae*)

Background

The genus *Lactococcus* was established as a separate genus from *Streptococcus* in 1985.[85] Generally, the lactococci were not associated with disease in humans or animals, although more recent data suggest otherwise,[86] and the original isolation of the *L. garvieae* was made from bovine mastitis.[87] However, "streptococcal" infections in marine fish had been reported for a number of years, predominantly in Japan,[46] and the isolates responsible were subsequently ascribed to a new species, *Enterococcus seriolicida*, based predominantly on phenotypic characteristics.[61] In 1993, the close similarity of *E. seriolicida* to *L. garvieae* isolated from disease outbreaks in trout showing similar symptoms in Europe (Spain and Italy) was determined by biochemistry and 16s rRNA sequence homology.[42] Subsequently, two groups working independently demonstrated that *E. seriolicida* was a junior synonym of *L. garvieae* based on DNA-DNA hybridization, with similarities of 77% under optimum or high stringency.[41,86]

Whilst *E. seriolicida* and *L. garvieae* were shown to be the same species by DNA-DNA hybridization, there are distinct differences. The Japanese

isolates were almost exclusively from marine fish, with the only freshwater isolates coming from eels. In contrast, the European isolates are almost exclusively freshwater, generally from trout.[41,61] Additionally, they are serotypically different when analyzed with rainbow trout antiserum.[88] All the European freshwater isolates fall into one serotype, whilst all the Japanese fall into a second serotype and this serotypic variation is a result of differing polysaccharide capsules.[88] Previously, two serotypes had been reported in Japan, KG+ and KG−. However, it has subsequently been demonstrated that the KG+ serotypes are capsule deficient[78,89] and avirulent.[90] Capsule deficient Japanese isolates are serotypically identical to European capsule deficient isolates.[88]

More recently, *L. garvieae* has been identified by 16s rRNA sequencing and PCR as a significant pathogen of farmed freshwater prawn (*Macrobrachium rosenbergii*) in Taiwan.[47] The serological relationship of these isolates to the Japanese and European isolates has yet to be determined. There, geographical location would suggest a close relationship to Japanese isolates. However, the fact that they were isolated from freshwater prawns suggests that they may be more akin to the European freshwater isolates.

L. garvieae can be divided into three distinct groups, based on RAPD analysis:[91] Spanish, Portuguese, English and Turkish isolates formed one group, French and Italian formed a second, whilst Japanese formed a distinct third group.[91] Generally there is high genetic diversity between *L. garvieae* isolates and further work is required to determine where Australian and Taiwanese isolates fit within the group, along with how the genetic variability translates into physical differences.

Host–pathogen interactions, virulence and vaccination

Interaction with the trout immune system

Interaction with the rainbow trout immune system is now quite well understood. All isolates of *L. garvieae* fix rainbow trout complement, regardless of serotype, or presence of capsule, though virulent capsulate isolates fix less complement than avirulent capsule-negative isolates.[88] Addition of homologous antibody increases complement fixation by

capsulate isolates, but has no effect on complement fixation by capsule-negative isolates.[88] In spite of complement fixation, virulent isolates are resistant to both normal and immune serum.[78] This may result from inhibition of the membrane attack complex by *L. garvieae*, similar to that reported for some species of Streptococcus,[92] or it may simply reflect the inability of the bound complement to penetrate the combined thickness of capsule and cell wall.

Resistance to immune serum *in vitro* does not translate to the situation *in vivo*. Passive immunization of rainbow trout with homologous antiserum results in protection against infection by *L. garvieae*.[78] Protection results from increased phagocytosis and killing of *L. garvieae* by rainbow trout macrophages in the presence of immune serum. Opsonization and phagocytosis are not complement dependent as heat-inactivated antisera are as effective as fresh antisera at increasing bactericidal activity of macrophages.[75] This indicates that there may be Fc receptors on rainbow trout macrophages that can interact directly with the Fc region of the bound antibody. This leads to a further interesting faculty of fish pathogenic *L. garvieae*; they are able to bind trout immunoglobulin in the wrong orientation. Both European and Japanese serotypes express proteins that are capable of binding the Fc region of trout immunoglobulin and this may assist in reducing phagocytosis by trout macrophages by effectively preventing interaction with Fc receptors on the macrophage surface.[75]

Interactions with the immune system of yellowtail

The interactions of *L. garvieae* with the humoral immune system of yellowtail may be similar to those described for rainbow trout; passive immunization with sera raised against capsulated isolates of *L. garvieae* confers strong protection in yellowtail, coupled with rapid bacterial clearance.[93] Studies on the interaction between *L. garvieae* and yellowtail macrophages revealed that capsule decreased opsonophagocytosis, with capsulated (KG2) strains being phagocytozed at half the rate of non-capsulated (KG1) isolates of *L. garvieae*.[94] However, as the study was conducted by microscopy, there was no indication of the ability of the phagocytes to kill the bacteria.[94] In an earlier study, the same authors

showed opsonization with normal yellowtail serum had no effect on chemiluminescent response of yellowtail macrophages against capsulate isolates, but increased the chemiluminescent response against non-capsulated isolates. However, the authors were using non-immune serum for opsonization and thus looking only at the opsonizing effect of complement, not antibody.[89] Studies in rainbow trout indicated that it is antibody, not complement, which is important for opsonization and increased killing of capsulated *L. garvieae* by rainbow trout macrophages,[75] and maybe this is also the case in yellowtail.

Interaction with the immune system of giant freshwater prawns

The interaction between *L. garvieae* and the giant freshwater prawn (*M. rosenbergii*) has been studied as a tool to measure the effects of various environmental parameters on the ability of *M. rosenbergii* to clear the pathogen, rather than as direct studies on host–pathogen interactions.[95–98] However, these studies do shed some light on what may be responsible for resistance to *L. garvieae* by *M. rosenbergii*. In essence, factors that decrease the prophenol oxidase system [determined by reduction in phenoloxidase (PO) activity], reduce hemocyte numbers and reduce phagocytic activity decrease the resistance of *M. rosenbergii* to *L. garvieae*. Low levels of copper (0.1 mg/ml) were shown to decrease PO activity over the 96-hour assay period,[95] but this did not decrease the resistance of *M. rosenbergii* to *L. garvieae*. Higher levels of copper had a significant effect on susceptibility with greatly increased mortality recorded at 0.4 mg/ml copper. This level of copper exposure was associated with increase in respiratory burst activity, and decreased PO. Another interesting effect was the response of the hyaline cells. In controls, the hyaline cell count increased by almost 50% over the sampling period, reflected identically in the total hemocyte count (THC). However, no such increase was noted in the *M. rosenbergii* exposed to copper.[95] It may be that the copper reduces the ability to respond to stress through increased THC, rather than having a direct effect on the present numbers of hemocytes. Nitrite had no effect on THC or PO activity, but did reduce phagocytic activity, resulting in increased mortality.[96] Additionally, during A, D1/D2 and D3 stages of the moult cycle, increased susceptibility to *L. garvieae* was attributed

to reduced phagocytic activity.[97] Hypoxia and changes in salinity that reduced phagocytic index also reduced resistance, while high resistance to *L. garvieae* was recorded under conditions which reflected high phagocytic efficiency.[98] Indeed, anything which decreases phagocytic activity of hemocytes appears to increase susceptibility to *L. garvieae*, and suggests that phagocytosis rather than PO is most significant in clearing *L. garvieae*, perhaps indicating a strong parallel between the mode of clearance in crustaceans and teleost fish.

Virulence factors

Whilst the capsule is clearly essential for full virulence, data on other putative virulence factors of *L. garvieae* are relatively scant. Some work was conducted on effects of extracellular products (ECPs) from S*treptococcus* spp. (probably *L. garvieae*) on yellowtail.[99] Crude ECPs pre-injected into yellowtail prior to intramuscular challenge with low virulence isolates greatly increased the mortality and clinical symptoms compared with fish which received the challenge alone.[99] In a later study, ECPs were fractionated by gel filtration chromatography and administered orally or percutaneously to determine toxicity. Whilst none were toxic orally, certain fractions induced exophthalmus and petechiae of the gill opercula when given percutaneously. When one of the fractions was given simultaneously with oral challenge with Streptococcus, persistence of bacteria in the gut was increased.[100] There was however, no further characterization of the biochemical nature of these fractions.

More recently, surface appendages similar to fimbriae have been observed. In other bacteria, these are associated with attachment and invasion of host cells. However, strains in which fimbriae were identified had a thinner capsule, and whilst more virulent than capsule-negative isolates, were less virulent than thickly capsulated isolates.[101] The role of these appendages in pathogenicity of *L. garvieae* is therefore yet to be determined. Siderophore production has been reported in *L. garvieae* under certain growth conditions,[102] though the nature of the siderophores was not determined. Expression of siderophore was dependent upon nutrient level rather than iron content, in that siderophores were detected in supernatants from iron-depleted medium, and heme-enriched medium,

but not from nutrient-rich medium.[102] The same authors reported expression of novel proteins during *in vivo* growth, but the precise protein profiles could not be replicated in culture. The function of the *in vivo*-expressed proteins remains to be determined.

Vaccination

In rainbow trout, passive immunization studies have shown that antibodies raised against capsulated isolates of *L. garvieae* confer high protection against subsequent challenge, whereas antibodies against non-capsulated isolates do not, and high agglutinating titers were raised against both capsulated and non-capsulated isolates. This suggests that capsule is the key protective antigen in trout isolates as surface proteins of capsulated and non-capsulated isolates have been shown to be identical.[78] In contrast, in yellowtail, protection was achieved regardless of the presence or absence of capsule.[93] Indeed, higher agglutinating titers were achieved when non-capsulated isolates were used, and cross-protection against capsulated isolates was noted.[93] It would appear, therefore, that the protective antigens in the Japanese serotype isolates are not capsular in nature and are located in the cell wall of both capsule-negative and capsule-positive isolates,[93] with the low agglutinating titers recorded for the capsule positive isolates indicating a degree of masking of the antigens by the capsule. In spite of this major difference between the Japanese yellowtail and European trout isolates, it appears that binding of antibody leading to improved phagocytosis and killing by macrophages is the main means of protection against *L. garvieae* in both yellowtail and rainbow trout. However, the nature of the antigens of importance in Japanese serotypes are yet to be determined.

Gram-negative Pathogens

Photobacterium damselae subsp. *piscicida* (*Ph. damselae*)

Background

Pasteurellosis, caused by *Ph. damselae*, continues to be one of the most devastating bacterial diseases in world mariculture. The broad host range, ubiquitous distribution, widespread antibiotic resistance and lack of

reliably effective vaccines combine to ensure that *Ph. damselae* remains a major concern for farmers and vaccine companies.

Formerly known as *Pasteurella piscicida* based predominantly on biochemical characteristics and subsequent numerical taxonomy, the phenotypic characteristics of *Ph. damselae* have been reviewed elsewhere.[103,104] Recent advances in the taxonomy of this organism have focused on molecular techniques. Phylogenetic analyses of small subunit rRNA sequences and whole DNA relatedness lead to the organism being reascribed to the family *Vibrionaceae* as *Ph. damsela* comb. nov.[105] then subsequently renamed *Ph. damselae* (addition of terminal "e") following general correction of epithets in 1997.[106] *Ph. damselae* subsp. *piscicida* may be readily distinguished from *Ph. damselae* subsp. *damselae* through absence of flagella.[105] Serologically, *Ph. damselae* subsp. *piscicida* is highly homogeneous regardless of strain origin,[107] and fatty acid methyl ester[106] and lipopolysaccharide[107] composition are identical. However, distinctions can be made at the molecular level by AFLP[109] and restriction fragment length polymorphism (RFLP)[108] depending on geographical origin of the isolates, with European isolates falling into one molecular subgroup, whilst Japanese isolates fall into a second.[109,110] More recent AFLP studies have revealed Israeli Mediterranean isolates may be distinguished within the European subcluster.[111]

Host–pathogen interactions, virulence and vaccination

Interaction with host humoral immunity In yellowtail, specific antibody to *Ph. damselae* has been recorded in cultured fish with peaks early in the season (June/July) and a secondary response through October/November.[112] The authors of this study speculated that the secondary response was protective as disease was lower during this period. However, it is likely that this probably reflected the increased size of the fish in October/November (277–361 g) compared with June/July (20–29 g). Other studies have shown that sea bass and sea bream, for example, are more resistant to experimental infection at this size.[113,114] Furthermore, studies in our own laboratory have shown no protection of survivors from experimentally infected fish during subsequent re-challenge

experiments (unpublished data). Antibody response to *Ph. damselae* has also been characterized in sea bass (*Dicentrarchus labrax*).[115,116] Following immunization of juvenile sea bass by intraperitoneal injection with inactivated bacterin, primary antibody secreting cell response in the head kidney and spleen occurred at approximately 528 degree-days post-vaccination.[115] The secondary response was both faster and greater in magnitude, in terms of numbers of antibody secreting cells, occurring approximately 150 degree-days following secondary stimulation.[115] By immersion, the response differs, with the primary organ for antibody secreting cells being the gill.[116] Extremely high numbers of antibody secreting cells were detected in fish of 0.1, 2 and 5 g, but significantly higher numbers occurred in the 2 and 5 g fish compared to the 0.1 g fish.[116] The response was also significantly faster in the older group, (5 g), with peak antibody secreting cells in the gills occurring at about 150 degree-days in 5 g fish compared to in excess of 300 degree-days in the younger groups.[116]

In terms of the antigens recognized following immunization, differences have been reported between those recognized by sea bass, compared to mice and rabbits.[117] Furthermore, the inactivation method, or use of live bacteria also leads to detection of differing components.[117]

Interaction with host macrophages

There are several studies documenting the interactions of *Ph. damselae* with fish macrophages. In sea bream, peritoneal exudate cells (PECs) isolated from fish of 20–30 g were able to kill *Ph. damselae* during the first 24 hours of exposure *ex vivo*.[114] However, in smaller fish (0.5 g), the PECs were not able to kill *Ph. damselae* and the resulting infection was lethal within five days.[114] Similarly, peritoneal macrophages isolated from 200 g sea bream were rapidly bactericidal against *Ph. damselae*, regardless of the presence or absence of capsule, although capsulated isolates were phagocytozed at a much lower rate.[118] *Ph. damselae* was also killed by sea bass and rainbow trout macrophages *in vitro*[119] and this may be explained by reports that *Ph. damselae* is highly susceptible to killing by reactive oxygen species.[119,120] The observations of susceptibility to phagocytosis and killing by phagocytes indicate that

Ph. damselae must avoid phagocytosis in order to cause disease in fish. Whilst the capsule provides some resistance to phagocytosis,[118] and there is now strong evidence that this bacterium is capable of colonizing and surviving within non-phagocytic cells,[121,122] perhaps the most intriguing means of avoiding phagocytosis is the recent report of a secreted product which triggers apoptosis in sea bass macrophages.[123]

Virulence factors

Protease, phospholipase, lipase and hemolysins have been reported as major toxins of *Ph. damselae*.[103] However, the discovery of a polysaccharide capsule[124] and the confirmation of the facultative intracellular survival of *Ph. damselae* in host non-phagocytic cells[121,122,125] have lead to the most significant recent advances in our understanding of the pathogenicity of this bacterium.

Ph. damselae expresses a periplasmic iron co-factored superoxide dismutase and a cytoplasmic heme catalase.[120] These are constitutively expressed and do not provide protection against exogenous superoxide anion[120] which would be encountered during phagocytosis by fish macrophages. Fish pathogens that are able to withstand phagocytic attack, such as *A. salmonicida,* have an adaptive antioxidant response that can be upregulated upon exposure to reactive oxygen species.[126]

The lack of such an adaptive antioxidant response in *Ph. damselae* is reflected by the fact that it is unable to withstand killing by sea bass or sea bream macrophages.[113,118] Thus *Ph. damselae* has evolved means of avoiding host macrophages. There is now conclusive evidence that *Ph. damselae* is able to invade and colonize non-phagocytic cells.[121,122,125] Internalization within epithelial cells appears to be a specific process as saturation kinetics have been demonstrated, indicating potential competition for cell-surface receptors. Further evidence of the involvement of specific receptors has been reported, including inhibition of invasion by sugars[121] and specific antibody,[122] and the demonstration that heat killing, but not UV killing of *Ph. damselae* may prevent internalization in fish epithelial cells.[17] It has also been shown that the capsule has no effect on invasion of fish cells.[17]

Identification of surface components which may be involved in the internalization process is of major interest. Incubation of EPC and SBL

cell lines with certain sugars inhibited invasion by *Ph. damselae*,[121] suggesting that carbohydrate structures such as glycoproteins on the surface of *Ph. damselae* may be required for interaction with carbohydrate receptors on the host cell surface. In a more recent study, lectins were used to investigate the structures of carbohydrates/glycoproteins on the surface of *Ph. damselae* that may be required for internalization.[127] Lectins which recognized alpha-linked mannose inhibited invasion of SBL cells by up to 50%.[127] However, the most pronounced inhibition of internalization of *Ph. damselae* by SBL cells was noted with *Sophora japonica* agglutinin, which binds beta-configured N-acetyl galactose. In contrast, *Dolichos biflorus* agglutinin, which binds alpha-configured N-acetyl galactose had no effect on internalization. Sea bass antibodies against *Photobacterium* also inhibited invasion.[122] Lectins and antibody, which inhibited internalization were used to stain Western blots of *Ph. damselae* whole cells, outer membrane protein (OMP) fractions or extracellular products (ECP). This study identified two proteins: a 97 kDa OMP and a 52 kDa ECP protein were stained by antiserum, *S. japonica* agglutinin and Con A, but not by *Dolichos biflorus*, suggesting that these proteins may be involved in internalization.[127]

Capsular polysaccharide

An additional means of avoiding the bactericidal effects of the hosts' macrophages may be the antiopsonic exopolysaccharide capsule which has been shown to reduce phagocytosis, but not killing, by sea bream peritoneal macrophages.[118] Indeed, an increase in capsular expression following growth in polysaccharide-rich media resulted in increases in virulence of *Ph. damselae* of up to 4 log.[124] Electron microscopic studies have revealed that expression of capsule is reduced under iron-limited growth conditions[128] and also decreases with age of the culture.[128] The carbohydrate composition of capsular polysaccharide of *Ph. damselae* will to a degree depend upon the growth conditions. However, it is known to contain sialic acid,[129] which may account for its antiopsonic properties. The capsule may have additional roles besides reduction in phagocytosis and it has also been reported to have a minor role in hemin binding

by *Ph. damselae*.[130] Acquisition of iron by *Ph. damselae* is currently not well understood, and although it has been reported that *Ph. damselae* may use hemin or hemoglobin as its sole iron source,[131] failure to detect siderophores by the authors (unpublished) and other workers[132] suggests that other uptake mechanisms must be involved. Indeed, evidence for cell surface protein receptors has been demonstrated as treatment of whole cells with proteinase K-reduced hemin binding.[130] However, even following protease treatment, *Ph. damselae* retained the ability to bind some hemin and this was demonstrated to be by capsular polysaccharide, though with lower affinity than the surface proteins.[130]

Vaccination

Many potential vaccine strategies have been proposed for *Ph. damselae* and these have been previously reviewed.[103,133] However, few of these ideas have been carried forward to commercialization. One exception is DI21 vaccine produced by Hypra, Gerona, Spain. This vaccine is based on ECP-supplemented formalin-inactivated cultures of *Ph. damselae* and has been reported to be effective in experimental studies in combination with *Vibrio alginolyticus* bacterin.[134] Other vaccines have been described in a recent workshop,[135,136] most of these are based on ECP-enriched or capsular polysaccharide-enriched formalin-inactivated cultures.[136] Efficacy of these types of vaccine is mixed, and duration of immunity is short.[136,137] However, short term protection may be achieved by immersion and oral delivery against immersion challenge[136] but not against challenge by intraperitoneal injection.[136] This probably reflects the stimulation of antibody secreting cells in the gill by this route of administration.[115,116] As this appears to be a major route of entry of the pathogen,[138] these antibodies may prevent colonization. Indeed, antibodies and lectins that bind proteins expressed in OMP and ECP are capable of blocking entry into fish epithelial cells. Upregulating these during culture and using the inactivated cultures as vaccines was effective. Vaccines expressing the 97 and 52 kDa proteins at levels about four-fold above those expressed in exponential TSB cultures as determined by densitometry of SDS-PAGE gels were quite effective when given by immersion, giving RPS of approximately 50% compared to unvaccinated controls in sea bass, and up to 70% in yellowtail against

challenge by intraperitoneal injection.[127] It may be that the ECP-enriched vaccines, which will clearly contain excess of the 52 kDa protein, work in an identical manner.

Edwardsiella ictaluri (*E. ictaluri*)

Background

E. ictaluri is the causative agent of enteric septicemia of catfish (ESC), and represents the most serious disease in the catfish industry in the US.[138] Isolates of *E. ictaluri* are considered to by highly homogeneous in terms of biochemistry and serology,[139] although different serotypes have been reported outside the US.[141] However, there are no reports of molecular typing methods having been used to try to confirm this homogeneity. Plasmids have been sequenced, but this was to determine function rather than as an epidemiological tool.[140] As *E. ictaluri* has been found outside the US,[141,142] clearly there is a need for a more rigorous molecular screening to clarify the epidemiological situation.

Host–pathogen interactions, virulence and vaccination

Interaction with the immune system of channel catfish
Antibody response to *E. ictaluri* by channel catfish *Ictalurus punctatus* has been characterized and compared with that of blue catfish *I. furcatus*.[143] A significantly higher antibody response was recorded in channel catfish compared to blue catfish following challenge by intraperitoneal injection. No mortalities were recorded in blue catfish, but significant mortality (>80%) was recorded in channel catfish, in spite of the higher titer.[143] The authors speculated that *E. ictaluri* was not cleared as rapidly from channel catfish as from blue catfish, resulting in the high mortality and also high antibody titers. Rapid clearance from blue catfish would preclude the development of a high circulating antibody titer. Comparison of peripheral blood leukocytes isolated from ESC resistant and susceptible families of channel catfish revealed marginally higher percentages of T-lymphocytes in resistant strains.[144]

Additionally, resistant families displayed more macrophage aggregation in the spleen and posterior kidney throughout the challenge period.[144]

The role of non-specific immunity in resistance to *E. ictaluri* in channel catfish is not clear. Feeding with immunostimulants including killed *S. cerevisiae* and ß-1, 3-glucan increased neutrophil migration and phagocytosis, but had no effect on resistance to *E. ictaluri* infection.[145] It is likely that *E. ictaluri*, whilst phagocytozed, was not killed by the neutrophils, indeed this has been previously reported.[146] Corroboration of this observation can also be found in an evaluation of the responses of channel catfish neutrophils to *E. ictaluri*.[147] In this study, neutrophils were clearly demonstrated to phagocytoze *E. ictaluri*, however no intracellular killing was detected. In the presence of complement and serum, extracellular killing of *E. ictaluri* was observed, but there was no killing by neutrophils in the absence of these serum components.[147] In contrast, in an earlier study, killing of *E. ictaluri* by channel catfish peripheral blood mononuclear phagocytes *in vitro* was recorded. The killing pathway would appear to be oxidative as addition of superoxide dismutase reduced intracellular killing of *E. ictaluri* by 30–40%.[148] Both antibody and complement appeared to have a role in phagocytosis and activation of oxidative defences by phagocytes as determined by induction of chemiluminescence, in response to *E. ictaluri*.[148] The induction of chemiluminescence was increased by complement in the absence of specific antibody. Peritoneal macrophages would also appear to be bactericidal against *E. ictaluri*. Macrophages from both susceptible and immune catfish were bactericidal, but at high ratios of bacteria to macrophages, cells from immune populations were significantly more lethal.[149] The killing activity of macrophages from immune fish was further enhanced by opsonization with immune serum. In contrast, opsonization with immune serum significantly reduced the killing activity of macrophages from susceptible fish.[149] This may reflect the accelerated rate of phagocytosis of the opsonized bacteria. Whilst the highly bactericidal macrophages from the immune population are able to deal adequately with the higher numbers of phagocytozed bacteria, the less bactericidal macrophages from the susceptible fish may become rapidly over-run.

Virulence factors

E. ictaluri expresses long chain O-polysaccharides on its cell surface which is the immunodominant antigen.[150] An isogenic mutant of *E. ictaluri* which was unable to express the O-polysaccharide side-chain was avirulent in catfish[151] and the authors concluded that O-antigen was essential for virulence of *E. ictaluri*. Subsequent characterization of the O-polysaccharide mutant revealed that it was marginally but significantly more susceptible to killing by normal catfish serum,[152] but retained its resistance to killing by neutrophils. The authors also demonstrated that the increased susceptibility was not a result of cationic peptides in the serum as the mutant was no more sensitive to polymyxin B than the wild type.[152]

Significantly, *E. ictaluri* has been shown to be efficiently invasive in a range of mammalian and fish cell lines, and harvested catfish intestinal cells.[153] Mechanisms of invasion were investigated in mammalian cell lines and a role for actin polymerization was proposed based on inhibition of invasion by cytochalisin D to 0.03% compared to untreated controls.[153] Additionally, monodansylcadaverine (MDC), an inhibitor of receptor-mediated endocytosis, significantly reduced invasion (treated 0.04%, compared to 100% in untreated controls).[153] Colchicine, an inhibitor of microtubule formation, had no significant effect on internalization of *E. ictaluri*.[153]

Vaccination

Vaccination of catfish against *E. ictaluri* was reviewed in 1997.[138] This review focused predominantly on killed vaccine and subunits, but highlighted the fact that protection was not correlated with circulating antibody response.[138] The facultative intracellular nature of this pathogen gives credence to the idea that cell-mediated immunity may be more important in pathogen clearance.[138,153] Subsequent to this review, live attenuated vaccine technology has been substantially explored,[154–157] resulting in the use of a licensed product in catfish farms in the US (AQUAVAC-ESC, Intervet). The first attenuated vaccine study in *E. ictaluri* involved use of a *purA* mutant which is auxotrophic for adenine.[154] The mutant, which was 5 $\log_{10}$ less virulent than the wild type strain, retained its invasive capabilities and was able to persist in

catfish tissues for at least 48 hours. Adenine auxotrophs have not routinely been used in mammalian systems as the extremely low levels of adenine in mammalian tissues preclude the survival of the mutant for sufficient duration to elicit an immune response.[158] However, purine metabolism in fish differs from mammals and may afford the longer survival of the adenine auxotroph observed in the trials.[154] Protection offered by this attenuated vaccine was low, but significant.[154]

A modified live *E. ictaluri* vaccine has shown greater efficacy in catfish.[155–157] The RE-33 mutant is reported to be effective regardless of the catfish family.[155] It has also been used to vaccinate juveniles as young as seven days post-hatch. At this early age, mortalities in non-vaccinates reached 30%–36% following challenge by immersion, about 600 degree-days post-vaccination. In vaccinates, mortalities ranged between 8.1% and 12.8%, equivalent to RPS between 58% and 77%.[156] The same vaccine has also been used to vaccinate eyed-eggs of channel catfish.[157] Incredibly, single immersion vaccination *in ovo* resulted in high relative percent survival (87.9%) when challenged by immersion about 750 degree-days post-vaccination. The immune mechanisms which may be operating at this early age are unknown. Interestingly, boosting the fish vaccinated *in ovo* seven days post-hatch resulted in a significant decrease in survival compared to fish that only received the primary vaccination as eyed eggs.[157]

Subunit vaccines against ESC in catfish have also been investigated.[159] Proteins expressed during infection of catfish by *E. ictaluri* were identified and cloned. These were delivered as recombinant subunits expressed in *E. coli*. However, whilst all vaccines gave high protection against challenge, the empty vector control resulted in the highest protection with an RPS of almost 90%. Thus the efficacy of such vaccines remains in question. Recently, surface proteins, identified by biotinylation and subsequent extraction by affinity chromatography using a streptavidin column, have been investigated for their potential role in internalization.[160] Antibodies raised in rabbits against these proteins excised from SDS-PAGE gels were able to significantly inhibit internalization of *E. ictaluri* in fathead minnow cell lines.[160] It may be that these are good candidates for potential subunit vaccines as antibodies able to block internalization may be protective in the early stages of infection.

Attenuated and subunit vaccines have been explored for their ability to elicit cell-mediated immunity against this facultatively intracellular pathogen. However, the immune response can be enhanced by adjuvanting. Indeed, cholera toxin (CT) and its non-toxic B-subunit (CTB) have been used to increase mucosal immunity.[161] *E. ictaluri* whole cells were conjugated to CT or CTB using N-succinimidyl 3-(2-pyridyldithio) propionate. Conjugation to both CT and CTB significantly increased the antibody response when given intraperitoneally, compared to *E. ictaluri* alone, or conjugated to CT or CTB independently. There was no effect of cholera toxin adjuvant on antibody response when given by the oral or rectal route. Whilst the live attenuated vaccines look promising in the laboratory, further research is required into other types of vaccine and potential adjuvants that may be delivered by immersion or oral route to juvenile catfish.

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Chapter 2

Viruses of Fish

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Introduction

Viruses that cause disease in fish represent nearly all of the animal virus families and their numbers are increasing. In 1981, only 16 fish viruses had been isolated in cell culture and an additional 11 had been observed by electron microscopy.[1] By 1988, the number of fish viruses isolated in tissue culture had increased to 34 and an additional 25 had been visualized but not yet isolated.[2] In 1993, another 35 new viruses had been identified[3] and tentatively classified as members of 13 virus families:[3,6–9] the DNA-containing Iridoviridae and Herpesviridae families and the RNA-containing Picornaviridae, Birnaviridae, Reoviridae, Rhabdoviridae, Orthomyxoviridae, Paramyxoviridae, Caliciviridae, Togaviridae, Nodaviridae, Retroviridae and Coronaviridae families. The most recent review of Essbauer and Ahne[5] lists at least 125 viruses of fish and an additional 36 that have been visualized but not isolated. The only animal virus families not represented in this group were the Bunyaviridae, Flaviviridae, Poxviridae and Parvoviridae.

The fish viruses have received considerable attention recently as outbreaks of viral diseases in wild and cultured populations of fish have had serious effects on fish stocks. A herpesvirus outbreak reduced the pilchard (sardine) fishery in southern Australia by 10% in 1995. In late 1998 and early 1999, a second mortality event again affected pilchard stocks. This "kill" was more extensive and an estimated two-thirds of the pilchard biomass was lost as a result of the virus infection, a major impact on the pilchard fishery in Australia. In the United States, a kill of approximately 1000 large-mouth bass occurred in the Santee Cooper

Reservoir of South Carolina in 1995. A new iridovirus, large-mouth bass virus (LMBV), was discovered as the etiologic agent and by 2003, the disease had spread to the bass fishery in 17 states.[10] In 2001, infectious salmon anemia virus (ISAV) was discovered in an aquaculture pen in Cobscook Bay, Maine. This discovery of this orthomyxovirus-like virus, a serious viral pathogen of Atlantic salmon in Europe, in the US forced the farmers to destroy about 2.6 million fish and cost the Maine salmon industry (valued at more than US $100 million) about US $24 million (USDA APHIS estimates). Viral diseases in fish can be economically devastating and fisheries (micro) biologists are working to characterize these viruses in order to develop appropriate vaccines.

The following review comprises an overview of the present knowledge of RNA and DNA viruses of fish. Classification and nomenclature of the viruses described is based on the Seventh Report of the International Committee on the Taxonomy of Viruses.[6]

RNA Viruses of Fish

Single-stranded RNA (Minus) Viruses

Rhabdoviridae

Among the most serious viral pathogens of aquacultured fishes are the RNA viruses: ISAV, infectious pancreatic necrosis virus (IPNV), infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV). These pathogens top the list of most dangerous viral pathogens because they can cause serious economic losses to fish farmers who raise salmon and trout. The salmonid fish aquaculture industry is a major player in the world fish market contributing 51% of the global seafood market (Agriculture and Agri-foods Canada, 2001), and it is no surprise that these viruses are considered among the most serious aquaculture pathogens.

Fish viruses that belong to the Rhabdoviridae are among those that were first isolated as salmon pathogens in the middle of the 20th century, and for this reason are well described. The physical map of

the single-stranded RNA (ssRNA) genome, which is negative sense (minus strand), is ordered from the 3' end as follows: leader-N-P-M-G-NV-L, where N stands for the nucleoprotein gene, P for phosphoprotein gene, M for the matrix protein gene, G for the glycoprotein gene, NV for the non-virion protein gene, and L for the virion RNA polymerase gene. The presence of the NV gene is what characterizes this group of viruses from the other Rhabdoviridae and led to their classification as a new genus, the Novirhabdovirus for Non-Virion rhabdovirus. The members of this genus are all fish viral pathogens and include: IHNV (the type strain for the novirhabdovirus), VHSV, hirame rhabdovirus (HIRRV) and snakehead rhabdovirus (SHRV). SHRV is the only virus isolated from warmwater fish, *Ophiocephalus striatus* or the common striped snakehead, and we expect that other novirhabdoviruses will be isolated as more warmwater fish species are brought under culture (see Table 1).

Reverse genetic analysis of the IHNV, SHRV and VHSV NV gene has, to date, not been able to uncover a function for NV.[11,12] Virus mutants constructed with the NV missing show no change in the virus replication process in tissue culture cells and there appears to be no difference in the infectivity of the virus in fish.[11]

The complete genome sequences for IHNV,[13] VHSV[14] and SHRV have been determined (Johnson, Bell and Leong, GenBank Accession No. AF 147498) and different parts of the genome have been sequenced for a number of other fish rhabdoviruses. A comprehensive analysis of the genetic relatedness of these viruses was reported by Ahne *et al.*[15] In this phylogenetic analysis, only the amino acid sequences of the viral N and G proteins were considered. For both proteins, the phylogenetic relatedness of the viruses was confirmed. IHNV most closely segregated with HIRRV. Interestingly, SHRV and VHSV were more closely related than either was related to IHNV or HIRRV. The other fish rhabdovirus, spring viremia of carp virus (SVCV), does not contain an intervening gene between its glycoprotein and L genes and thus, does not belong in the genus Novirhabdovirus. It clusters with the Vesiculovirus genus that includes vesicular stomatitis virus (VSV).

Table 1 Rhabdoviruses of fish.*

Novirhabdoviruses
Infectious hematopoietic necrosis virus (IHNV)
Viral hemorrhagic septicemia virus (VHSV)
Snakehead rhabdovirus (SHRV)
Hirame rhabdovirus (HIRRV)
Possible members: eel virus B12, eel virus C26
Vesiculoviruses
Spring viremia of carp virus (SVCV)
Possible members: pike fry rhabdovirus (PFRV), eel virus American, ulcerative disease rhabdovirus (UDRV)
Uncharacterized fish rhabdoviruses
Brown trout rhabdovirus
Carpione rhabdovirus
Chinese sucker rhabdovirus
Eel rhabdoviruses (B44, C30, D13)
Eel virus European
Perch rhabdovirus
Pike-perch rhabdovirus
Rhabdovirus anguilla
Rhabdovirus salmonis
Rio Grande perch rhabdovirus

*Adapted from Essbauer and Ahne.[5]

Infectious hematopoietic necrosis virus (*IHNV*)

IHNV is a highly contagious viral disease that affects salmon and trout at temperatures between 8–15°C.[16] The entry of the virus into the host tissues is made through the gill lamellar cells and the cells lining the oral cavity. Studies by Drolet *et al.*[17] have shown that IHNV enters through the epithelial cells where it first replicates and then exits to the blood stream to travel to the lamina propria lining the intestinal wall and the lamina propria in the anterior kidneys. There, viral growth is explosive and results in extensive necrosis of the hematopoietic tissue of the anterior kidney. It is this classic histological sign that gives the virus its name, infectious hematopoietic necrosis virus. Virus infection

in young fry can lead to the death of 100% of the fish in an affected hatchery or pond site. Those fish that do survive release the virus for approximately 45 days, after which time there is no recoverable infectious virus. Nevertheless, there is ample evidence that the virus persists in these fish since the tissues from survivors are positive for IHNV L, G and N genes by polymerase chain reaction (PCR) amplification and electron microscopic immunogold staining of their kidney tissues reveal positive staining truncated viral structures.[4,18,19]

The role of virus carriers in the maintenance of the virus in the environment is complicated by the fact that infectious virus is not easily isolated from survivors of the virus infection.[19] Early observations by Don Amend and colleagues indicated that rainbow trout, that had survived an IHNV infection, remained virus-free until sexual maturation when virus could be detected in the ovarian/seminal fluids.[20] Unfortunately, these findings have not been confirmed by other investigators (Bootland and Leong, unpublished data).[21] In the wild when IHNV survivors (steelhead, sockeye or chinook salmon) return to freshwater upon sexual maturity, virus prevalence in the population is low and rises with time into the spawning run. This has suggested to some that a few infected animals have spread the virus to other members of the spawning run or that the fish are all infected at the same time and the different virus levels are a reflection of infecting virus dose and/or the fishes' immune state. No matter what the reason, the overriding considerations are whether the fish were virus carriers that are able to vertically transmit the disease to their offspring or whether there are other IHNV reservoirs in the environment that could potentially contribute to the infection of these fish. The answers to these questions are important because fisheries biologists have instituted a "culling" program to discard fertilized eggs from parents that have tested positive for IHNV. If carrier fish do exist and can serve as nidi of infection, then culling is justified. However, if IHNV carriers do not exist, then the loss of genetic material and the costs involved in the screening process are not acceptable.

Currently, the majority of evidence suggests that survivors of an IHNV infection do carry the virus for long periods of time and can

potentially transmit the virus, albeit at a very low rate, to their progeny. The practice of treating eggs with a disinfectant during the water hardening process appears to prevent the transmission of the virus to the progeny and indicates that most of the vertical transmission observed for IHNV is due to surface contamination with the virus.

Prevention of IHNV epizootics have relied heavily on appropriate quarantine restrictions, iodophor treatment of eggs, and most recently, experimental vaccines.[16] Recent studies have shown that a DNA vaccine encoding the viral glycoprotein gene is highly effective as a prophylactic treatment to prevent the lethal effects of IHNV infection in rainbow trout (*Oncorhynchus mykiss*), chinook salmon (*Oncorhynchus tshawytscha*) and Atlantic salmon (*Salmo salar*). The salmon aquaculture industry awaits the licensing of these vaccines. IHNV is an Office of International Epizooties (OIE) notifiable disease agent.

Viral hemorrhagic septicemia virus (*VHSV*)

VHSV is the causative agent of a hemorrhagic septicemia of European rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), grayling (*Thymallus arcticus*), whitefish (*Coregonus nelsoni*), northern pike (*Esox lucius*) and turbot (*Scophthalmus maximus*). In this case, the virus affects juvenile fish where outbreaks in hatcheries can lead to 90–100% mortality; adult fish are also killed by this virus. Disease normally occurs at temperatures between 4–14°C in the spring when water temperatures are fluctuating. A typical infection in rainbow trout results in exopthalmia, bleeding in the skin and fin bases, and darkening of the skin of infected fish. Infectious virus is shed in the feces, urine and sexual fluids.

The virus was once thought to be confined to European fish until Ted Meyers and Jim Winton discovered that marine fish, Pacific cod (*Gadus macrocephalus*), were also infected with a VHSV strain that was serologically indistinguishable for that obtained from dying rainbow trout in Europe.[22] VHSV has also been found in dying Pacific herring (*Clupea harengus pallasi*), Pacific hake (*Merluccius productus*) and walleye pollock (*Theragra chalcogramma*) in Alaska. In Europe, the virus has been isolated

from several marine species in the Baltic Sea, Kattegat, Skagerrak and North Sea.[23] More recent studies using molecular tools to sequence the Pacific Ocean isolates have shown that the VHSV from wild marine species in the Pacific Ocean are genetically distinct from the European freshwater isolates. In fact, infection trials with rainbow trout fry have shown that the pacific marine isolates are not pathogenic in rainbow trout.[24] The Pacific isolates are highly pathogenic in Pacific herring[25] and the European marine isolates are pathogenic in turbot fry.[26]

The spread of VHSV in Europe is controlled by regulatory agencies that govern the movement of trout eggs and fry. This program has restricted the spread of the virus from Norway's very large Atlantic salmon aquaculture industry although it is routinely found in the Netherlands and Denmark and in wild fish in coastal waters of Norway. A DNA vaccine for VHSV has been developed.[27] VHSV remains an Office of International Epizooties notifiable disease.

Hirame rhabdovirus (*HIRRV*)

The culture of hirame or Japanese flounder (*Paralichthys olivaceus*) in Japan is an economically important aquaculture activity. In 1998, disease caused the hirame aquaculture industry to lose approximately US $250 million (Hirono, personal communication). Most of the loss was due to HIRRV. The virus can infect ayu (*Pleuroglossus altivelis*) as well as salmonid fish.[28] Infected fish exhibit hemorrhages at the base of the fins, in the musculature and internal organs, and there is extensive necrosis of the hematopoietic tissue in the anterior kidney.

Japanese flounder infected with HIRRV express Mx mRNA in leukocytes and the tissue of internal organs.[29] The Mx expression is an indication of interferon induction and is expected with this rhabdoviral infection. The finding has been confirmed by real time PCR amplification (Hirono, personal communication); yet, microassay analysis that examined 871 different *P. olivaceous* genes did not show Mx or interferon induction with HIRRV infection. Instead, such genes as tumor necrosis factor (TNF), T-cell receptor genes and interferon regulatory factors 1 and 2 were induced.

No vaccines or other forms of treatment have been reported for HIRRV in hirame.

Snakehead rhabdovirus (*SHRV*)

There are two rhabdoviruses that are associated with an epizootic ulcerative syndrome (EUS) in estuarine warmwater fish species. These viruses are: SHRV and ulcerative disease rhabdovirus (UDRV). SFRV was isolated from snakehead fish (*Ophicephalus striatus*)[30] and URDV was isolated from a freshwater eel (*Fluta alba*).[31] Both viruses are serologically distinct from other known rhabdoviruses and from each other.[32] The entire genome for SHRV has been sequenced; it belongs to the genus Novirhabdovirus[33] and a phylogenetic analysis of the SHRV G gene indicates that it is more closely related to the G genes of VHSV and IHNV than to SVCV.[34] Mutant SHRV with a deleted NV gene was found to be no different from wildtype virus in cell culture assays of virus infectivity and virulence. Further analysis of the mutant virus *in vivo* was conducted in zebrafish and no difference in virulence *in vivo* between wildtype SHRV and SHRV with the NV gene missing was detected (Alonso *et al.*, unpublished data).

The role of SHRV and UDRV in the pathology of EUS is still questionable. Frerichs[35] found that less than 5% of fish examined during a EUS epizootic contained virus by infectivity assays. Also, experimental infection of snakehead with URDV did not produce any lesions. We replicated this experiment in wildtype zebrafish and found that five out of 15 fish injected with SHRV did produce a hemorrhagic lesion at the site of the injection two weeks after the injection at 28°C (Leong, unpublished data).

Spring viremia of carp virus (*SVCV*)

SVCV was first identified as the etiologic agent of an acute hemorrhagic disease in common carp (*Cyprinus carpio*) in Europe.[36] Typically, the virus produces hemorrhages in the skin, gills and anterior eye chamber, swollen abdomen, exophthalmia and darkening of the skin. Internally,

the signs are hemorrhages in the body cavity, swim bladder and intestines. The disease can infect koi, a cultured variety of common carp highly prized by aquarium fish hobbyists, crucian carp (*Carassius carassius*), grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*), goldfish (*Carassius auratus*), tench (*Tinca tinca*) and sheatfish (*Silurus glanis*).

The virus was once thought to be confined to Europe, where the virus has a substantial impact on the production of carp with estimated losses of 10–15% of year-old carp, about 4000 tons annually. The virus has been isolated from diseased fish in the Middle East, Asia, and recently, North and South America. The outbreaks in the US have raised concerns that indigenous fish species in the minnow family (Cyprinidae), some of which are endangered species, may be susceptible to SVCV. Experimental infections in roach (*Rutilus rutilus*), pike (*Esox lucius*), guppy (*Lebistes reticulates*), pumpkinseed (*Lepomis gibbosus*), zebra danios (*Brachydanio rerio*) and golden shiners (*Notemigonus crysoleucas*) have produced disease. The broad host range of the virus is a concern to fish disease health specialists.[15]

The virus grows optimally between 16–17°C, a narrow temperature range in experimentally infected common carp. Temperatures from 11–15°C and 17–26°C produce lower mortalities and longer average times for mortalities to occur in infected carp. These results correlate with the observations made in Europe that SVCV outbreaks occur in the spring when water temperatures begin to rise above 15–18°C. The virus is shed with feces and urine by clinically infected fish and by carriers; thus, horizontal transmission is thought to occur primarily through the water.

Detection of the virus has relied on tissue culture assays using EPC cells (cell line derived from a tumor of the common carp, *Epitheliosum papulosum cyprini*), although serological assays such as virus neutralization, enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody (IFA) test are available. Reverse transcriptase PCR (RT-PCR) methods have been used to identify SVCV in the laboratory.[37] SVCV remains a notifiable disease under the current guidelines of the Office of International Epizooties. Although there is

no available vaccine for SVCV, there are indications that a DNA vaccine will induce specific immunity in common carp to SVCV infection (Shelkunov, personal communication).

The sequence analysis of a 550-base pair region of the SVCV glycoprotein gene from different virus isolates identified four genogroups, I to IV. Genogroup I contained the SVCV group with members whose glycoprotein gene sequence diversity varied from 82% to 100% identity. All members of genogroup I were serologically indistinguishable. Genogroup IV contained those viruses formerly assigned to the pike fry rhabdoviruses. These isolates showed a high degree of nucleotide sequence identity to each other (>93.7% identity). Only one strain, the PFRV reference strain 4, whose glycoprotein sequence identity was less than 80% to the other members of genogroup IV, was assigned to genogroup III. A grass carp isolate (V76) which was also previously identified as PFRV, shared less than 70% nucleotide sequence identity with both PFRV F4 and representatives of genogroup IV and was assigned to genogroup II.[37] Although the physical map of the SVCV/PFRV group of viruses resembles that of the genus Vesiculovirus, efforts are now underway to determine whether the aquatic vesiculoviruses should be formally recognized as species within the Vesiculovirus genus, or whether they have sufficient unique properties to place them in a separate genus with the family Rhabdoviridae.[38]

Pike fry rhabdovirus (*PFRV*)

The etiological agent for an acute hemorrhagic disease of pike fry (*Esox lucius*) in Denmark was isolated in 1972 by de Kinkelin's group in France.[39] The virus has since been isolated from a variety of freshwater fish in Europe. A rhabdovirus antigenically related to PFRV was isolated from wild common bream (*Abramis brama*) during a disease outbreak with high mortality in Northern Ireland during May 1998. At the same time, healthy farmed rainbow trout and brown trout (*Salmo trutta*) were isolated on the same stretch of river. Eleven months later, healthy wild bream and roach (*Rutilus rutilus*) on the same river system in

Northern Ireland were found to contain the same virus. This virus can be distinguished serologically from SVCV, but both antigenic and sequence data analysis suggests that PFRV is closely related to SVCV.

Paramyxoviridae

In 1985, Jim Winton described an enveloped, pleomorphic RNA virus with an approximate diameter of 125–250 nm and a single helical genome with a diameter of 18 nm and a length of 1000 nm.[40] The virus had been isolated from chinook salmon (*Oncorhynchus tschawytsha*) in CHSE-214 (chinook salmon embryo cells) and it produced syncytia in these cells, a characteristic of paramyxoviruses. The virus also showed a tendency to form persistent infected cells *in vitro*[41] with the tissue culture lines CHH-1 (chum salmon heart), CHSE-214, KO-6 (kokanee salmon ovary) and CSE-119 (coho salmon embryo). No pathogenic effects of virus infection have been identified in chinook salmon.

The second fish paramyxovirus that caused epidermal necrosis in black sea bream (*Acanthopagrus schlegeli*) larvae was reported by Miyazaki *et al.* in 1989.[42] The affected sea bream larvae had necrotic lesions on the body surface, fins, gills, intestinal and oral mucosa. The virus was never cultured *in vitro*, but electron microscopy showed enveloped particles with the characteristics of paramyxoviruses.

A more recent report of a paramyxovirus from Atlantic salmon post-smolts suffering from gill disease in Norway was made by Kvellestad *et al.*[43] Their study showed that it took nine weeks before the cytopathic effects (syncytia formation) of the virus infection appeared in RTgill-W1 cells. The virus replicated productively in these cells and electron micrographs show budding particles at the cell plasma membrane. The virus had a pleomorphic envelope ranging in size from 150–300 nm and the helical viral nucleocapsid was approximately 17 nm thick. Replication occurred at 6–21°C. The authors have suggested the name Atlantic salmon paramyxovirus (ASPV). No *in vivo* infections were carried out and thus, the role of this virus in gill disease remains unknown.

Orthomyxoviridae

The first identification of infectious salmon anemia (ISA) in the US was made in early 2001 after a diagnostic investigation was conducted on Atlantic salmon smolts dying in netpens in Cobscook Bay, Maine. The virus was identified in moribund fish from a cage with fish that had been dying at the rate of 150 fish per day. Biosecurity efforts called for the isolation of the affected cage and site, daily removal of dead fish for landfill burial, and slaughter of all fish in the affected cage. Increased surveillance of neighboring cages and disinfection of equipment was initiated. As the disease progressed throughout the area, fish farmers were forced to destroy about 2.6 million fish at a cost of US $24 million. The causative agent was ISAV, an orthomyxovirus-like agent that is a serious problem in Norway where the virus has been known since 1985.

Clinical signs of ISAV infection include lethargy, swelling and hemorrhaging in the kidneys and other organs, protruding eyes, pale gills, and darkening of the caudal portion of the gut. Internally, the infected fish has anemia, ascites, congestion, and enlargement of the liver and spleen. Susceptible hosts include Atlantic salmon, sea trout, rainbow trout and Atlantic herring that may pick up the virus from sea lice (*Lepeophtheirus salmonis*) or by ingestion of infected feces.[44] The virus has been isolated and grown in tissue culture where foci of syncytia are formed like virus plaques. Previously, the virus was thought to grow only in SHK-1, a cell line derived from the Atlantic salmon head kidney.[45] However, maintenance of the cell cultures under more acidic conditions enhances the formation of the syncytial foci in CHSE-214 cells as well.[46] The temperature optima for virus replication is 15°C. Detection of the virus is based on tissue culture isolation but PCR and IFA tests are available for confirmation.

ISAV has been tentatively identified as a member of the Orthomyxoviridae based on its segmented ssRNA genome and pleomorphic enveloped virion. Sequences have been reported for all eight RNA segments of the ISAV genome. Segment 1 encodes PB2, a component of the virion RNA polymerase; segment 2 encodes PB1; segment 3, the nucleocapsid protein NP; segment 4, the RNA

polymerase PA; segment 5, acetylcholinesterase P3; segment 6, hemagglutinin; segment 7, proteins P4 and P5; and segment 8, proteins P6 and P7. The proteins P4 and P5 may be the ISAV counterparts to the membrane proteins M1 and M2 of influenza A virus. The proteins P6 and P7 may be related to the non-structural proteins NS1 and NEP of influenza A virus.[47] The hemagglutinin of ISAV does hemagglutinate fish red blood cells in particular; ISAV does not hemagglutinate mammalian red blood cells.

A comparative analysis of the sequence of the PB1 gene with other members of the Orthomyxoviridae indicates that ISAV is more closely related to influenza viruses than to the Thogoto viruses. However, the distance between the ISAV and the other members of the Orthomyxoviridae is so large that it is probable that the investigators will seek classification of ISAV into a fifth genus to be added to the already existing genera: influenzavirus A, B and C and Thogoto-like viruses.

There are other less well-characterized orthomyxovirus-like particles that have been identified in European eels (*Anguilla anguilla*). The eel viruses (A1B, EV1 and EV2) were isolated in an investigation of the causative agent for stomatopapilloma "cauliflower disease" by Nagabayashi and Wolf in 1979.[48] The virus was grown in fathead minnow cells (FHM) at 10–15°C and formed syncytial plaques. Electron micrographs indicated an orthomyxovirus-like appearance. Further studies to determine whether the genome of the virus was similar to the Orthomyxoviridae were not conducted.

Single-stranded RNA (Plus) Viruses

Picornaviridae

Picorna-virus like particles have been observed in fish since 1988, as reported by Moore *et al.*[49] on the isolation of a picornavirus from landlocked rainbow smelt (*Osmerus mordax* Mitchell) in New Brunswick, Canada. The fish showed no signs of clinical disease and was obtained as part of their routine monitoring efforts. The virus grew in CHSE-214 cells and produced plaques of syncytial cells as early as four days post-inoculation. Viral particles that were observed in the cytoplasm

were non-enveloped, spherical and measured 20–30 nm in diameter. Transmission studies in brook trout provided no evidence of disease although the virus was isolated from all ten of the inoculated fish.

Since that first report, picorna-like virus particles have been reported in tumor-like lesions on the fins of adult European smelt (*Osmerus eperlanus*),[50] in moribund Atlantic salmon juveniles in an epizootic in Washington State, USA,[51] and in the ovarian fluids of cutthroat trout (*Oncorhynchus clarki*), brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*).[52]

In 1989, a small picorna-like virus infection of the brain and retina caused mortalities in 15-, 17- and 18-day-old larvae from a hatchery in North Queensland.[53] The pathogen responsible has since been identified as a piscine nodavirus and the disease has been named "viral nervous necrosis" (VNN). The clinical signs of this disease include uncoordinated darting, corkscrew swimming, pale coloration, anorexia and wasting.[54] The disease can be controlled in hatcheries by not recycling culture water, chemical disinfection of influent water and larval tanks between batches, and reduction of larval stocking densities to not more than 15 larvae per liter (preferably less than 10/L) (Anonymous, 1995).

Coronaviridae

The Coronaviridae is composed of two genera, Coronavirus and Torovirus. The family is characterized by pleomorphic, enveloped virions of 120–160 nm diameter with distinct surface peplomers. The nucleocapsid is helical and consists of a single, linear positive sense ssRNA. In fish, coronavirus-like agents have been identified in color carp ana-aki-byo (*Cyprinus carpio*) in Japan.[55] These investigators report the isolation of a coronavirus in EPC cells and the experimental transmission of the virus to experimental inoculated carp. The same pathological signs were observed, i.e. dermal ulceration, necrotic cells in the hematopoietic tissue, spleen and intestines, and mortality.

A viral agent tentatively classified as a coronavirus in fish was reported by Sano *et al.*, at the International Fish Health Conference in 1988. This virus was isolated from laboratory-held common carp dying with

erythematous skin on the abdomen.[56] The virus was isolated in FHM cells from the kidney, liver and spleen of moribund fish. The optimal temperature for growth was 20°C. The virus was identified as a coronavirus based on its enveloped virion of 60–100 nm, buoyant density of 1.21 g/ml, and resistance to IudR. No other examination of the viral genome was conducted. The disease could be transmitted to carp fry by immersion in water containing the cultured virus.

Caliciviridae

There appears to be only one report of calicivirus isolation in fish.[57] The caliciviruses, isolated from opaleye (*Girella nigrigans*), was designated as a San Miguel sea lion virus types 7 (SMSV-7) and 6 (SMSV-6), and found to produce identical vesicular exanthema in experimentally infected swine. Among the viruses of fish, the caliciviruses appear to have the broadest host range, crossing the species barrier to infect sea lions, trematodes, dolphins, chimpanzees, humans and swine.[58]

The caliciviruses have non-enveloped, icosahedral virions measuring 27–40 nm in diameter. The genome is characteristically one segment of ssRNA, positive sense, with a 3' polyadenylated tail. The seventh report of the International Committee on the Taxonomy of Viruses[6] lists four genera in the family: Lagovirus, Norwalk-like viruses, Sapporo-like viruses and Vesivirus. The calicivirus isolated from opaleye is listed with the Vesivirus.

Togaviridae

The fish viruses in the family Togaviridae are all grouped in the Alphavirus genus. There are no members in the Rubivirus genus whose members include the mumps virus. Most of the alphaviruses are arthropod borne and are typically maintained in natural cycles involving transmission by an arthropod vector among susceptible vertebrate hosts. However, the newly identified salmonid viruses, salmon pancreas disease virus (SPDV)[59] and sleeping disease virus (SDV),[60] are not known to have arthropod vectors. These viruses are small, spherical, enveloped viruses with a genome consisting of a single strand of positive sense RNA. The 5' end of the genome is capped and the 3' end contains

a polyadenylated tail. The non-structural protein genes are encoded in the 5' end of the genome and the structural proteins are translated from a subgenomic mRNA co-linear with the 3' end of the genome. The virus replicates in the cytoplasm and virions mature by budding through the plasma membrane where virus-encoded glycoproteins are assimilated. The glycoproteins, E1 and E2, make up the peplomers on the surface of the virion. E2 contains most of the neutralizing epitopes, and E1 has more of the conserved, cross-reactive epitopes.[61]

Pancreas disease of farmed Atlantic salmon was first described in Scotland in 1984[62] and similar disease syndromes have been reported in North America[63] and Norway.[64] This disease is associated with runting and production losses in first-year salmon smolts of up to 50%. The causal agent, SPDV, was first identified in tissue culture by Nelson *et al.*[65] and shown to cause pancreas disease in experimentally infected Atlantic salmon juveniles.[66]

Sleeping disease syndrome describes a disease in freshwater-reared rainbow trout who lie on their sides at the bottom of the tank when infected.[67] The etiological agent was isolated from diseased animals in France and grown in tissue culture cells, CHSE-214 and RTG-2.[60] The virus, SDV, was cloned and the sequence of the viral genome was determined. SDV is an alphavirus of the family Togaviridae. Its relation to SPDV was further characterized by Weston *et al.*[68] who found that the two viruses were 91.1% identical over their complete genomes at the nucleotide level. Both viruses cause the similar histological lesions of the pancreas, heart and muscle of their respective species and it was suspected that the two viruses were related. This view was confirmed when immune cross-protection was demonstrated in rainbow trout.[69] Virus neutralization tests also indicate that the two viruses belong to the same serotype.[68] In this study, the authors have concluded that SPDV and SDV are closely related isolates of the same virus species for which the name salmonid alphavirus has been proposed.

Retroviridae

The family Retroviridae consists of seven genera of avian and mammalian viruses that are grouped by common morphology, genomic structure

and host range. The piscine retroviruses did not fit into any of these classifications and a new genus, Epsilon retroviruses, was established to include the piscine retroviruses: walleye dermal sarcoma virus (WDSV), walleye epidermal hyperplasia virus type-1 (WEHV-1), walleye epidermal hyperplasia virus type-2 (WEHV-2) and snakehead retrovirus (SnRV). All of these viruses have been molecularly cloned and sequenced. There are also numerous reports of C-type particles of about 110–150 nm in Atlantic salmon with tumors of the swim bladder and epidermal papilloma. Electron micrographs of C-type particles have been found in the epidermal papillomatosis of European smelt (*Osmerus eperlanus*) and in the cells cultured from neurofibromas of damselfish (*Pomacentrus partitus*).[70] A retrovirus has also been suggested as the etiological agent for a plasmacytoid leukemia in chinook salmon.[71,72]

Ron Sonstegard was among the first investigators who identified a retrovirus-like agent in lymphosarcoma of northern pike (*Esox lucius*) and muskellunge (*Esox masquinongy*).[73] He, in collaboration with T. Papas, found that lymphosarcoma lesions contained a reverse transcriptase-like DNA polymerase that had a temperature optimum of 20°C.[74] Despite all of this early evidence, it was not until 1992 when Martineau *et al.*[75] reported the first molecular evidence for a piscine retrovirus. They molecularly cloned a type-C retrovirus from walleye dermal sarcoma. These tumors are formed on the surface of adult walleye (*Stizeostedion vitreum*) in the fall and regress in the spring. The genome of the virus (13.2 kb) was larger than all other known retroviruses at the time. The sequence analysis of the WDSV contained three additional open reading frames (Orf), Orf-C at the 5' terminal end, and Orfs -A and -B at the 3' terminal end. Recent studies indicate that Orf-A encodes a D-cyclin homologue (retroviral cyclin) that locates in the nucleus of tumor cells in interchromatic granule clusters.[76,77] Orf-C is expressed in regressing tumors when full length viral RNA is synthesized. Nudson *et al.*[78] has shown that the Orf-C encodes a cytoplasmic protein that targets the mitochondria and is associated with apoptosis. The function of Orf-B, which is distantly related to Orf-A, is unknown. The protease gene for WDSV has also been characterized for its preferred cleavage sites that contain glutamine in the P2 position.[79] The WDSV reverse transcriptase is temperature sensitive and is rapidly

inactivated at temperatures greater than 15°C, a finding that corresponds to the virus adaptation for growth in a coldwater fish species.[80]

Two additional retroviruses have been cloned from epidermal hyperplasias on walleye, and the genome sequences indicate that they are distinctly different from each other (77% identity) and from WDSV (64% identity). The retroviruses, WEHV-1 and WEHV-2, also have Orf-C in the leader region of the virus at the 5' end of the viral genome, and Orfs -A and -B in the 3' end right after the envelope gene. Orfs -A and -B are homologues of cyclin-D. Orf-C is highly conserved with WDSV and its function is still unknown. All of the walleye retroviruses do produce lesions in naïve walleye juvenile fish from a cell-free filtrate of homogenized tumor tissue.

The complete nucleotide sequence and transcriptional analysis of SnRV was described by Hart *et al.* in 1996.[81] The proviral genome is arranged in a typical LTR-gag-pol-env-LTR retrovirus organization. There are three additional ORFs: ORF-1 encoding a 52-amino acid (aa) protein (5.7 kDA); ORF-2 encoding a 94-aa protein (11 kDa); and ORF-3 encoding a 205-aa protein (24 kDa). BLAST searches for possible homologues of these proteins do not produce any meaningful matches and their functions remain unknown. The SnRV genome differs from the retroviruses of walleye because SnRV has no 5' encoded ORF. The pathogenicity of SnRV has also not been determined.

Nodaviridae

Nodaviruses are composed of two separate genera, Alphanodavirus and Betanovirus. Betanoviruses occur only in fish hosts and the type species is striped jack nervous necrosis virus (SJNNV).[6] Nodaviruses possess non-enveloped virions of approximately 30 nm length. The capsid is composed of 180 viral proteins and the genome consists of two molecules of positive sense ssRNA.[6] These strands of RNA, RNA1 and RNA2, have been completely sequenced in SJNNV[82,83] and in greasy grouper nervous necrosis virus (GGNNV).[84] RNA1 encodes the viral replicase and RNA2 encodes the capsid proteins. Additional fish nodaviruses for which RNA2 segments have been sequenced include

Dicentrarchus labrax encephalitis virus (DIEV)[85] and Atlantic halibut virus (AHV).[86]

By comparing full length RNA1 and RNA2 sequences as well as partial sequences obtained from more than 20 other betanovirus RNA2 sequences, the betanoviruses have been classified into four different groups.[87] These groups are the SJNNV, tiger puffer NNV (TPNNV), barfin flounder NNV (BFNNV) and red grouper NNV (RGNNV).

Betanoviruses are the causative agents of neurological disorders in a number of fish species. The first report of disease attributable to a nodavirus was described by Yoshikoshi and Inoue in 1990,[88] affecting young Japanese parrotfish (*Opellegnathus fusciatus*). These authors were the first to use the term "viral nervous necrosis" to describe infection by these viruses. Infection causes nervous necrosis, encephalopathy[89,90] and retinopathy[91] in fishes and leads to abnormal swimming behavior, darkened or abnormally light color, and massive mortalities in hatchery-reared larval and juvenile fishes.[53,92,93] Many species of fish have been reported to be susceptible to betanovirus infection[5] (Table 2). Vertical transmission has been documented in this group by Munday and Nakai.[54]

Presumptive diagnosis of betanoviruses may be done using light microscopy since typical cellular pathology occurs as vacuolation and necrosis of nervous tissue.[94] DIEV has been shown to grow in simian Cos1 cells, human Hela cells and sea bass larva (SBL) cells, although at low levels of infection.[85] Piscine nodaviruses can be propagated in the SSN-1 cell line derived from striped snakehead (*Ophicephalus striatus*).[95,96] The disadvantage to using this cell line is that it is infected with another fish virus, snakehead reovirus.[95] More recently, development of a cell line from grouper (*Epinephelus coiodes*) (GF-1) has been reported to have utility in culturing GNNV.[97]

Double-stranded RNA Viruses

Piscine viruses that have double-stranded RNA (dsRNA) genomes fall into two families, the Reoviridae and the Birnaviridae. The genus members of the Reoviridae include the Orthoreovirus, Orbivirus, Rotavirus, Coltivirus, Aquareovirus, Cypovirus, Fijivirus, Phytoreovirus

Table 2 Fish species affected by betanodaviruses.*

Nervous necrosis viruses
Striped jack (*Pseudocaranx dentex*)
Purplish amberjack (*Seriola dumerii*)
Barfin flounder (*Verasper moseri*)
Halibut (*Hippoglossus hippoglossus*)
Flounder (*Dicentrarchus labrax*)
Japanese flounder (*Paralichthys olivaceus*)
Turbot (*Scophthulumus maximus*)
Barramundi (*Lates calcifer*)
Japanese sea bass (*Lateolabrax japonicus*)
Redspotted grouper (*Epinephelus akaara*)
Kelp grouper (*E. moara*)
Sevenband grouper (*E. septemfasciatus*)
Greasy grouper (*E. tauvina*)
Tiger puffer (*Takifugu rubripes*)
Sea bream (*Sparus aurata*)
Shi drum (*Umbrina cirrosa*)
Japanese parrotfish (*Oplegnathus fasciatus*)
Rock porgy (*Sebasticus marmoratus*)
Uncharacterized fish nodaviruses
Atlantic halibut (*Hippoglossus hippoglossus*)
Malabar grouper (*E. malabaricus*)

*Modified from Munday and Nakai (1997).[54]

and Oryzavirus. Members of the Reoviridae are non-enveloped with icosahedral to spherical virions with one to three distinct capsid shells. The size of the virions range from 60–80 nm in diameter and the genome consists of ten, 11, or 12 segments of dsRNA. The reoviruses that infect aquatic animals are grouped in the genus Aquareoviruses and are characterized by a double capsid shell, 11 dsRNAs and seven structural proteins. The Birnaviridae have single-shelled non-enveloped capsids with genomes of two segments of dsRNA. There are three genera in this family: Aquabirnavirus, Avibirnavirus and Entomobirnavirus. The names of each genus denotes the type of host for each taxon.

Reoviridae

John Plumb isolated the first finfish reovirus, golden shiner virus (GSRV), from golden shiner (*Notemigonus crysoleucas*) in 1979.[98] Since then, several reovirus-like agents have been reported for piscine, molluscan and crustacean hosts. They all have 11 segments of dsRNA and grow at temperatures that reflect their host range. The species have been grouped into six genotypes (aquareoviruses A through F) based on RNA–RNA hybridization profiles (see Table 3). Like other reoviruses, the aquareoviruses can withstand pH 3 and are ether resistant.

Most of the aquareovirus isolates are non-pathogenic or not very virulent in their host species. Grass carp hemorrhage virus (GCV) is the exception and is likely the most pathogenic aquareovirus.[99] The GCV strain was isolated from a grass carp (*Ctenophyngodon idellus*) in the People's Republic of China.[100] This virus causes severe hemorrhagic disease in grass carp, affecting about 85% of infected fingerling and yearling populations.[101]

The complete genome sequence for any species of aquareovirus has not been determined. However, the sequence has been determined for segment 1 which encodes a putative guanylyl/methyl transferase. Segment 2 encodes the RNA-dependent RNA polymerase. Segment 3 encodes a dsRNA binding protein with NTPase and helicase activity. Segment 10 encodes the external capsid protein.

Birnaviridae

There are three genera in the family, Birnaviridae: Aquabirnavirus, Avibirnavirus and Entomobirnavirus. The birnaviruses have been grouped by their host range. The virions of a typical birnavirus are about 60 nm in diameter with a single-shelled, non-enveloped icosahedral capsid. The birnavirus genome consists of two segments of dsRNA. The larger segment A encodes a polyprotein containing the virion capsid protein VP2, an autocatalytic protease NS, and an internal capsid protein VP3 in the physical order VP2-NS-VP3. There is an additional 17 kDa protein encoded in a second reading frame at the

Table 3 Species and tentative species of aquareoviruses.*

Aquareovirus A
American oyster reovirus
Angelfish reovirus
Atlantic salmon reovirus HBR
Atlantic salmon reovirus ASV
Atlantic salmon reovirus TSV
Chinook salmon reovirus DRC
Chum salmon reovirus CSV
Geoduck clam reovirus CLV
Herring reovirus HRV
Masou salmon reovirus MS
Smelt reovirus
Striped bass reovirus
Aquareovirus B
Chinook salmon reovirus B
Chinook salmon reovirus LBS
Chinook salmon reovirus YRG
Chinook salmon reovirus ICR
Coho salmon reovirus CSR
Coho salmon reovirus ELC
Coho salmon reovirus SCS
Aquareovirus C
Golden shiner reovirus
Aquareovirus D
Channel catfish reovirus
Aquareovirus E
Turbot reovirus
Aquareovirus F
Chum salmon reovirus PSR
Coho salmon reovirus SSR
Aquareovirus “G”
Grass carp hemorrhage reovirus

Table 3 (*Continued*)

Tentative species of Aquareovirus
Chub reovirus
Hard clam reovirus
Landlocked salmon reovirus
Tench reovirus

*Modified from van Regenmortel *et al.* (2000).[6]

5' end of RNA segment A, and it has been shown to be a novel anti-apoptosis gene of the Bcl-2 family.[102] The segment B encodes the virus RNA-dependent RNA polymerase. There is no evidence of 5' capping of any of the viral mRNAs.

Infectious pancreatic necrosis virus (*IPNV*)

Infectious pancreatic necrosis (IPN) is a highly contagious viral disease of salmonid fish. The disease most characteristically occurs in fry of rainbow trout, brook trout (*Salvalinus fontinalis*), brown trout, Atlantic salmon and several species of Pacific salmon. In salmonid fish, the virus causes an acute gastroenteritis and destruction of the pancreas. The signs of the disease are typically darkening, a pronounced distended abdomen and a spiral swimming motion. The virus has also been associated with disease in Japanese eels (*Anguilla japonica*) where it causes a nephritis, menhaden (*Brevoortia tryrranus*) where it causes a "spinning disease," and in yellowtail fingerlings (*Seriola quinqueradiata*). A birnavirus has been associated with hematopoietic necrosis causing high mortalities in turbot (*Scophthalmus maximus*) with renal necrosis, and birnaviruses have been isolated from clams exhibiting darkened gills and gill necrosis. A non-typical apoptosis has been observed in cultured cells infected by IPNV.[103]

Transmission of the virus can occur via the feces of piscivorous birds and the virus shed by virus carriers who survive an IPNV infection. Survivors of an IPNV outbreak become IPNV carriers and continue to shed the virus for life. The majority of IPNV isolates are antigenically related and belong to one large serogroup A[104,105] (see Table 4). There

is only one virus in serogroup B. The serological classification is included here because reference is still made to this classification in the literature. More recent studies using sequence comparisons of the deduced VP2 amino acid sequence.[105] This study identified six genogroups.

Table 4 Serological classification of aquabirnaviruses.*

Name of virus	Abbreviation	Origin	Serotype	Genogroups (type)
Serogroup A				
West Buxton	WB	USA	A1	1(4)
Dry Mills	DM	USA	A1	1(4)
VR299	VR299	USA	A1	1(3)
Buhl	Buhl	USA	A1	1(1)
Reno	Reno	USA	A1	1(2)
64-93	64-93	USA	A1	1(1)
90-11	90-11	USA	A1	1(1)
91-114	90-114	USA	A1	1(1)
91-137	91-137	USA	A1	1(1)
Sjarup	SP	Denmark	A2	5
Fr10	Fr10	France	A2	5
Fr21	Fr21	France	A2	5
Oyster virus 2	OV2	UK	A2	5
Snakehead DPL	DPL	Thailand	Not typed	
Abild	Ab	Denmark	A3	3
Eel virus	EEV	Japan	A3	3
Eel virus	E1S	Taiwan	A3	3
Perch virus	PV1	Taiwan	A3	3
Clam virus	CV-HB1	Taiwan	A3	3
Hecht	He	Germany	A4	6
Tellina (bivalve)	TV-2	UK	A5	4
Canada 1	C1	Canada	A6	4
Canada 2	C2	Canada	A7	2
Canada 3	C3	Canada	A8	2
Jasper	Ja	Canada	A9	1(4)
Norway	N1	Norway	A10	5
Serogroup B				
Tellinavirus	TV-1	UK	B1	

*Hill and Way (1995); Blake *et al.* (2001).

Genogroup 1 (equivalent to serotype A1) had four subgroups: genotypes 1, 2, 3 and 4.

With the increased culture of marine species of fish, there have been increasing reports of mortalities in yellowtail (*Seriola quinqueradiata*) and amberjack (*Seriola dumerili*) in Japan from marine aquabirnaviruses.[106] The first isolation of an aquatic birnavirus from fish in Tasmania, Australia was reported by Crane *et al.* in 2000.[107] They found the virus in Atlantic salmon held in netpens in Macquarie Harbor on the west coast of Tasmania. The isolate was closely related to IPNV FR21 and N1, which suggests that the virus was imported to Australia.

Several vaccines have been developed for IPNV and include bacterially produced capsid protein and a DNA vaccine. The protein vaccine has been fairly effective in reducing the lethal effects of IPNV infection in Atlantic salmon in Norway. However, control methods still reply on quarantine and certification of eggs/fry as being disease free.

Novel Fish RNA Viruses

As new fish viruses are described, it is no surprise that novel isolates are being identified. Granzow *et al.*[108] identified a new RNA virus by electron microscopy that shares some similarities with rhabdoviruses, baculoviruses and coronaviruses. This virus was isolated from a white bream (*Blicca bjoerkna* L.) and possesses an unusual morphology. The virion is bacilliform (like some rhabdoviruses) with an envelope containing coronavirus-like spikes. This new virus has a rigid rod-shaped nucleocapsid, a feature found in baculoviruses. Morphogenesis of viral progeny occurs only in the cytoplasm of infected cells. Similar virus isolates have been previously described in the literature in grass carp (*Ctenophayngodon idella*),[109] blue crab (*Callinectes sapidus*),[110,111] European shore crab (*Carcinus maenus*),[112] fathead minnow[113] and shrimp (*Penaeus monodon*).[114]

DNA Viruses of Fish

In terms of emerging viral pathogens of aquacultured fishes, DNA viruses are becoming more recognized as causing serious disease outbreaks (OIE Fish Viral Disease List B) (see Table 5). In particular, fish iridoviruses identified as pathogens in the past ten years have increased dramatically[115,116] and most of the iridoviruses listed in Table 6 are not even listed in the Seventh Report of the International Committee on the Taxonomy of Viruses.[6] In Hetrick and Hedrick's review of new finfish viruses,[3] there were 17 distinct herpesviruses isolated or observed in fish. A web-based review of the literature increases that number and also reveals how many different fish species are affected by herpesvirus infection.

Adenoviridae

Adenovirus particles have been associated with a variety of fish lesions but to date no individual fish adenoviruses have been identified to the genus level. Recently, it has been proposed that white sturgeon adenovirus

Table 5 Fish viral diseases reported to the Office of International Epizooties (OIE).

Diseases listed as "notifiable" (list B)	Etiologic agent
Channel catfish herpesvirus disease	CCHV
Epizootic hematopoietic necrosis	EHNV, ECV, ESV
Infectious hematopoietic necrosis	IHNV
Oncorhynchus masou virus disease	OMV
Spring viremia of carp	SVCV
Viral hemorrhagic septicemia	VHSV
Other fish viral diseases listed as "significant" by the OIE	Etiologic agent
Infectious pancreatic necrosis	IPNV
Infectious salmon anemia	ISAV
Red sea bream iridovirus disease	RSIV
White sturgeon iridovirus disease	WSIV
Nervous necrosis virus disease	SJNNV, GGNNV, BFNNV, DIEV, TPNNV, LcEV

Table 6 Fish iridoviruses.

Lymphocytiviruses
Lymphocystis
Lymphocystis virus-1 (LCDV-1)
Lymphocystis virus-2 (LCDV-2)
Ranaviruses
Epizootic hematopoietic necrosis (EHN) virus group
Epizootic hematopoietic necrosis virus (EHNV)
European sheatfish virus (ESV)
European catfish virus (ECV I–III)
Santee-Cooper ranavirus (SCRV) group
Large-mouth bass virus (LMBV)
Guppy virus (GV-6)
Doctor fish virus (DFV-16)
Red sea bream iridovirus (RSIV) group
Red sea bream iridovirus (RSIV)
Sea bass iridovirus (SBIV)
Grouper iridovirus (GIV)
Ranavirus-like iridoviruses
Turbot iridovirus
Pike-Perch iridovirus
Cod iridovirus
White sturgeon iridovirus (WSIV) group
White sturgeon iridovirus (WSIV)
Uncharacterized fish iridoviruses
Goldfish iridovirus (GFV-1,2)
Carp iridovirus
Eel iridovirus
Cichlid iridovirus
Gourami iridovirus
Angelfish iridovirus
Infectious spleen and kidney necrosis virus (ISKNV)
Erythrocytic necrosis virus (ENV)

be classified as a separate (fifth) genus from other adenoviruses (Atadenovirus, Mastadenovirus, Aviadenovirus and Siadenovirus) based on phylogenetic analysis of a conserved region of the adenoviral DNA polymerase gene.[117] The proposed name for this new genus has not been suggested. Viral particles are non-enveloped, have an icosahedral morphology and are 70–90 nm in diameter. The genome is composed of a single dsDNA molecule and approximately 40 proteins.[6] Although various genomic sequences have been obtained from other adenoviruses, only one DNA polymerase, hexon and protease sequence has been archived from a fish adenovirus[118,119] (white sturgeon).

Atlantic cod were the first fish in which adenovirus infection was described.[119] White sturgeon have also been affected by adenovirus infection and an initial outbreak of disease ravaged fry in the Sacramento River in California in 1984. Infection is generally characterized by epithelial hyperplasia and enlarged cell nuclei have been observed to contain as many as 200 viral particles.[120] Additionally, lympholeukemia has been observed in Japanese red sea bream (*Pagrus major*) and papillomas have been observed in dabs (*Limanda limanda*).[55,120]

To date, attempts to propagate fish adenoviruses in cell culture have been unsuccessful, although a number of cell lines and strategies have been employed.[4]

Herpesviridae

There are nine genera of Herpesviridae that have been assigned to three subfamilies, Alphaherpesvirinae, Betaherpesvirinae, Gammaherpesvirinae and an unassigned genus "Ictalurid-herpes-like virus."[5] To date, only one of the fish herpesviruses, channel catfish virus (CCV), has been assigned to a subfamily, Alphaherpesvirinae (see Table 7).[121] Common morphologic features of herpesvirus include enveloped virions ranging in diameter from 100 to 200 nm. The virion contains a single linear molecule of dsDNA. The icosahedral capsid is 100–110 nm in diameter and is composed of 162 capsomeres.[6]

Herpesviruses have been reported in shark, channel catfish, carp, eels, salmon, trout, northern pike, muskellunge, white sturgeon, turbot,

Table 7 Herpesviruses identified in fishes.

Herpesviridae
Acipenserid herpesvirus (AciHV-1,2)
Anguillid herpesvirus (AngHV-1)
Eel herpesvirus formosa (EHVF)
Cyprinid herpesvirus (CyHV-1,2)
Koi herpesvirus (KHV)
Esocid herpesvirus (EsHV-1)
Percid herpesvirus (PeHV-1)
Pleuronectid herpesvirus (PlHV-1)
Salmonid herpesvirus (SalHV-1,2,3)
Yarname tumor virus (YTV)
Viral epidermal necrosis of flounder (VENF)
Ictalurid herpes-like viruses
Ictalurid herpesvirus (IcHV-1)
Uncharacterized herpesviruses
Carp nephritis and gill necrosis virus (CNGV)

walleye, flounder, angelfish, redstriped rockfish and various smelt.[5] A variety of pathologies are associated with herpesvirus infection in fish, although the most common characteristics are papillomas of skin and fin.[122,123] Other signs may include hyperplasia, hypertrophy, necrosis of epidermal cells and edema in the gills.[124] Microscopic examination of infected fish and fish cell lines reveals pycnosis, syncytia and Cowdry type-A intranuclear inclusions.[5]

Channel catfish virus (*CCV*)

First isolated by Fijan in 1968, CCV was characterized as a herpesvirus based on analysis of viral morphology by Wolf and Darlington in 1971.[125] Channel catfish (*Ictalurus punctatus*) is the natural host of CCV, with young of the year fry and fingerlings being the most susceptible to infection.[126] Blue catfish (*Ictalurus furcatus*) can be

infected experimentally, but to date other closely related species (brown and yellow bullheads and European catfish) are resistant to experimental CCV infection.[127–129] Natural infections are transmitted horizontally via gills or intestine[130] and in experimental conditions, via waterborne exposure, injection and cohabitation.[127,131,132] Vertical transmission has been documented to occur using cloned *Eco* RI fragments of CCV DNA.[133] To date, CCV is the only fish herpesvirus for which the complete genome has been sequenced[134] and comparison of the 134,226 bp genomes reveals little sequence homology with herpesviruses of higher vertebrates. Davison suggests a separate evolutionary origin for fish herpesviruses based on this molecular evidence.[134]

Salmonid herpesviruses (*SalHV*)

Salmonid herpesvirus (SalHV-1) was originally described in the early 1970s at the Winthrop (Washington) National Fish Hatchery, and prior to being identified as a virus was termed Winthrop agent.[2] The agent was later identified as a virus and termed *Herpesvirus salmonis.*[135,136] The genome has been characterized by endonuclease mapping, cosmid cloning, DNA hybridization and targeted DNA sequencing experiments.[137] SalHV-1 shares homology with at least 18 CCV genes, the only fish herpesvirus for which the entire genome sequence is known.[137]

White sturgeon herpesviruses (*WSHV*)

White sturgeon herpesvirus (WSHV-1) is associated with epidermal hyperplasia followed by necrosis of tissue.[138] WSHV-1 can be cultured in a white sturgeon skin cell line (WSSK-1) and as with other herpesviruses, infection *in vitro* is characterized by appearance of syncytia in cultures two to four days post-inoculation.[138] Another strain, WSHV-2, was reported in 1990[139] to be different than WSHV-1 based on serological assays and differences in *in vitro* growth characteristics. WSHV-2 has also been seen to occur in a latent carrier state in white sturgeon.[139]

Uncharacterized herpesviruses

One of the most recently described fish herpesvirus, the carp nephritis and gill necrosis virus (CNGV), is highly contagious and extremely virulent.[140] CNGV has only been seen to affect koi and common carp populations and is not infectious in other fish species such as *Carassius aurata, Bidyanus bidyanus, Hypophthalmichthy molitrix, Ctenopharyngodon idella* and *Oreochromis* spp., even in cases where long-term cohabitation with diseased fish was performed.[141] More recently, it has been suggested that this virus is not a herpesvirus based on sequence comparisons of CNGV thymidine kinase gene sequences with those previously archived in the GenBank database.[142]

Iridoviridae

There are four genera of Iridoviridae, including two that cause infection in fishes (Lymphocystivirus and Ranavirus). At times, Goldfish Virus 1-like viruses have been described as a fifth genus,[143] although this review will refer to these viruses as unclassified iridoviruses. Most iridoviruses are enveloped and possess an icosahedral capsid ranging from 120 to 350 nm in diameter. Virions contain a single linear molecule of dsDNA molecule and up to 36 polypeptides, including proteins with enzymes activities, e.g. protein kinase, nucleotide phosphohydrolase, ribonuclease, deoxyribonuclease and protein phosphatase.[6] Particles may contain a dense core within two non-cellular membrane-derived envelopes and have filaments extending from the vertices (Lymphocystivirus),[144] or may have an electron dense coat enclosing a clear zone and nucleoid core that appears trilamellar by electron microscopy (Ranavirus).[145] The main structural component is the major capsid protein and this is highly conserved across virus families Asfarviridae, Iridoviridae and Phyconaviridae.[6]

Genome analyses has been published for a number of fish iridoviruses, including lymphocystis virus (LCDV),[145] epizootic hematopoietic necrosis virus (EHNV)[146] and infectious spleen and kidney necrosis virus (ISKNV),[147] allowing for additional characterization of the individual viral genes encoded by these iridoviruses.

Piscine iridoviruses are easily propagated in several different tissue culture cell lines, including fish cell lines derived from brown bullhead, bluegill fry, chinook salmon, channel catfish, common carp cell lines, fathead minnow, rainbow trout, pike, white sturgeon, and mammalian cell lines from hamster, cow, pig and monkey.[148–153] Viruses replicate in tissue culture at temperatures ranging from 15°C–28°C and mature virus particles may be observed in the cytoplasm 7–12 hours post-infection.

Lymphocystis virus (*LCDV*)

One of the most easily identified viral infections in fish is caused by LCDV. Infection is characterized by a gross hypertrophy of cells on the skin and fins of fish. Infection is benign in that mortalities are rarely observed in infected fish. Lesions generally heal spontaneously after maturing into a growth that appears wart-like on the skin of the body, fins, tail and gill. At maturity, LCDV lesions are generally 100 μm in diameter but can sometimes exceed 1 mm. LCDV has been identified in over 140 species of fish from freshwater, estuarine and marine fishes.[2] The genome of LCDV-1 is 102.7 kbp and sequence analysis reveals that the genome encodes a DNA-dependent RNA polymerase II subunit, zinc-finger proteins, a helicase and a GTP phosphohydrolase.[6] The genome of LCDV-2 is smaller, 98 kbp, established by restriction endonuclease analysis,[6] and sequence analysis shows the genome encodes a thymidine kinase, ATPase, DNase and RNase.[6] SDS-PAGE analysis of LCDV polypeptides indicates approximately 33 different polypeptides can be separated.

LCDV is currently unculturable and the wart-like surface lesions are considered pathognomic for the infection. Electron microscopy may be performed to confirm presence of LCDV, or PCR can be applied to amplify a region of the major capsid protein (MCP) to diagnose infection.[154] A monoclonal antibody has been generated against a 29 kDa protein present in the cytoplasm of infected cells around the lymphocystis nodules of plaice and dab that can be used for ELISA diagnosis.[155]

Epizootic hematopoietic necrosis virus (*EHNV*)

The epizootic hematopoietic necrosis group of Ranaviruses cause a severe systemic necrosis of hematopoietic tissue in fish as well as frogs, and numerous fish species are affected by this group of highly pathogenic viruses.[5] The first observance of EHNV was reported by Langdon *et al.*[145] in perch. EHNV is notifiable to the Office of International Epizooties and the major viruses affecting fish in this group are EHNV (affecting rainbow trout and redfin perch), European sheatfish virus (ESV) and European catfish virus (ECV I–III). Although these fish viruses are distinguishable from frog virus (FV-3) by examination of viral protein synthesis patterns, RFLP analysis and MCP sequences, all four viruses cross-react with a monoclonal antibody specifically generated against an epitope of FV-3 MCP.[156,157] This observation serves to emphasize that using only serological, molecular, or pathological criteria to define viruses to species level is insufficient.[157]

Santee-Cooper ranaviruses (*SCRV*)

Although the SCRV group is named after the original site in South Carolina, USA, from which this type of virus was first described,[157] molecular evidence now supports that the virus more likely originated in Florida. A comparison of RFLPs and the DNA sequence of a portion of the MCP gene from the iridovirus isolated from the Santee-Cooper Reservoir outbreak and a viral isolate from a large-mouth bass obtained in 1991 revealed identical sequences.[158] There are three large-mouth bass virus (LMBV) isolates that have been identified from distinct sources in North America and they differ genetically as well as phenotypically.[10] It has been proposed that these isolates are, in fact, different strains based on differences in band patterns by amplified fragment length polymorphisms (AFLP) and in virulence, although they tend to grow in cell culture at about the same rate and have sequence homology at two loci of different degrees of evolutionary stability.[10] There are three viruses currently assigned to this group[6] — LBMV, doctor fish virus (DFV-16) and guppy virus (GV-6). The only pathological sign that fishes are infected with SCRV is an enlarged, inflamed swimbladder.[5]

Red sea bream iridoviruses (*RSIV*)

The RSIV group is comprised of iridoviruses that affect red sea bream (RSIV) as well as sea bass (SBIV) and grouper (GIV). This group of viruses was first isolated in 1990 during an outbreak causing mortality in juvenile red sea bream in Japan.[159] Over 25 different fish species from the orders Perciformes, Pleuronectiformes and Tetradontifomes are reported to be RSIV-susceptible.[5] Application of direct DNA amplification of the viral polymerase by PCR provides a reliable diagnostic for detecting RSIV.[160]

White sturgeon iridoviruses (*WSIV*)

The WSIV group was first described in white sturgeon raised in hatcheries in North America.[51] It has been suggested that the virus may be enzootic in wild white sturgeon throughout the Pacific Northwest of North America.[161] The virus especially affects juvenile white sturgeon less than one year of age.[161] WSIV is the only fish iridovirus believed to be transmitted both vertically as well as horizontally from broodstock.[162] Russian sturgeon and Siberian sturgeon have also been reported to be affected by an iridovirus similar to WSIV in Northern Europe.[163]

Unclassified iridoviruses

Unclassified fish iridoviruses include infectious spleen and kidney necrosis virus (ISKNV),[164] erythrocytic necrosis virus (ENV), goldfish iridovirus (GFV-1,2),[165] and a variety of iridoviruses identified as causing disease in carp, eel, cichlids, gouramis, angelfish and flounder.[143] Of historical interest is the fact that ENV was originally ascribed a protozoan parasite etiology in the late 1800s and early 1900s.[166] The first report of fish ENV was by Laird and Bullock in 1969,[167] who published a survey of marine finfish blood films collected in 1958–1961 that were described as having piscine erythrocytic necrosis (PEN). Although limited to light microscopy, Laird and Bullock described virus-like particles in the films and illustrated these particles in camera lucida drawings.

Polyomaviridae

Polyomaviruses are non-enveloped viruses that possess an icosahedral capsid comprising of 72 capsomeres. Virions contain a circular double-stranded DNA molecule and five to ten proteins. Polyomaviruses are not included in the current taxonomy of lower vertebrate viruses[6] although they have been reported to occur in hybrid swordtails (*Xiphophorus* spp.) and winter flounder (*Pseudopleuronectes americanus*).[2,168] The only recent observation of polyoma-like virus particles in another fish species was reported during an epidemiological survey of white sturgeon.[161]

Summary

Viral pathogens are significant contributors to disease outbreaks in wild and farmed fish populations. The importance of viral pathogens is due, in part, to the fact that there are few prophylactic measures that can be used by the fish farmer or wildlife manager to control the disease and because the losses at a hatchery facility can be catastrophic. We must begin to understand how these viruses are maintained and transmitted in the environment and develop a clearer vision with regards to the role the environment has on immune resistance to viruses in fish.

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Chapter 3

Innate Immune Recognition of Pathogens in Teleost Fish

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Introduction

Innate immunity refers to the germline encoded systems that allow molecular and pattern-based recognition of non-self cells. It is an ancient and universal form of immunity in animals. All metazoa have an innate immune system that provides protection from infection by pathogens. In the invertebrates and simple chordates such as the tunicates, there is no adaptive immunity involving antibodies, for which gene rearrangements and clonal expansion greatly increase the range of recognition and effectiveness. But those animals are able to resist infection and this underscores the power of the innate system. Vertebrates have an adaptive immune system capable of generating antibodies, although there is always a reliance on the innate system for the initial recognition and destruction of pathogens and for pathogen presentation and priming of the adaptive immune system.[1]

Teleost fish are a uniquely informative model system for the study of innate immunity. Like other vertebrates, many fish species such as rainbow trout (*Oncorhynchus mykiss*) have a fully functional adaptive (clonal, antibody-based) immune response.[2] But there are limitations to the flexibility and scope of this response in other fish species. For example, cod (*Gadus morhua*) appear incapable of a specific immune response in spite of high resting immunoglobulin levels.[3] Moreover, the adaptive immune responses of some species appear sensitive to temperatures at the low end of their natural temperature ranges and are therefore not always fully functional.[4,5] Fish are expected to rely

greatly on innate immunity for protection from disease, perhaps more so than birds or mammals. Among the teleostei, there are extremely diverse species that cover a remarkable phylogenetic distance. The scope for study and understanding of innate immunity in fish is greater, but the task more daunting, because of the heterogeneity of this group. Furthermore, the life histories of different fish species, which include exposure to a range of abiotic factors such as salinity and temperature and susceptibility to a multitude of different pathogens, must engender considerable diversity in innate immune function and requirement within this group. Fish are also intriguing model organisms because their innate immune system is very similar to the well-studied mammalian one, such that it is easy to map one onto the other in terms of proteins and processes, but they differ in important aspects as well. The differences are most likely to provide new understanding of fish protection from disease as well as novel insight into the mammalian system by virtue of its divergence.

The immediate and fundamental event in immunity is the molecular and pattern-based recognition of non-self cells. Some proteins act exclusively as sensors for non-self cells and either recruit other proteins to destroy them or act as opsonins by tagging the cells for uptake by macrophages or related leukocytes. Other proteins detect and destroy foreign cells directly. Still others involve a number of processes in pathogen destruction. In every case, the distinction between self and non-self is the pivotal event in eliciting host protection from infection. Detection of non-self involves the molecular recognition of distinct structures that can range from the dense mannan arrays of fungi to the unusual glycolipids of bacterial membranes. In many instances, the arrangements (patterns) of surface molecules on pathogenic cells, as much as their nature, serve to distinguish them from the host and these are commonly referred to as pathogen-associated molecular patterns (PAMPs). The proteins that carry out these recognition roles are the sentinels of the innate immune system.

The cellular and humoral aspects of innate immunity in fish have been the focus of several excellent reviews.[6–9] This chapter does not attempt to recapitulate those reports. Some of the newest and most

intriguing developments in our understanding of innate immunity in fish involve immediate recognition of pathogens and their ensuing destruction. This chapter therefore highlights a number of specific recognition proteins and processes following recognition events that have been significant areas of recent discovery in fish innate immunity.

Antimicrobial Proteins and Peptides

Antimicrobial peptides are small, soluble peptides that interact with pathogen surfaces and disrupt their integrity. A wide variety of antimicrobial peptides have been identified to date and they include diverse sequences and structures. Many of these peptides are cationic as a result of the constellation of positively charged residues that mediate their interaction with the predominantly negatively charged membrane surfaces of microbial cells. The peptides are also generally rich in hydrophobic residues, which may facilitate their interaction with the lipid bilayer. Several antimicrobial peptides appear monophyletic, with different forms of a peptide or even entirely different suites of peptides present in related animal species.[10] There are several mechanisms of action proposed for antimicrobial peptides. The majority appear to associate on the membrane surface and to displace the outer bilayer.[11] Others have been shown to span the bilayer and associate to form a channel, which would depolarize the target cell.[11] A few peptides have been proposed to cross the bilayer and interact with microbial DNA in a manner similar to histones in eukaryotic chromatin, thereby disrupting transcription and/or replication.[12,13] Antimicrobial peptides generally interact with bacterial, fungal or protozoan cells and many recognize all three.[14] The antimicrobial peptides are a highly efficient means of innate immunity because they destroy pathogens directly, upon recognition and binding. There is also evidence that certain peptides can promote other immune processes and tissue repair through their actions as signaling molecules in host response.[15,16] Among fish species, a number of peptides and larger proteins with antimicrobial activity have been identified. They are summarized in Table 1 and discussed here.

Table 1 Overview of extracellular antimicrobial peptides in teleost fish.

Protein	Species	Structure	Specificity	Expression	Unusual features	References
Pardaxin	Mosses Sole	Cationic, amphipathic α-helix	Gram-positive bacteria Gram-negative bacteria	Skin mucus gland	Shark repellant	17, 18
Moronecidin/ Pleurocidin	Winter flounder Yellowtail flounder American plaice Atlantic halibut Hybrid striped bass	Cationic, amphipathic α-helix	Gram-positive bacteria Gram-negative bacteria Fungi Yeast	Skin Intestine	Salt-resistant	19, 20, 22
Misgurnin	Loach	Cationic, amphiphilic α-helix	Gram-positive bacteria Gram-negative bacteria Fungi	Unknown		25
Hepacidin	Hybrid striped bass Winter flounder Atlantic salmon	Disulfide-rich	Unknown	Liver Spleen and others	Transported internally to different tissues	22, 23
Hipposin/ Parasin	Amur catfish	Histone H2A N-terminal fragments	Gram-positive bacteria Gram-negative bacteria Fungi	Skin		30
Unnamed 1	Coho salmon	Histone H1 N-terminal fragment	Gram-negative bacteria (with lysozyme)	Serum (origin unknown)		27
Histone-like proteins	Channel catfish Rainbow trout Atlantic salmon	Histones (H1, H2 types)	Gram-positive bacteria Gram-negative bacteria Fungi Protozoa	Skin Liver		31, 33, 34
Unnamed 2	Rainbow trout	Ribosomal protein S30 N-terminal fragment	Gram-positive bacteria	Skin		26

Pardaxin

The first reported antimicrobial peptide in fish was pardaxin, a 33-residue peptide secreted into the skin mucus of Moses sole, *Pardachirus marmoratus*.[17] Pardaxin is 33 residues in length and is largely helical, although it is interrupted by a proline hinge. This peptide was first identified through its pore-forming properties in metazoan cell membranes and then, interestingly, was found to act as a shark repellent.[17] It was later shown to be highly active in the destruction of Gram-positive and negative bacteria.[18] In its native form, this peptide cannot be considered as an efficient recognition system for non-self because it has been shown to be toxic to cells of more complex organisms and had demonstrated hemolytic activity.[18] For this reason, pardaxin has been widely considered to be generalized toxin, rather than a specific antimicrobial agent. Moses sole are not likely to be affected by these activities because they secrete the peptide into the mucus from an external gland and they would therefore not be exposed to dangerous levels. Peptide engineering revealed that the removal of the 11 residues from the C-terminal end of pardaxin resulted in enhanced antibacterial activity with a concomitant loss of activity in a hemolysis assay,[18] suggesting that these are different activities, at the molecular level. This example highlights the subtlety of self/non-self discrimination by these peptides.

Pleurocidins and Moronecidins

Winter flounder (*Pleuronectes americanus*) and other righteye flounders produce a 25-residue cationic peptide called pleurocidin. First isolated and sequenced from flounder skin mucus, pleurocidin was found to have a sequence similar to ceratotoxin from the medfly (*Ceratitis capitata*) and dermaseptin from the frog (*Pyllomedusa sauvagii*).[19] The sequence predicted an amphipathic, alpha-helical structure and antibacterial assays showed a wide spectrum of activity.[19] Genomic and cDNA cloning along with expression analysis revealed that winter flounder have a multigene family encoding isoforms with sequence microheterogeneity and that two of the four pleurocidin genes are

expressed predominantly in skin whereas the other two are preferentially expressed in intestine.[20] Southern blot analysis of genomic DNA revealed multigene families of pleurocidin-related genes in Atlantic halibut (*Hippoglossus hippoglossus*), yellowtail flounder (*Pleuronectes ferrugineus*) and American plaice (*Hippoglossoides platessoides*) whereas gadoid and osmerid cDNA showed no detectable signal.[20] A similar peptide has been reported in hybrid stripped bass (*Morone chrysops* × *M. saxatilis*). This peptide, named moronecidin, is a 22-residue cationic alpha-helix and it is found in the skin and gills.[21] The peptide sequence and overall gene organization appeared comparable to pleurocidin and alignment of their peptide sequences revealed substantial similarity, suggesting that these are homologues. Although bacterially challenged fish were used for moronecidin isolation and cloning, expression studies did not reveal strong inducibility and moronecidin mRNA was detected in a number of tissues from healthy (non-challenged) controls.[21] Pleurocidin peptide and cDNAs were all obtained from healthy winter flounder, suggesting that exposure to bacteria is not required for their synthesis. Therefore, these peptides can be considered to be a first line of host defence in fish. Pleurocidin might provide pathogen recognition with direct destruction and, in many cases, is likely to save the fish the cost of a more involved acute phase response.

Hepcidin

Hybrid striped bass, winter flounder and Atlantic salmon (*Salmo salar*) have all been shown to express hepcidin, a disulfide-rich peptide.[22,23] Hepcidins in these species were 19 to 27 residues in length and cationic. In all cases, the hepcidins were expressed in liver but expression in other tissues was also detected. A single hepcidin peptide was identified in bass, two in salmon and three isoforms appear to be present in flounder. Southern analysis suggests a multigene family tightly clustered on the flounder genome.[23] Although the presence of hepcidin transcripts in a cDNA library from healthy salmon implies constitutive expression, the gene appears to be highly expressed in response to infection. In bass, up-regulation was suggested to be approximately 4500 fold.[22]

In salmon, hepcidin was among the clones identified in libraries of up-regulated genes from spleen and liver in response to infection.[24] Up-regulation in response to infection was further confirmed for both salmon genes in several tissues.[24]

Misgurnin

The sequence of a 21-residue peptide isolated from loach (*Misgurnus anguillicaudatus*) predicted an amphiphilic alpha-helix but CD spectra suggested a random coil in aqueous solution.[25] Like several other antimicrobials, misgurnin might adopt a helical confirmation once docked on the cell surface. This peptide was found to be more effective on a concentration basis than frog magainin-2 against fungi as well as Gram-positive and negative bacteria.[25] Since whole fish were used in the isolation and no cDNA sequence has been obtained, the tissue distribution and location of synthesis are not known. Similarly, the regulation of expression of this peptide remains unknown.

Peptides Derived from Ribosomal Proteins

A 6.7 kDa antimicrobial peptide from skin of rainbow trout was found to be highly active against Gram-positive bacteria.[26] Partial N-terminal sequencing revealed similarity to the N-terminal sequence of ribosomal protein S30 from various species. It therefore seems possible that other species might employ S30-derived peptides as antimicrobials.[26] The production of antimicrobial peptides by processing of larger proteins is a common occurrence and suggests a highly effective means of generating two functions from a single protein.

Histones and Derivatives

A number of antimicrobial agents are derived from histones in various vertebrate species. Among fish, an unnamed peptide from coho salmon (*Oncorhynchus kisutch*) was identified as deriving from the N-terminus

of histone H1[27] and two related peptides, parasin I of Amur catfish (*Parasilurus asotus*) and hipposin of Atlantic halibut, have been identified as N-terminal fragments of H2A.[12,27,28] The 26-residue H1-derived peptide was isolated from the sera of bacterially challenged coho salmon. Synthetic forms of the peptide were not active against the bacterial species tested, but the peptide was found to potentiate the effect of pleurocidin and lysozyme as well as crude extracts from coho salmon that contain lysozyme.[27] Although a direct effect cannot be ruled out, it would appear that the histone H1 peptide plays a synergistic role with other soluble agents *in vivo*.[27] This will also be discussed in the context of the lysozymes in a later section of this chapter. Hipposin was purified from the mucus of healthy halibut and found to be 51 residues in length and highly cationic. It was active against a variety of Gram-positive and negative bacteria.[28] The 19-residue parasin I was discovered in the mucus of injured catfish and was very similar to buforin I, a 39-residue H2A-derived peptide in toads.[29] Parasin I was found to be directly active against Gram-positive and negative bacteria as well as fungi, and showed minimal inhibitory concentrations far lower than the well-studied frog-derived peptide, magainin-2, against many different bacteria.[12] The mechanism by which parasin I is produced from a larger protein has been found to be cleavage by the protease cathepsin D. This protease, in turn, is activated by matrix metalloprotease-2, which is known to be elevated during immune response or tissue repair.[30] It seems likely that hipposin would be produced in the same way. It will be interesting to determine whether a similar proteolytic system generates the trout peptide from ribosomal proteins. If that were the case, multiple antimicrobials could be produced from a number of different proteins in a single cleavage process.

Antimicrobial activity has also been ascribed to whole histones or histone-related proteins. Three antimicrobial proteins were isolated from the skin of healthy channel catfish (*Ictalurus punctatus*) and found to be active against bacterial and fungal fish pathogens.[31] The most abundant protein was very similar to histone H2B, having a molecular mass of 13,549 Da and a 20-residue N-terminal partial sequence that was identical to trout histone H2B at all but two positions.[31]

Histone H2B-like proteins from the skin of hybrid striped bass and from catfish were also shown to have antiprotozoan activity against *Amyloodinium ocellatum*, a parasitic dinoflagellate that causes mortalities in tropical aquaculture.[32] Furthermore, this protein appeared to be modulated by stress in channel catfish, with levels diminishing in stressed fish.[33] This response to stress could allow pathogens normally controlled by this protein to proliferate, which may contribute to the well-documented relationship between stress and disease in this fish species and others.[33] In healthy Atlantic salmon, no antimicrobial activity was detected in acid extracts of skin. Liver, stomach and intestine revealed strong activity, however, and purification of an active agent from liver revealed histone H1. The molecular mass was 20,734 Da and a peptide mass fingerprint showed four individual hits against rainbow trout H1. Furthermore, a partial internal sequence precisely matched rainbow trout histone H1 residues at the same positions.[34] This histone was only tested against *E. coli*. Its activity against bacterial and fungal pathogens of fish remains to be investigated. Overall, the *in vivo* relevance of whole histones to innate immunity in fish is unclear. Studies in mammals have shown, however, that whole histones have significant antimicrobial roles aside from their traditional DNA binding and gene regulation activities.[35,36]

Lysozymes

Lysozymes are prevalent among plants and animals as key effectors in innate immunity.[37] These enzymes are also referred to as muramidases. Lysozymes directly recognize the cell walls of Gram-positive bacteria by cleaving the ß(1–4) linkage between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan. There are two major forms of lysozyme in vertebrates and, reflecting the predominance of birds as a source of the enzyme, they are named chicken (c) and goose (g) types. The two types have divergent amino acid sequences in birds and they are expressed in different tissues.[38]

Lysozyme activity has been widely reported and studied in fish. Activity has been found in various tissues that are rich in leukocytes

such as head kidney and at sites where there is a significant risk of bacterial infection including skin, gills, eggs and gastrointestinal tract.[39] Gene or protein sequences have also been obtained for several fish lysozymes. C-type lysozyme cDNAs have been obtained from leukocytes of carp (*Cyprinus carpio*),[40] kidney of rainbow trout,[41] and a gene encoding Japanese flounder (*Paralichthys olivaceus*) c-type lysozyme has been characterized.[42] A c-type lysozyme has also been reported in macrophages of zebrafish (*Danio rerio*).[43] The only g-type lysozyme sequences reported outside the class Aves have been in fish. Healthy Japanese flounder express a g-type lysozyme gene in several different tissues, including head kidney, spleen, kidney, skin, muscle, heart and brain, and this protein showed activity against various fish pathogens.[44] This is consistent with the multitude of reports on lysozyme activity in many tissues and fluids of fish, and it is likely that the source of much of the reported activity is g-type lysozyme. Although the zebrafish lysozyme appeared constitutive, lysozyme gene expression is frequently elevated in response to infection. G-type lysozyme gene expression of Japanese flounder was shown to be up-regulated in heart, intestine and whole blood following treatment with *Edwardsiella tarda*.[44] C-type lysozyme was also among the up-regulated genes identified using subtractive hybridization for genes expressed in liver of rainbow trout exposed to *Vibrio anguillarum*[45] and spleen of Atlantic salmon exposed to *Aeromonas salmonicida*.[24]

Trout c-type lysozyme has been found to be active against a number of fish pathogens, including several Gram-negative species, in contrast to hen egg white (c-type) lysozyme.[46] Later work, however, showed the activities of flounder c and g types to be comparable to hen lysozyme overall, with specific differences in activities against certain species.[44] The most relevant difference was a somewhat greater activity of fish c-type lysozyme than either the fish g-type or the hen lysozyme against fish pathogens *Pasteurella piscida* and *V. anguillarum*. Different assay formats may have contributed to these contrasting results. Lysozyme should have an effect on Gram-negative bacteria if the outer membrane is breached such that the enzyme can reach and destroy the cell wall. A recent study suggests that lysozyme and other agents may be able

to act in concert, perhaps achieving that outcome. Activities of Coho salmon skin extracts containing lysozyme and hen egg white lysozyme against *V. anguillarum* were potentiated by histone-derived peptides which had no activity on their own.[27] The interesting aspect of this finding with respect to lysozyme is that it suggests that the potentiation of lysozyme activity by the histone peptide could result from a transient or permanent peptide-induced break in the outer membrane of this Gram-negative species allowing lysozyme to reach the cell wall and destroy the bacteria. The histone peptides are able to permeabilize the *V. anguillarum* membranes,[27] which supports this mode of lysozyme potentiation.

Complement C3

The complement system comprises a large number of serum proteins that react with one another to opsonize pathogens and induce inflammation and pathogen cell destruction. Two of the three pathways of complement activation involve direct recognition of pathogens. These are the mannose-binding lectin (collectin) pathway, which is discussed elsewhere in this chapter, and the alternative pathway. The alternative pathway involves the direct binding and recognition of foreign cells by complement protein C3, which triggers two processes in immune protection. Upon recognition of a foreign surface, C3 can be cleaved into components C3a and C3b, which respectively mediate inflammatory processes and covalent cell surface binding. The bound C3b can opsonize the foreign cell for uptake by phagocytes and can also trigger direct destruction of the cell by a cascade of reactions leading to formation of a membrane attack complex and ensuing cell lysis. C3b can also associate with the component b of factor B to form the C3b convertase, thereby contributing to the local processing of C3 and further binding of C3b molecules to the pathogen.[47,48]

Numerous complement proteins have been identified in fish. And, there are multiple isoforms of a key protein that mediates pathogen recognition. Rainbow trout were found to have three isoforms of C3 that differed in their binding to complement activators including yeast

zymozan, *E. coli* and erythrocytes.[49] The gilthead sea bream (*Sparus aurata*) showed even more diversity with five different isoforms of C3 that showed similar variation in function.[50,51] Zebrafish have three different isoforms[52] whereas medaka (*Oryzias latipes*) have two.[53] Moreover, a BLAST search of the pufferfish (*Fugu rubripes*) genome[54] (*http://ensembl.fugu-sg.org/Fugu_rubripes/blastview*), using rainbow trout C3a as the query sequence, clearly reveals three C3 isoforms located on different sequence scaffolds. The fish proteins are all homologous to their mammalian counterparts and appear to work through the same mechanism. Nonetheless, the paralogs of C3 could allow either greater specialization of function or increased overlap in activity, either of which would enhance the effectiveness of the innate immune recognition of pathogens.

Pentraxins

Pentraxins are so named because they are pentameric proteins formed of five identical subunits. These proteins, along with many of the complement components, are the classical acute-phase reactants. They are synthesized in liver and secreted to plasma where levels can vary dramatically in some vertebrate species in the hours following infection. The two proteins recognized as pentraxins are serum amyloid P (SAP) and C-reactive protein (CRP).[6] In humans, levels of CRP can rise so dramatically in response to infection that blood CRP is frequently used to determine the degree of inflammation in patients.[55] CRP binds to phosphorylcholine on the surfaces of Gram-positive and negative bacteria in a Ca^{2+}-dependent manner.[56–58] Recognition of phosphorylcholine-like groups on the surfaces of fungi and a nematode (*Ascaris lumbricoides*) have also been reported.[59] Phosphorylcholine groups on the surfaces of host cells appear to be in a form that does not interact with CRP. Binding by CRP mediates the killing of pathogens because it is an opsonin for monocytes and it can activate the complement cascade by the classical pathway through C1q.[54] CRP also has lectin-like properties, binding galactan structures.[60] Studies on transgenic mice carrying the human CRP gene have shown enhanced

resistance to *Streptococcus pneumoniae*, demonstrating the relevance of this protein in mediating recognition and bringing about an immune response.[55] Another pentraxin, SAP, binds to host cell components, suggesting a role in clearing debris after tissue injury. This pentraxin also binds lipopolysaccharide (LPS) from bacteria.[61] When SAP binds to certain strains of bacteria, there is no ensuing host response and as such it acts as an anti-opsonin, reducing phagocytosis and killing of bacteria through innate immune processes.[62] Therefore, although SAP binds bacteria, it might not have a protective role in innate immunity.

There have been numerous reports of pentraxins in fish. Functional studies, such as the initial report by Baldo and Fletcher[59] and others, have shown bacterial precipitation activities that can be inhibited by phosphorylcholine, suggesting CRP to be present.[63,64] However, analyses of partial sequences from various species have not allowed clear classification of these proteins as SAP or as CRP.

Accordingly, one group has named the pentraxin they have isolated, which has CRP activity, phosphorylcholine-reactive protein rather than classify it specifically as CRP.[65] Comparisons of protein sequences derived from pentraxin cDNAs from livers of Atlantic salmon and rainbow trout with known metazoan pentraxins did not allow definitive identification of the fish proteins as CRP or as SAP.[66] It is therefore possible that the salmonid pentraxin is an ancestor of both proteins.[66] In Arctic charr (*Salvelinus alpinus*), expression of this pentraxin did not respond to infection by *A. salmonicida*.[66] It was, however, identified in products of subtractive hybridization as an up-regulated gene in liver during infection of rainbow trout with *V. anguillarum*.[45] Therefore, as in mammals, the pentraxins may be regulated by infection in some fish species but not in other closely related ones. A pentraxin with a partial sequence similar to SAP was also found to be highly up-regulated in liver, head kidney and spleen of Atlantic salmon infected with *A. salmonicida*.[24] This pentraxin is likely to be a distinct protein from the one cloned earlier. Another pentraxin cloned from carp was most similar to a pentraxin fusion protein, which is an unusual pentraxin derivative known in frog, *Xenopus laevis*.[67] These differences in response to

infection do not necessarily reflect different roles or effectiveness. In mammals, it is the resting, constitutive expression of CRP that predicts effectiveness of innate immunity.[55] None of the proteins for which sequences are determined have been assayed for CRP-like activity although this activity is known to occur in fish. Functional studies on the expressed proteins will be needed in order to determine the source of the CRP-like activity.

Lectins

Proteins that bind to carbohydrates are referred to as lectins. Whereas plant lectins have been the focus of study for several decades, animal lectins have only become well known in the last few years. Many microbes have carbohydrate-rich surfaces with sugar compositions and surface patterns that differ in composition or spacing from those found on host cells. Lectins that recognize the specific carbohydrate patterns of bacteria, viruses or fungi serve as a mode of distinguishing these cells as potential pathogens. Since several similar carbohydrates are present on host cell surfaces and on soluble glycoproteins, lectins must frequently recognize the distinct PAMPs of carbohydrates on pathogen surfaces rather than simply the presence/absence of a particular sugar. Therefore a requisite feature of many of these lectins is the oligomeric structure.

The C-type lectins are so named because they share a carbohydrate-recognition domain that requires Ca^{2+} binding in order to adopt an active conformation. The carbohydrate-binding site of the C-type carbohydrate-recognition domain (CRD) is situated directly on the major Ca^{2+} binding site. Several C-type lectins play roles in innate immunity by recognizing PAMPs. A major soluble recognition protein in mammalian serum is the mannan-binding lectin (MBL). It is a "collectin," having a collagenous domain that trimerizes and a CRD. These, in turn combine to form larger bouquet-like structures that mediate carbohydrate PAMP recognition.[68] MBL binds bacteria, fungi and several viruses, and subsequently brings about their destruction either by activating complement or by opsonization.[69] There are also several receptors

containing C-type CRDs, including some that bind pathogens and mediate their uptake into macrophages or other cells.[70,71] Although some receptors have roles in pathogen recognition, these interactions are often exploited by pathogens for infection of those cells.[72,73]

The ficolins are a family of lectins structurally related but non-homologous to the C-type lectins. These proteins have a collagenous domain similar to the collectins but have a fibrinogen-like CRD and they recognize carbohydrate PAMPs in a Ca^{2+}-independent manner.[74] These have not yet been identified in fish. Using ficolin-1 from the frog (*Xenopus laevis*) as a query sequence, a BLAST search of the pufferfish genome[54] at *http://ensembl.fugu-sg.org/Fugu_rubripes/blastview* identified several putative homologous proteins, of which one or more may be ficolins, but further analysis would be required for positive identification. In contrast to the ficolins, other families of lectins with possible roles in immunity have been characterized in fish species, although they are less well known, or unidentified, in mammals.

Collectins

A direct homolog of the mammalian and avian collectins was identified in three cyprinid fishes including zebrafish, goldfish (*Carassius auratus*) and carp.[75] cDNA clones were obtained from liver of all three fish; however, the spleen was found to be the major site of synthesis among tissues examined in carp.[75] The encoded proteins had the invariant N-terminal Cys that mediates the interchain disulfide linkage of the trimers, an extensive collagenous domain and a C-terminal CRD typical of collectins. A four-residue spacer interrupts the collagen helix in the fish MBP, as was found for other mannan-binding lectins. An intriguing difference between these collectins and the avian and mammalian forms is their predicted carbohydrate specificity. C-type lectins that bind mannose and related sugars have an EPN tripeptide motif within their Ca^{2+}/carbohydrate-binding site and this sequence is invariant among all collectins except the teleost ones. The three fish collectins have a QPD tripeptide in this position instead.[75] In the C-type CRD, EPN has been shown to mediate binding to mannose and related

carbohydrates whereas the QPD motif generates recognition of galactose and related sugars.[76] The QPD motif in the fish collectins suggests that their primary ligand is galactose or a derivative thereof. But the QPD motif is also present in CRDs that do not bind sugars, such as the antifreeze proteins of Atlantic herring (*Clupea harengus*) and rainbow smelt (*Osmerus mordax*).[77] So, carbohydrate ligand specificity of the fish collectins will require experimental analysis. Determination of the precise carbohydrate specificity of the teleost collectins will provide insight into the significance of this divergence of collectins among fish, birds and mammals. The biological roles of the fish collectins have not been examined but they would be expected to function similarly to the avian and mammalian collectins in activating complement and opsonizing pathogens, based on their similarity to those proteins. The mannose-binding lectin-associated serine protease (MASP) mediates the activation of complement following collectin binding to pathogens. Homologues of this mammalian protein have been found throughout the vertebrates including teleost fish[78] suggesting that collectins can activate complement in fish as they do in mammals.

C-type Lectins Without Associated Domains

Several lectins from fish have a single CRD with no associated domains and they do not appear to be orthologs of the collectins. One such protein is the Atlantic salmon serum lectin.[79] Sequence analysis and genomic sequence organization suggest this lectin to be most closely related to the pancreatic stone proteins and fish antifreeze proteins, which belong to the C-type lectin family but do not bind carbohydrate.[79] At least five isoforms of this lectin are produced and it appears to be encoded by a closely linked multigene family.[79] Unlike most serum lectins, the predominant site of synthesis of this lectin is kidney although the specific cells of origin are unknown. The salmon lectin is functionally similar to the mammalian collectins. It binds mannose and related carbohydrates and it has the EPN motif that is characteristic of mannose-type binding.[79,80] The possibility of complement activation by this lectin has not been investigated but it has been shown to form large oligomers,

as the collectins do, and it binds and opsonizes *A. salmonicida*.[80,81] This implies a PAMP recognition role similar to other serum lectins, although the ligand on bacteria is not yet known. A lectin that appears related to this one has been isolated from the blood of rainbow trout by affinity chromatography on Sepharose™.[82] There is only limited N-terminal sequence available for this protein but it aligns with the salmon lectin and the two proteins form identical laddering patterns on non-reducing SDS-PAGE.[80,82] This trout lectin was also identified as a rainbow trout plasma protein binding to *A. salmonicida* LPS in a chromatography assay, which suggests functional similarity to the salmon lectin.[83]

Galactose-binding C-type lectins have been isolated from the skin[84] and gills[85] of Japanese eel (*Anguilla japonica*). Like the salmon and trout lectins, these lectins are CRDs with no associated domains. The gill lectin was identified by subtractive hybridization as a gene more highly expressed in fresh than salt water and this was confirmed by Northern blotting. Two isoforms were identified with minor sequence variation.[85] The protein appears to bind galactose and its derivatives, as suggested by a QPD motif and confirmed by affinity chromatography. The subunits of this lectin form dimers, which assemble into octamers. Although this lectin has not been shown to recognize pathogens, the multimeric structure would allow surface pattern recognition. Moreover, the increased expression of this lectin gene upon transfer of fish from salt water to freshwater, where a larger number of pathogens are thought to be present, would be consistent with this role.[85] The lactose-binding lectin in skin is homologous to the gill lectin but it only associates as dimers and can remain monomeric.[84] The carbohydrate binding activity and specificity of this lectin are highly unusual among C-type lectins in that it is unaffected by EDTA, suggesting no Ca^{2+} dependence, and it recognizes lactose by means of an EPN mannose-type binding motif.[84] It was shown to agglutinate bacteria *in vitro* and to suppress their growth.[84]

Carp may produce a similar single-domain lectin in leukocytes. A full-length cDNA clone encoding a lectin CRD was obtained by PCR cloning, following subtractive hybridization designed to isolate genes preferentially expressed during inflammation by leukocytes in the

peritoneum.[86] An EPN motif suggests binding to mannose or related sugars but no studies have been done on the protein. This lectin is homologous to the salmon serum lectin and the eel skin and gill lectins and, like them, it is a single CRD without other domains. However, it is unlikely to be an ortholog of the skin, gill or serum lectins above since none of them are known to be expressed in leukocytes. The soluble C-type lectins of fish are aligned in Fig. 1, showing common residues and the QPD/EPN motif that is in the Ca^{2+} and carbohydrate-binding site.

Related C-type lectins named 2-1 and 2-2 were identified among infection-specific subtracted cDNAs from the livers of rainbow trout treated with *V. anguillarum*.[45] C-type lectin 2-1 was also obtained as a subtraction product in the liver, spleen and head kidney of Atlantic salmon infected with *A. salmonicida*.[24] C-type lectin 2-2 was also identified as a subtraction product up-regulated in response to interferon or the viral hemorrhagic septicemia virus.[87] The up-regulation in response to *A. salmonicida* in Atlantic salmon suggested by the subtraction result was confirmed in spleen and kidney of this fish species by RT-PCR.[24] The clones obtained by subtraction are short and no

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Carp lectin           -----WTNFGVQCYKFFSRSTSWIAAERNCIEEHANLASVHNEEENDFLMGLLP
Salmon lectin SSL2    -----WFQFGSRCFMFVETARSWPLAERHCVSLGANLASVHSSADDQFLQAIAG
Eel lectin 1          -----WKGFNGCCYKHFDLLKNWREAEFYCMIRGGHLASVHSNVEYQFLRELNK
Eel lectin AJL-2      -----WVEHKNRCYLHVAEKKTWLDAELNCLHHGGNLASEHSEDEHQFLKDLHK
Carp collectin        -SVGHFRQVGQKYYITDGVVGTFDQGLKFCKDFGGTMVFPRTSAENQALLKLVV

Carp lectin           STT---KRCWLGVQDAVEEGQWLWSDGT----PYDYSNWCSNEPNNLN-VENCG
Salmon lectin SSL2    CKTGAFSTTWIGGFDAVQDRLWFWSDGS----EFDYQNWAKGEPNNSGGREPCI
Eel lectin 1          ASDPQDSMFWIGLTDIRKEGTWVWSDGS----AVDFTTWNPGQPDDWQGNEDCV
Eel lectin AJL-2      GSDDP---FWIGLSAVHEGRSWLWSDGTSASAEGDFSMWNPGEPNDAGGKEDCV
Carp collectin        SSGLSSKKPYIGVTDRETEGRFVNTEGK----QLTFTNWGPGQPDDYKGLQDCG

Carp lectin           EINWTSDRCWNDASCSTSMGYVCAKDCELCSRPVPQLP
Salmon lectin SSL2    VINWGDEYRWNDIKCGNSFPSVCSKRICEIQKN
Eel lectin 1          HANVPEQKNWNDVDCSTPYRFICALRSNAAGK
Eel lectin AJL-2      HDNYGGQKHWNDIKCDLLFPSICVLRMVE
Carp collectin        VIEDSG--LWDDGSCGDIRPIMCEIDNK
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Fig. 1 Alignment of CRD portions of soluble C-type lectins from fish. The alignment was done using ClustalW. [129] Residues identical among all lectins shown are shaded in grey. Residues in the Ca^{2+} and carbohydrate binding site that normally direct specificity toward mannose- or galactose-type carbohydrates are shown in white on black. Sequences are as follows: Eel lectin 1[85] (GenBank AB060539), Eel lectin AJL-2[84] (AB050703), Salmon lectin SSL2[79] (AY191314) and carp collectin[75] (AF227737).

full-length clones are available. Therefore, it is not clear whether lectins 2-1 and 2-2 are single domain proteins or if they have additional regions such as collagenous domains or transmembrane sequences that would imply different biological functions. A role in immunity or inflammation is suggested, however, by their independent isolation in these subtraction studies involving three different pathogens and two host species.

Fucolectins

Lectins binding to fucose have been isolated from the serum of Japanese eel (*Anguilla japonica*) for use as anti-H hemagglutinins for blood typing and histochemistry for many years. Recently, these fucolectins have been characterized more fully and reveal a potential role in innate immunity in the fish that produce them. Sequencing of cDNAs from liver and gill libraries revealed three liver isoforms and four gill isoforms of the eel lectin.[88] LPS treatment resulted in increased levels of lectin mRNA in cultured eel hepatocytes and increased lectin levels in the culture medium, compared with untreated controls.[88] The response to LPS suggests an immune role for fucolectins. Although recognition of bacteria by the eel lectin has not been investigated, a homologous lectin from horseshoe crab (*Tachypleus tridentatus*), which is named tachylectin, is known to bind the O-antigen of LPS.[89] Further suggestion of an innate immune role is derived from experiments in Atlantic salmon showing that a homolog of tachylectin is up-regulated in response to *A. salmonicida* infection.[24] Recent crystal structure determination of the fucolectin from European eel (*A. anguilla*) revealed a novel protein fold and highlighted key residues that differ among isoforms that may generate diverse pathogen-specific recognition properties.[90]

Rhamnose-binding Lectins

A number of rhamnose and galactoside-binding lectins have been reported in fish. These have been primarily identified in eggs of steelhead trout (*O. mykiss*), white-spotted char (*Salvelinus leucomaenis*) and catfish (*Silurus asotus*).[91–93] An interaction with egg yolk proteins, unrelated

to immunity, has been suggested for the steelhead trout egg lectins[91] and analysis of the interaction of a rhamnose-binding lectin from eggs of coho salmon with several strains of *A. salmonicida* showed no antibacterial activity.[94] Rhamnose-binding lectins from steelhead trout have, however, been shown to bind bacterial LPS and lipoteichoic acid.[93] The biological roles of these lectins remain unclear but bacterial recognition in innate immunity appears to be a plausible function.

Toll-like Receptors

Toll-like receptors (TLRs) recognize the PAMPs of microorganisms and they induce the expression of inflammatory cytokines and antimicrobial genes through the activation of nuclear factor kappa B (NF-κB),[95,96] as outlined in Fig. 2. There are nine TLRs in *Drosophila*[97] and ten TLRs have been described to date in humans.[96,98] The TLR superfamily is still growing and these receptors are found in many species throughout the animal and plant kingdoms. All TLRs contain leucine-rich repeat (LRR) motifs of the extracellular domain, a transmembrane domain and the Toll/interleukin-1 receptor domain (TIR) in the cytoplasmic region of the receptor.[99] The cytoplasmic TIR domain is highly conserved among species and is also found in IL-1 and IL-18 receptors.[100,101] The signaling pathways from TLRs are broadly conserved in mammals. The extracellular regions of TLRs are variable and they are thought to define the specificity of pathogen recognition, although the exact biochemical mechanism of recognition has not yet been determined. TLRs 2 and 4 have been reported to recognize components of Gram-negative bacteria, such as LPS and peptidoglycan in mice.[102] In *Drosophila*, double stranded viral RNA is recognized by TLR3,[103] flagellin in bacterial flagella by TLR,[104] a lipoprotein found in mycoplasma by TLR6,[105] and unmethylated bacterial CpG DNA by TLR9.[106] In fish, a number of TLR genes and cDNAs have been identified. The genome sequence of the pufferfish revealed clear orthologs of mammalian TLRs 2, 3, 5, 7, 8 and 9.[107] The pufferfish genome also encoded a TLR with equally high sequence identity to mammalian TLRs 1, 6 and 10. Moreover, two TLRs named 21 and

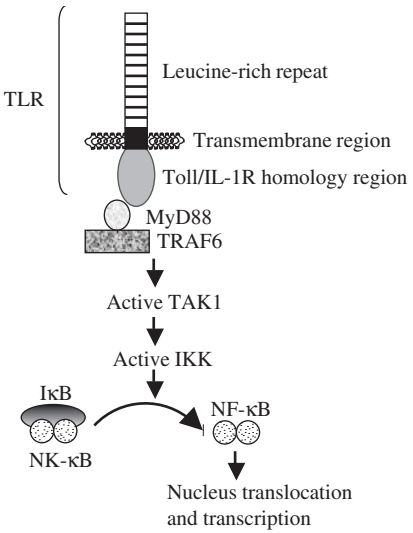

Fig. 2 Toll pathway signalling, as outlined by Takeda and Akira.[130] Upon stimulation of TLR by exogenous ligands, the N-terminal region of TIR domain recruits two adaptor molecules MyD88 and TRAF6. This complex activates two kinases, a MAP kinase named TAK-1 and IκB kinase (IKK), through subsequent phosphorylation. The activated IKK then inactivates IκB by phosphorylation, thereby releasing the transcriptional factor NF-κB into the nucleus. NF-κB activates the transcription of immune and inflammatory genes. Abbreviations: IκB, inhibitor of κB; IL-1R, interleukin 1 receptor; MAP, mitogen-activated protein; NF-κB; nuclear factor kappa B.

22 were found and these appear to be unique to fish.[107] RT-PCR analysis showed all of these receptors to be expressed in liver and each one to be expressed in at least four other tissues of healthy pufferfish.[107] Studies in salmonids have revealed one expressed TLR. A TLR5 cDNA was identified in subtraction products enriched for genes from Atlantic salmon liver in response to infection by *A. salmonicida* [24] and was also found to be highly up-regulated in infected liver by cDNA microarray analysis (Douglas *et al.*, unpublished). Pufferfish appear to have two TLR5 paralogs, of which one is a cell surface receptor and the other is a soluble protein.[108] Because the salmon sequence is incomplete, it is not yet possible to determine which of these types it is but preliminary results suggest that it is the soluble TLR5 (Tsoi, unpublished). A shorter

TLR sequence was identified among subtraction products from liver of rainbow trout injected with *V. anguillarum*.[45] The sequence did not span regions that would allow identification of receptor type but it nevertheless supports the presence of inducible TLRs in fish. The only TLR not detected in fish to date is TLR4. In mammals, this TLR primarily recognizes LPS.[108] It appears that this mode of LPS recognition is absent or highly divergent in fish.

A protein implicated in mammalian TLR function was also revealed in fish. A homolog of the mammalian LPS-binding protein and bactericidal permeability-increasing protein, that is likely to be an ancestor to both, was identified in rainbow trout.[109] In mammals, a key role of the LPS-binding protein is to bind LPS in plasma and deliver it to CD14 on cells of the myeloid lineage, and this in turn facilitates recognition of LPS by TLR4.[108,110] To date, no CD14 homolog has been identified in fish. Taken together with the absence of TLR4, this implies a different role for the LPS-binding protein homolog in fish. Functional studies on the protein will be required in order to determine whether it binds LPS and, if so, what the immunological effect of that recognition might be. Fish may rely more strongly on different mechanisms for the recognition of this structure. Perhaps other PAMP-recognizing proteins, such as C-type lectins, assume this LPS-binding and recognition role in fish.

Fish Innate Immune Proteins in Marine Biotechnology

Among the recognition proteins that are reviewed here, several may become valuable tools for preserving or promoting the health of wild and cultured fish species. They may find applications in fish health monitoring or assessment, in broodstock selection or for use as therapeutants. Transgenic fish harboring specific innate immune proteins have been developed for use in culture. Some innate immune proteins may also be useful in vaccines.

Health and Toxicology Monitoring

Innate immune proteins and processes in fish have been used as bioindicators for various monitoring purposes. The two major applications are ecotoxicology and fish health.

A growing area of study that employs fish innate immune proteins and processes is ecotoxicology (reviewed in Bols *et al.*[111]). Work done in this area has been wide-ranging and divergent, in terms of species used, measurements made, methods used and ecotoxicants tested.[111] One significant challenge in the effective use of immunotoxicology will be to relate changes in immune function to differences in susceptibility to disease.[111] For example, lysozyme has been used for monitoring but there is no known incidence of a direct relationship between lysozyme levels and disease resistance in fish. It will therefore be interesting to determine whether some of the antimicrobial proteins, collectins or other innate immune effectors will be shown to respond to specific ecotoxicants. Compromises in expression or function of these have been shown to predict disease resistance in other animals[15,69] and may also do so in fish. If such proteins were sensitive to ecotoxicants, they would provide systems in which impairment would directly relate to disease resistance.

Monitoring of fish health status requires indicators for stress and disease. Acute phase proteins that are highly inducible in a particular species, such as the pentraxin in Atlantic salmon[24] might be useful indicators of infection in fish by virtue of their elevated expression during infection. Conversely, proteins that are not induced by the infection process itself but respond to stress in fish would be ideal for monitoring stress status in fish independently of disease status. The antimicrobial histone-like protein has emerged as ideal indicator for this purpose in channel catfish. As noted elsewhere in the chapter, levels of this protein in skin extracts decreased in response to chronic stress whereas there was no evidence for a response to acute stress.[33] Chronic stress is a predisposing factor to disease of fish in aquaculture.[112] Therefore, although it is not known to what extent low levels of the histone-like protein affect disease susceptibility, its response to stress makes it a potentially valuable marker in fish health and toxicology.

Broodstock Selection for Aquaculture

A logical approach to broodstock selection for a particular trait is to construct a genetic map using microsatellites and then to identify the loci that contribute to the characteristic that is being sought.[113] The first application of this approach in fish health identified two quantitative trait loci associated with resistance to infectious pancreatic necrosis (IPN) in rainbow trout.[114] In a complementary approach to genetic marker-assisted selection, innate immune proteins that contribute directly to innate immunity can be used to predict disease resistance in individual fish. For example, it is known that decreased levels of functional MBL predict increased susceptibility to common infectious diseases in humans.[69] If the abundance of collectin mRNA or functional collectin protein were found to have the same predictive value in fish as in humans, it could be added to the suite of markers, genetic and otherwise, that predict disease resistance. For selection purposes, however, it would be even better if the gene tested were constitutively expressed rather than inducible, otherwise it would be difficult to distinguish an individual with constitutively high levels of a protective protein from one with an elevated level in response to infection. Whereas the highly inducible histone-like protein appears ideal for monitoring, it would not be useful in broodstock selection because this inducibility would mask genetically encoded differences in constitutive expression. In contrast, the pentraxin that is non-inducible[66] would be ideal for brookstock selection purposes if its resting state were predictive of disease resistance. An advantage to protein markers is that dipstick-type tests can be developed using specific antibodies and these would be convenient for use at aquaculture sites and for field use. The limitation, at this time, is that little is known about the counterparts of key mammalian proteins in fish and their predictive value.

Therapeutants for Fish

A number of good vaccines are available for use in fish but in some target species, for specific pathogens, and under certain conditions, vaccines are either unavailable or give variable results. Furthermore,

the use of antibiotics in aquaculture has declined although the requirement for effective protection of fish from infection has not changed. Bacterial, fungal, viral and parasitic diseases of fish frequently cause losses in the aquaculture industry. There is therefore a need for novel, effective vaccines and therapeutants that would prevent diseases or mitigate their effects. Soluble effectors of innate immunity are interesting candidates for these purposes.

Investigation of the therapeutic potential of antimicrobial peptides for fish has begun. Experiments involving intaperitoneal injection of *V. anguillarum* in coho salmon were used to assess the effectiveness of two synthetic antimicrobial peptides.[115] Continuous pump-mediated injection of either amidated synthetic pleurocidin or a synthetic hybrid peptide derived from the sequences of two insect peptides, cecropin and mellitin, resulted in greater survival time and greater overall survival rate in the salmon.[115] Nevertheless, the effectiveness of antimicrobial peptides as therapeutants for fish may be compromised by bacterial resistance, even if a broad-spectrum peptide is chosen. Strains of *A. salmonicida* that have an A-layer are far less sensitive to killing by cecropins than are A-layer negative strains.[116] Therefore, virulent A-layer positive strains of *Aeromonas* spp. might effectively infect fish, even if high levels of antimicrobials are produced endogenously or through therapy or transgenic means. A further challenge with the use of antimicrobials is concentration-dependent lytic activity against host cells. At low concentrations, antimicrobial peptides are lytic against bacteria or other pathogens but have no such effect against metazoan cells. However, at higher concentrations, certain peptides are strongly hemolytic,[17] and this could be toxic to the fish. Therefore, choice of natural peptides or engineering of suitable peptide sequences for therapy would need to take this potential risk into consideration. Finally, the protective effects of antimicrobial peptides during infection may involve more than their direct interaction with bacteria. Cathelicidins and defensins in mammals have been shown to signal host systems to initiate various arms of immune defence and host tissue repair.[117] If their counterparts in fish have similar activities, the potential of these peptides for immune enhancement would be very significant.

In order to produce highly effective vaccines, it is crucial that the fish recognize the target antigen. An obvious way to promote antigen recognition is to ensure efficient opsonization. Several bacterial recognition proteins such as the soluble C-type lectins, complement C3 or the pentraxins have been cloned from fish. If these are used as part of the vaccine protein, they might enhance opsonization, as this is their endogenous role in fish. One of these proteins, the Atlantic salmon serum C-type lectin, has been successfully expressed as a recombinant fusion protein in *E. coli* (Hudson *et al.*, unpublished). A vaccine antigen from a fish pathogen could be cloned in-frame with the lectin and expressed as a larger fusion protein. If the lectin still functions as an opsonin with the added antigenic peptide, it could be a very effective vaccine construct. This might be a viable option with one or more of the soluble opsonins that recognize pathogens.

Transgenic Fish

Several of the proteins that recognize pathogens as non-self and destroy them may be interesting candidates for increasing disease resistance through transgenic technology. As noted above, mice that are transgenic for human CRP show enhanced resistance to infection by *Streptococcus pneumoniae*.[118] Studies on mice made transgenic for human mannose-binding lectin showed that this lectin played a more important role than antibodies in initial defence against *Candida albicans*.[119] If fish were made transgenic to express higher levels of one or more of these proteins, they might have increased resistance to infection. It would be important, however, to establish that elevated levels of these proteins were not harmful to the animal. Early work on characterization of rainbow trout lysozyme was done with the intention of producing transgenic fish harboring extra copies of the lysozyme gene.[46] Transgenic Atlantic salmon that harbor this rainbow trout lysozyme gene were produced, and analysis of their serum lysozyme levels is underway (Fletcher and Hew, personal communication). This technology would offer a straightforward way to enhance natural disease resistance of fish and thereby further reduce antibiotic and chemical use in aquaculture. However, public anxiety over

the use of this technology has made it an unpopular choice for enhancing disease resistance and well-being of cultured fish.

Fish Innate Immunity in the General Life Sciences and Medicine

Mammalian (and specifically medical) research into immunological effectors has generally preceded, and therefore instructed, the development of fish immunology. But there are refreshing examples in fish innate immunity that are sufficiently unique or novel to lead mammalian immunology in new directions or toward new perspectives. For example, the salt-resistant alpha-helical antimicrobial peptides of species such as hybrid striped bass may have unusual therapeutic value in human medicine.[21] In fact, pleurocidins have been suggested to be intriguing candidates for antimicrobial therapy of cystic fibrosis patients because the high NaCl levels in their lung mucosa inhibits the endogenous antimicrobial peptides.[20,120]

The most striking example of a fish study leading comparative immunology and human medicine in new directions has centered on mast cell function. The immunological roles of mast cells were thought to be directed predominantly toward allergy and eliciting inflammation by secretion of mediators.[121] But recently, intracellular antimicrobial peptides named piscidin were discovered in the mast cells of hybrid striped bass.[122] These 22-residue peptides were found to have moderate hemolytic activity and broad spectrum activity against fish pathogens.[122] The piscidins were not mentioned previously in this chapter because they are intracellular and therefore not involved in the initial recognition of pathogens. However, this discovery of potent antimicrobial peptides in mast cells suggested that these cells might contribute directly to the destruction of pathogens. This finding in fish immunology set the stage for discovery of direct antimicrobial roles of mast cells and is thereby having a significant impact in medical immunology.[123]

Fish studies also have an impact on our general understanding of immune recognition. The question of foreignness and the innate immune system can gain a wider and more accurate definition from fish adaptations.

Ice crystals can grow in fish below their freezing temperature in cold oceans. Many polar and cold temperate fish species produce ice-binding antifreeze proteins as protection from freezing. The antifreeze proteins of smelt and herring are close homologs of fish single-domain C-type lectins[79,124] noted earlier but they have evolved an ice-binding site in place of the lectin carbohydrate-binding site.[125] The recognition by these proteins of ice crystals (water arrays) instead of pathogens with carbohydrate arrays is clearly a variation on molecular recognition in innate immunity. Furthermore, ice crystals can cause cell damage and leakage in the fish, which would elicit a "danger signal" that may assist in triggering an immune response.[126] Indeed, an acquired immune response, leading to anti-ice antibodies in fish exposed to ice, appears to take place in cold ocean fish.[127] These findings would suggest that the very definition of pathogen might need to be expanded to include abiotic structures if we are to consider pathogens at the molecular level, as does the immune system.

An interesting aspect of immune protection in fish, by contrast to other vertebrates, is innate diversity. Whereas the more derived vertebrates have expanded and refined their adaptive immune responses, it appears that fish and other poikilothermic vertebrates have primarily relied upon the diversification of their innate mechanism.[128] Diversity and specialization in complement C3 isoforms appears to be a strategy in fish to expand innate immune recognition capabilities.[128] Isoform frequency and diversity in the Atlantic salmon serum C-type lectins[79] might represent a similar adaptation. These findings suggest different possibilities for immune therapy in immunocompromised individuals. Moreover, just as fish have multiple immune strategies, the variation is compounded by the divergent nature of this vertebrate class. Fish are an extremely heterogenous group with a multitude of life histories and physiological parameters. Therefore, they present a variety of different models for innate host defence against infection.

Considerations for Future Work

Genomic resources and functional genomics tools are expanding our opportunities to appreciate fish innate immunity. Mining of the pufferfish

genome has led to new perspectives on the TLRs.[107] The lack of a counterpart to TLR4 in the pufferfish genome or the presence of a type VII immune-active serum lectin in Atlantic salmon with no clear mammalian ortholog suggest differences in the mechanisms of pathogen recognition in fish that might shed insight into these functions in mammals. Large-scale gene expression analyses in various species by means of subtractive hybridization have revealed genes and processes that are key to immune protection.[24,45,86] Further analysis of fish gene expression using cDNA microarrays is underway in several laboratories and will be uniquely informative in defining the overall transcriptional response to pathogens, particularly for the initial stages involving innate immunity. As proteomic and metabonomic technologies become more refined, these will contribute a much greater understanding of the dynamics of proteins and metabolites that perform innate immune functions. These large-scale approaches will be particularly useful in mapping the complementary or synergistic effects of alternative proteins or processes in this system. For example, the soluble lectins and the TLRs appear to have complementary PAMP recogition roles and the relationships between their roles can be better determined if their relative gene expression and protein levels can be coordinately assessed. Large-scale paralog analysis can also be undertaken for innate immune multigene families in fish using genomic information to better define the multitude of isoforms for specific proteins and salient differences in their structures or expression patterns. In cases such as the lysozyme-antimicrobial peptide synergy,[27] coordinate expression and/or localization can be examined to determine whether they act together in more than a random fashion in the fish. Taken together, these new approaches could provide an integrated view of innate immune pathogen recognition with better definition of the relative roles of different components and their interaction. It will also allow a more rational approach to the selection of key pathogen recognition proteins for new biotechnology in fish.

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Chapter 4

Molecular Mechanisms of Interactions Between *Aeromonas hydrophila* and Hosts

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Introduction

The genera *Aeromonas* comprises Gram-negative rods which are facultative anaerobic, oxidase-positive and generally motile by polar flagella. They are resistant to vibriostatic agent O/129 and do not require NaCl for growth.[1] Although the genera are currently classified in the family *Vibrionaceae*, Colwell *et al.*[2] suggest that they should be classified within their own family, the *Aeromonadaceae.*

Aeromonas spp. are predominately pathogenic to poikilotermic animals, including amphibians, fish and reptiles.[3] However, mesophilic aeromonads are emerging as important human pathogens because they are now implicated as the etiologic agent in numerous clinical situations, involving immunocompetent individuals of all ages, not just immunocompromised patients.[4,5] Mesophilic *Aeromonas* spp. are a complex bacterial group widely isolated from clinical, environmental, food and moribund fish samples, being considered important pathogens of fish and opportunistic human pathogens. *A. salmonicida* is the etiological agent for furunculosis of salmonid fishes, while *A. veronii* and *A. hydrophila* causes the bacterial hemorrhagic septicemia of cultured warmwater fish.[3] *Aeromonas* are ubiquitous water-borne bacteria that causes a wide variety of human infections,[6] including septicemia,[7] wound infections, meningitis[8] and pneumonia,[9] however they are most frequently associated with cases of gastroenteritis,[10,11] although the role of many aeromonads strains isolated

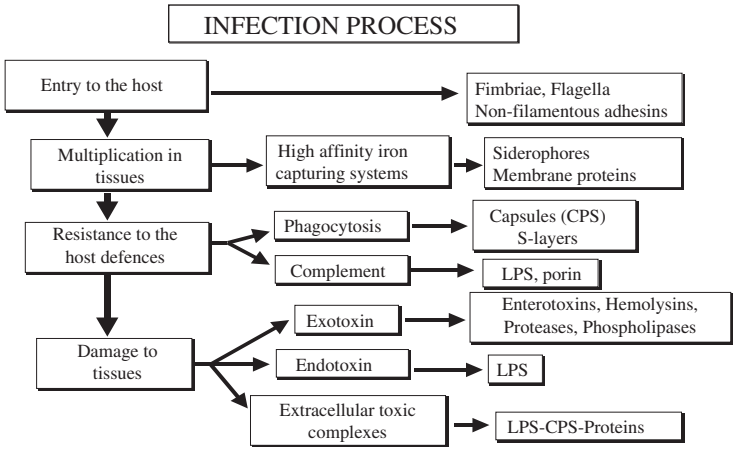

Fig. 1 The various steps in the *Aeromonas* spp. infection process.

from feces as gastrointestinal pathogens still remains controversial because of the lack of a good animal model.[11] Among the 14 species of *Aeromonas* isolated today,[3] *A. hydrophila* (HG1, HG3), *A. caviae* (HG4) and *A. veronii* bv. *sobria* (HG8/10) have commonly been isolated from human infections, which account for 85% of all clinical specimens.[12] *A. caviae*, in particular, has been reported as the most prevalent pediatric enteropathogenic species of the genus.[13]

A number of putative pathogenic determinants have been reported for aeromonads, including toxins, adhesins and invasins, and they interact at different levels to produce disease. Figure 1 shows the various steps of the *Aeromonas* spp. infection process, as well as the molecules/surfaces involved. In order to understand how some molecules/structures from *Aeromonas* could act like pathogenic factors, we review in this chapter the most important pathogenic significance of them (Fig. 1).

Fimbriae and Other Adhesins

Kirov[14] has reported that adhesion is an essential virulence factor for aeromonads which infect through mucosal surfaces or cause gastroenteritis disease. Among the most adhesive strains in the genus to cultured

Table 1 *Aeromonas* adhesions.

	Family		*Aeromonas* spp.	References
Filamentous				
Fimbriae				
S/R fimbriae			*A. veronii* bv. *sobria*	17
L/W fimbriae	Pili type IV	Bfp	*A. veronii* bv. *sobria*	22
			A. caviae	23
		Tap	*A. hydrophila*	24
	Pili type I		*A. hydrophila*	28
Flagella			*A. caviae*	35, 41
			A. hydrophila	35, 47
Non-filamentous				
S-layer			*A. hydrophila*	29
LPS			*A. sobria*	104
			A. hydrophila	105
Capsule			*A. hydrophila*	67
			A. salmonicida	68

mammalian and human cell lines, *A. veronii* bv. *sobria* have been reported the most adhesive[14] and more than 30% of clinical isolates of *A. caviae* have also been reported to be adherent.[15,16] The least efficient adhesive to cell lines among the three species more commonly isolated from human infections are *A. hydrophila* (HG1, HG2 and HG3). Two kinds of adhesins have been described in mesophilic *Aeromonas* spp. — filamentous (fimbriae or pili) and non-filamentous (Table 1).

Filamentous Adhesins

We can divide the aeromonads fimbriae into different morphological types. The type of filamentous surface structure expressed and/or number of them in a cell vary according to the source of the isolate and the bacterial growth conditions.[17] In addition, growth at low temperatures (5°C > 20°C > 37°C) and in liquid medium favor overall fimbriae expression for a majority of strains from all sources, although very little is known about the factors influencing *Aeromonas* spp. fimbriae

expression. The two major groups of fimbriae in aeromonads are: short, rigid (S/R) fimbriae, which are numerous per bacterial cell, and long, flexible (L/W) fimbriae, which are fewer per cell. Both are observed in environmental and clinical isolates. S/R fimbriae shared common epitopes among different strains analyzed and are widely distributed (more than 95% of the strains tested have been shown to possess S/R fimbriae). It is the predominant type expressed on heavily piliated aeromonads such as environmental strains of *A. veronii* bv. *sobria*.[17] This fimbriae cause auto aggregation of bacteria but are not hemagglutinating and do not bind to intestinal cells.[18]

L/W fimbriae have shown a high degree of homology in their N-terminal amino acid sequence among the different strains. Moreover, analyses of this N-terminal sequence have revealed that they are type IV pili, an important appendix in order to bind epithelial cells of a variety of Gram-negative pathogens.[17] The pili are long and thin (4–7 nm) and are also hemagglutinins; among hemagglutinins of aeromonads species, some of them have been associated with fimbriae and others have been associated with outer membrane proteins.[19] L/W fimbriae are predominant on strains isolated from feces, in particular *A. veronii* bv. *sobria*,[20] despite being poorly piliated (<10 pili per cell).

It is important to point out that gastroenteritis-associated aeromonads can express at least two distinct families of type IV pili.[21] The predominant family among the fecal isolates of *A. veronii* bv. *sobria* and *A. caviae* are the bundle-forming pili (Bfp).[22,23] The second type has been described and cloned by Pepe *et al.*[24] — the type IV pili (*tap*) biogenesis gene cluster from *A. hydrophila*. This is widespread in *Aeromonas* spp. and its gene cluster is homologous to a number of other Gram-negative bacteria.[21] Tap differ from Bfp in their N-terminal amino acid sequence and in their molecular weight. In addition, Tap are not as significant as Bfp for intestinal colonization. Bfp are an important adhesin and colonization factor because purified long flexible Bfp expressed from *A. sobria* and *A. hydrophila* (HG1, HG2 and HG3) are able to adhere to human intestinal tissue. Furthermore, purified long flexible Bfp can block adhesion of the source bacteria to intestinal tissue.[23,25–27] Moreover, the conditions that showed maximal expression

of fimbriae are reported to be the same, showing optimal adhesion to cell lines. In addition, removal of the surface structures by mechanical or enzymatic means decreases (by 60%–80%) bacterial adhesive ability for some strains.[20]

Not all pili of flexible morphology are type IV pili; the amino acid sequence of one, purified from a clinical *A. hydrophila* (HG1, HG2, and HG3) isolate, showed homology with *Escherica coli* type I and Pap pilin.[28] It is composed of a novel amino acid polypeptide (mini pilin) not found in any other strains. Its significance as a colonizing factor remains unclear.

Non-filamentous Adhesins

Other non-filamentous structures have been reported to act, such as adhesins in aeromonads. Among these structures, the major adhesin is usually the monomer that constitutes the S-layer; their adhesive properties was previously studied.[29] The lipopolysaccharide (LPS) complex, the capsule (reviewed later) and outer membrane proteins have also been implicated as adhesins. It has been proposed that porins of *A. hydrophila* act as lectin-like adhesins for attachment of this strain to carbohydrate-rich surfaces, such as erythrocytes, and possibly the human gut.[14,30]

Flagella and Motility

Aeromonas are usually motile by means of polar unsheathed monotrichous flagellum, which is responsible for the swimming motility in liquid media. Despite this psicrophilic nature, *A. salmonicida* has been defined as being a non-flagellated and non-motile species in the genus. There have been reports suggesting that some strains of *A. salmonicida* express unsheathed polar flagella at a low frequency.[31] McIntoch and Austin[32] reported that incubation of *A. salmonicida* at supra-optimal temperatures (30°C–37°C and in broth medium with high viscosity) resulted in the expression of motility by polar flagella in about 1% of the cells observed.

The flagellum organelle consists of a complex membrane-associated structure composed of the basal body and the externally located hook and filament. Rotation of the flagella filament by the membrane-associated flagella motor apparatus results in propulsion of the bacteria. Flagella filaments can be simple homopolymers of a single flagellin subunit or they can be complex heteropolymers of multiple flagellins and represents the major component of this complex and biologically important organelle.[33]

Certain strains of *Aeromonas* are able to produce many peritrichous lateral flagella when cultured on solid surfaces,[34,35] but only 50% to 60% of mesophilic aeromonads among the species most commonly associated with diarrhoea are able to express this second type of flagella which is unsheathed and is required for *Aeromonas* swarming motility,[36] although the hyperflagellated *A. caviae* or *A. hydrophila* do not appear to have differentiated into multinucleated and elongated swarmer cells, which have been observed during swarming in other species.[37] The production of flagella involves the expression of over 40 genes and is thus a very costly commitment by the bacterium in terms of resources and energy.[33] It would therefore seem to be advantageous to a bacterium to produce flagella only when required. Moreover, to have two distinct flagella systems is relatively uncommon; this has also been observed in *Vibrio parahaemolyticus*,[38] *Azospirillum brasilense*,[39] and *Rhodospirillum centenum*.[40]

Different flagellins from *A. hydrophila* and *A. caviae* have been purified and they showed molecular masses around 30 kDa, although lateral flagellin is smaller than the polar flagellin.[35,41] Both flagella types showed a higher estimated molecular weight on SDS-PAGE than that predicted from the nucleotide sequence. This aberrant migration could be due to post-translational glycosylation of flagellins, which has been described in an increasing number of bacteria.[41] Four bacteriophages from *A. hydrophila* and *A. veronii* bv. *sobria*, using flagella as their primary surface receptor, have been isolated and characterized by Merino *et al.*[42] and Rubires *et al.*[43] All these bacteriophages were able to replicate in different mesophilic *Aeromonas* strains independently of the species or the O-serotype. However, any single strain was able to host at the same time two of these bacteriophages. Moreover *A. salmonicida*

strains, different *Vibrio* spp. strains, or more than 40 *Enterobacteriaceae* strains tested were resistant to them. In conclusion, the four bacteriophages could be very useful in initiating the phagotyping of mesophilic *Aeromonas* strains by their H-antigen (flagellum). Two different works have reported the cloned, sequenced and characterized flagellin polar loci from *A. salmonicida*,[31] and *A. caviae*.[41] Both loci possessed two tandem flagellin genes (FlaA and FlaB) and other flagellar genes. Moreover, Gavín *et al.*[35] have cloned, sequenced and characterized the flagellin lateral loci from *A. hydrophila* and *A. caviae*, which possess one (LafA) or two (LafA1 and LafA2) flagellins, respectively. These works argue for the production of a simple or complex flagellum in the genus. The two loci described, polar and lateral respectively, exhibit similar organization to that of *V. parahaemolyticus* despite the number of flagellins.[44,45] Moreover, the different flagellins in the genus shared high homology, a property present in flagellin genes. Gryllos *et al.*[46] have reported another cloned and sequenced flagellar locus, the *flm* operon, which contains two genes widely distributed in mesophilic aeromonads and involved in flagellum assembly, possibly through glycosylation of the flagellins or other flagella proteins. In *A. caviae* Sch3N, this operon possesses five genes and is also involved in the biosynthesis of the O-antigen LPS.

Motility and the presence of flagella have been related to different early aspects of bacterial pathogenesis, predominantly adherence and invasion of eukaryotic cells. A variety of studies have showed that motility and polar flagellum are required for adherence, the first step in colonization, and invasion of human and fish cell lines by mesophilic *Aeromonas* spp. strains.[11,41,46,47] These results were based on the fact that in unflagellated mutants, removal of flagella by mechanical shearing or agglutination by antiflagellin antibodies, reduce adherence and invasiveness. The induced hyperflagellation coincided with the highest adherence levels in *A. hydrophila* and *A. caviae*, suggesting that the lateral flagella increase the adherence in both strains. Moreover, these structures were required for the formation of biofilms,[35] that are known to be a particular feature of persistent infections.[48] The production of the lateral flagella from *Aeromonas* spp. strains has been linked to the

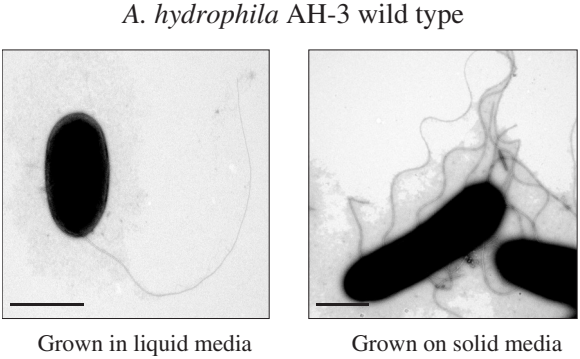

Fig. 2 Transmission electron micrographs of *A. hydrophila* AH-3 expressing only polar flagella and/or the induced lateral flagella (bar = 0.6 μm).

swarming motility which could mediate the formation of biofilms and increase the ability for invasion[48,49] (Fig. 2).

Siderophores

Competition for iron between a vertebrate host and an invading microorganism is one of the points on which the outcome of an infection is balanced. Owing to the presence of the host iron-binding proteins, e.g. heme, transferrin, lactoferrin or ferritin, iron is poorly available *in vivo*. In normal sera, the free iron concentration is far below the minimum required for the growth during an infection of most bacteria.[50] The bacteriostatic and bactericidal activity of serum and secretions is due to the thermodynamic capability of transferrin or lactoferrin to sequester iron[51] in addition to the complement system, although a great number of pathogens can acquisite this iron-sequestered in response to iron starvation. Aeromonads, as other bacterial pathogens, secretes siderophores, Fe (III) specific ligands of low molecular mass to obtain their supply of iron, although some siderophores may be inactivated by components of vertebrate serum. Aeromonads acquire also iron *in vivo* by direct contact between the host iron sequestering proteins and a binding protein of the bacteria.[52] Another alternative

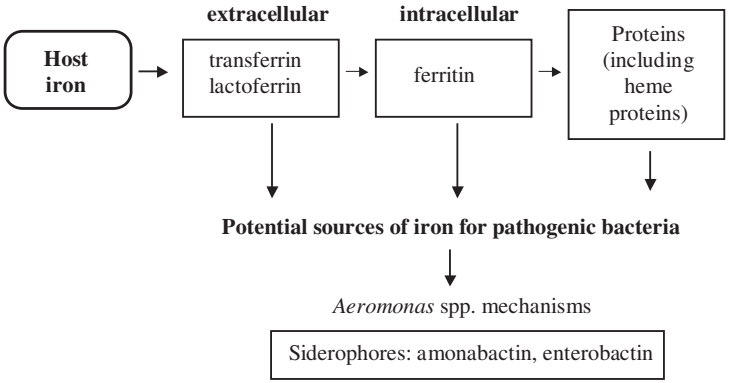

Fig. 3 Schematic representation of the host iron-binding proteins that can be potential iron sources for pathogenic bacteria. Aeromonads secrete siderophores to obtain their supply of iron during the infection process.

mechanism to obtain iron without intervention of a siderophore is to use heme, such as a source of iron primarily in the hemoglobin form. This mechanism requires hemolytic destruction of host cells to access iron in heme. We previously indicated the importance of hemolysins in the virulence of *Aeromonas* spp. (Fig. 3).

Most isolates of the mesophilic *Aeromonas* spp. produce either of the two iron-transporting siderophores, amonabactin and enterobactin, the indigenous siderophore of certain enteric bacteria;[53] however, a few strains produce no siderophore.[54,55] Amonabactin is the predominant siderophore in isolates phenotypically identified as *A. hydrophila* and *A. caviae*, while enterobactin is found in most members of the phenospecies *A. veronii* bv. *sobria*.[54] In addition, no isolate capable of producing both amonobactin and the enterobactin siderophores was identified. There is a correlation between the DNA hybridization group and the type of siderophore produced, conferring a possible useful characteristic in the separation of some of the genospecies of the genus *Aeromonas* and possibly in the evaluation of their potential virulence.[56]

Both siderophores in aeromonads are composed of catecholate (phenolate) groups based on 2,3-dihydroxybenzoic acid (DHB) — which form the chelating center — and aromatic amino acids.

Amonabactin is synthesized in two biologically active forms, each composed of bis-catechol, lysine, glycine and either tryptophan (amonabactin T) or phenylalanine (amonabactin P).[54] Therefore enterobactin is a cyclic triester of 2,3 dihydroxybenzoylserine.[53] Siderophore-dependent iron uptake systems contain another element in addition to the ferric chelating siderophore — a cell-associated apparatus which processes the ferrisiderophore for delivery of the metal to metabolism. The cell-associated components include a membrane-embedded receptor ferrisiderophore specific. Colligan and Colleman[57] have reported the ferrisiderophore receptor gene sequence of *A. salmonicida* which encodes an 86 kDa protein. Despite this, its function requires more study. Another siderophore receptor has been reported by Stintzi,[58] who found that *A. hydrophila* possesses a single amonabactin receptor that is able to recognize and transport an extraordinarily broad range of siderophores with chelating groups as varied as catecholate, hydroxamate or hydroxypyridonate. Moreover, this receptor uses a new kind of iron membrane transport mechanism, the shuttle mechanism, consisting of ligand exchange. This mechanism for iron acquisition allows the bacterium to steal iron from exogenous siderophores, providing *A. hydrophila* with great advantage *in vivo*, as it avoids the costly loss of secreted siderophores and provides the bacterium with the ability to rapidly acquire iron wherever it is encountered.[58]

Although there is not much information available on the biosynthesis and utilization siderophore genes in aeromonads, the genus has at least two distinct but functionally and probably evolutionarily related genetic systems for the biosynthesis and activation of 2,3-DHB: amonabactin- or enterobactin-producing species, respectively. Each of these systems differs from but is also functionally related to the 2,3-DHB operon (*entCEBA*) found in *E. coli*.[59] Borghouthi *et al.*[60] have reported the sequenced *amoA* gene from amonabactin, the first enzyme in the pathway from chorismic acid to 2,3-DHB, that showed only 58% identity to its homolog in *E. coli*, *entC*, but the deduced amino acid sequence product has a 79% carboxy-terminal similarity with EntC protein. Both operons, in amonabactin or enterobactin respectively, are preceded by an iron box sequence resembling the Fur repressor protein-binding site. Massad *et al.*[61]

have presented evidences which suggest that among the two types of *Aeromonas* spp. siderophores, amonabactin-producing strains can remove iron from host Fe-transferrin for microbial use while enterobactin is not functional in serum because serum albumin binds to this siderophore type.[62] Moreover, Stintzi and Raymon[52] have showed that amonabactin is not only able to use iron from transferrin but also from lactoferrin, proving this essential role of amonabactin in iron acquisition and therefore in the establishment of pathogenesis.

Capsules

The capsule is an extracellular polysaccharide enclosing the bacterium while remaining attached to the cell. It acts as a prominent antigen and plays important roles in pathogenicity of many bacterial pathogens. The capsular polysaccharide has been found to be present in some species of the genus when these were grown in a glucose-rich medium and also *in vivo*: *A. hydrophila* AH-3 (serogroup O:34), PPD134/91 and JCM3980 (both of serogroup O:18),[63,64] *A. veronii* bv. *sobria* AH-1 (serogroup O:11),[63] and in some strains of psicrophilic *A. salmonicida*.[65] Aeromonads are not only able to activate complement, their capsular polysaccharide has the capability of protecting bacterial cells from complement-mediated serum killing[64,66] because capsulated strains are able to inactivate the C3b deposit and as a consequence, reduce opsonization and impede phagocytosis. Moreover, the capsule plays a role in the adherence and invasion of fish cell lines.[67,68] In Aguilar *et al.*,[69] two capsular genes from *A. hydrophila* O:34 (*orf1* and *wcaJ*) have been found to confer serum resistance on *E. coli* K-12 strains, but there is little information on the genetics of capsular genes. Recently, Zhang *et al.*[64] described the gene cluster for capsule biosynthesis of PPD134/91, which can be divided into three regions, like group II capsule gene clusters of other bacteria including *E. coli*,[70] which represents all of the known capsule assembly systems seen in Gram-negative bacteria. This cluster consists of 13 open reading frames (ORFs) with high similarity to the genes encoding capsule transport and biosynthesis proteins, respectively.

S-layers

Two-dimensional paracrystalline surface protein arrays, or S-layers, are produced by a number of pathogenic bacteria, but in most cases their function is unknown.[71] Bacterial S-layers serve as the interface between the cell and its environment. For this reason, it can act as a permeability barrier and also provides protection for the cell. Moreover, they are positioned to play an important role in host-pathogen interactions.

The most studied *Aeromonas* spp. that possess an S-layer are: *A. salmonicida* (with an S-layer commonly known as A-layer) and *A. hydrophila*,[72,73] although *A. veronii* bv. *sobria* has also been reported to produce an S-layer.[74] To date, both mesophilic *Aeromonas* possessing S-layers belong to a single LPS serogroup — O:11.[75] This serogroup is commonly associated with human infections.[74] It is possible that other mesophilic aeromonads can produce S-layers.

The S-layers of *Aeromonas* spp. are composed of subunits of a single protein which self-assemble to form a tetragonal array surrounding the entire cell.[76] The surface location and high copy number of the S-layer protein subunits also mean that these subunits are a major surface antigen in the bacterial cell.[77]

The structural genes for the S-layers of *A. salmonicida* (*vapA*) and *A. hydrophila* (*ahsA*) have been cloned and sequenced,[78,79] and the purified proteins show a molecular mass in the range of 50–52 kDa,[76] moreover both reveal a classical signal peptide which is cleaved on translocation across the cytoplasm membrane. This secretion of the S-layer subunits requires the action of a number of secretion proteins which show homology to members of the general secretor pathways, but are specific for the S-layer subunits.[80,81] The tetragonally arranged S-layers produced by *A. hydrophila* and *A. veronii* bv. *sobria* are very similar morphologically to the *A. salmonicida* S-layers, with protein subunits that possess two morphological domains, a major domain connected to a lesser domain by a narrow connector. One unit in an array is formed by four subunits.[74,82] However, *A. salmonicida* appears to be genetically unrelated to these motile aeromonads; moreover, the protein subunit in *A. salmonicida* is translationally modified, probably phosphorylated such as the S-layer subunits from *A. hydrophila* and

A. veronii bv. *sobria*.[79] In contrast to *A. salmonicida*, in which the S-layer protein appears to be antigenically conserved, the S-layer proteins of mesophilic *Aeromonas* spp. reported are antigenically diverse.[83]

There are also significant differences between the role of the S-layer of *A. salmonicida* and *A. hydrophila* in the pathogenesis of these bacteria. The surface protein array of *A. salmonicida* is clearly demonstrated as an important virulence factor in most studies. Ishiguro *et al.*[84] showed that spontaneous S-layer mutants resulted in a dramatic decrease in lethality, and similar results were obtained with Tn5 mutants in specific S-layer proteins of the secretion pathway. Despite the fact that *A. hydrophila* S-layer may play a lesser role in virulence, Kokka *et al.*[85] suggest that this structure may play a role in systemic dissemination after invasion through the gastrointestinal mucosa as the S-layer possesses antiphagocytic activity. Moreover, in mesophilic aeromonads *A. hydrophila* and *A. veronii* bv. *sobria*, the presence of the S-layer increases the capability of adhesion to and colonization of mucosa. Also, the presence of S-layers renders the microorganism less susceptible to opsonophagocytosis.[86]

Other potential *in vivo* roles for the S-layers of *Aeromonas* spp. are to protect against the bactericidal effects of immune and non-immune serum,[87] and proteolysis, that can occur within phagolysosomes.[83,88] The S-layer from *A. salmonicida* also plays a role in colonization,[80] facilitates association with macrophages,[89] binds to a variety of extracellular matrix host proteins (such as collagen type IV, laminin and fibonectin[89,90]), as well as immunoglobulins M and G from different animal sources.

The study of the *A. salmonicida* S-layer has been used for diagnostic techniques, polymerase chain reaction (PCR) and hybridization,[92,93] as well as their use in attenuated vaccines.[94] Moreover, O:11 aeromonads have been detected in foods with an enzyme-linked immunosorbent assay (ELISA) using specific polyclonal antibodies against the S-layer.[95]

Endotoxin (LPS)

The lipopolysaccharide (LPS) is present in all Gram-negative bacteria and consists of a glucolipidic complex located in the outer membrane. The only phospholipidic portion, lipid A, is a high conserved structure

covalently ligated to the polysaccharidic complex, the LPS core, inner and more conserved than O-antigen that extends outward from the cell surface and is composed of repeating oligosaccharide units that vary among species.[96] The genus *Aeromonas* has been classified into 96 serogroups, 44 of which are based on the O-antigen LPS,[75] and a new extended serogrouping scheme by Thomas *et al.*[97] Serogroups O:11, O:16 and O:34 have special importance in human infections.[98] Among the few genetic studies on the LPS of aeromonads, Zhang *et al.*[64] recently reported the analysis of two O-antigen gene cluster. They have cloned and sequenced the cluster in the strains of *A. hydrophila* PPD134/91 and JCM3980, both belonging to serogroup O:18. This cluster possesses 17 ORFs transcribed in the same direction and organized into three classes of genes: some genes required for the enzymes involved in the biosynthesis pathways of nucleotide sugars, others for the glycosyltransferases, and genes for oligosaccharide or polysaccharide processing. Zhang *et al.*[64] have also studied the distribution of O-antigen from PPD134/91 among various serotypes of *A. hydrophila*, showing that different O-serotypes of this species shared some common features in these O-antigen clusters and that serogroups O:18 and O:34 are closely related. The O-polysaccharide from a virulent strain of *A. hydrophila* has been found to content rhamnose and glucosamine and to have an identical structure to that of the O-polysaccharide from *A. salmonicida*.[99] However in the *A. hydrophila* PPD134/91 strain, the sugar synthesis pathways identified in the O-antigen cluster were rhamnose and manose, respectively. Rhamnose is a component of surface polysaccharide present in many bacterial polysaccharides and highly conserved throughout all species.[100]

The virulence properties of LPS from *Aeromonas* spp. have different origins. Their lipid A, which seems to be very similar among Gram-negative bacteria,[101] has the general characteristics of the endotoxin from all these bacteria. It acts as a T-independent mitogen that produces polyclonal B-cell activation and a predominantly immunoglobulin M response. Gudmundsdóttir and Gudmundsdóttir[102] have reported that the LPS from *A. salmonicida* spp. *achromogenes*

(Asa) can induce inflammatory cytokines production in mice *in vivo* and *in vitro*, and exert anergic and mitogenic properties. Moreover, a wide variety of effects after injection in animals have been reported, including pyrogenicity, leukopenia followed by leukocytosis, septic shock, hemorrhagic necrosis of tumors, local Schwartzman reactions, diarrhoea and death.[85] It is important to point out that when Gram-negative bacterial infections lead to septic shock, endotoxins (LPS) are classically viewed as the prime initiators and many studies have shown that cytokines play important roles in pathogenesis[103] (Fig. 4).

On the other hand, different works have reported a variety of roles in pathogenesis of O-antigen LPS from mesophilic *Aeromonas* strains. This O-antigen acts as a prominent antigen and plays important roles in the pathogenicity of many bacterial pathogens. In *Aeromonas*, they act like an adhesin[104,105] and a colonization factor,[106] indicating that some *Aeromonas* spp. strains are important primary pathogens in gastroenteritis. Moreover, secretion of some exotoxins are dependent on the presence of O-antigen LPS,[107] which is implicated in resistance to the bactericidal activity of serum, although the strains can activate the complement, probably because the binding of C3b by O-antigen is sufficiently far away from the membrane to exclude the formation

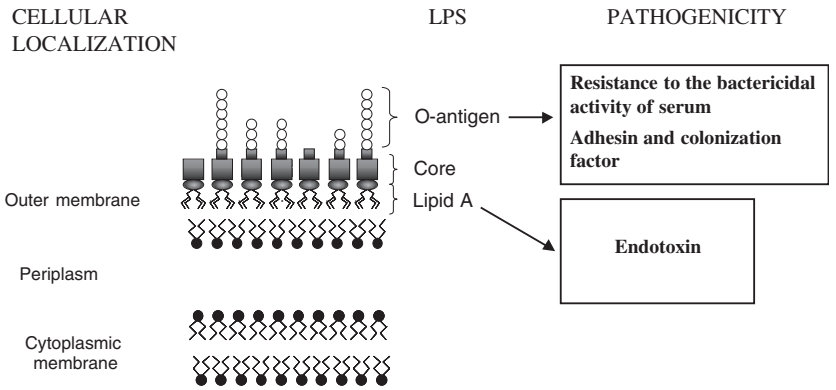

Fig. 4 The virulence properties of LPS from *Aeromonas* spp. and their different origins: lipid A or O-antigen.

of C5b-9 complex and prevent the formation of the complement membrane attack complex (MAC).[108] Alternatively, purified polysaccharides may randomly absorb some of the complement proteins and decrease part of the complement-mediated bactericidal activity, suggesting that O-antigen may serve as a double barrier to block complement-mediated bactericidal activity.[64] In addition, it is important to point out that polysaccharide chains in the smooth LPS, which are also known as the somatic antigen, from at least serogroups O:13, O:33, O:34 and O:44, showed a phenotypic change with important consequences for the expression of virulence determinants, expressing a smooth LPS (smooth and rough forms, with or without O-antigen, respectively) when they grow at 20°C in low or high osmolarity media and at 37°C only in high osmolarity medium. They express rough LPS (only the R-form LPS) when they grow at 37°C in low osmolarity medium.[69,107,109] When these strains infect humans, their O-antigen is strongly expressed in the human body temperature when osmolarity increases to values normally present, for example in the ileum lumen although is not expressed in the usual culture media at 37°C.[110] In mesophilic aeromonads, LPS with smooth ladder-like patterns predominates among clinical isolates.[111]

Secretion of Extracellular Enzymes and Toxins

Most aeromonads secrete a variety of extracellular enzymes and toxins, with different roles in pathogenicity. The majority of the protein secreted to the periplasm, outer membrane or extracellularly have classical N-terminal signal sequence in order to use the general secretor pathway.[112]

Exotoxins

Not all the strains in this genus produce all of the toxins described to date, and moreover if strains do possess particular toxin genes, these genes may be expressed only under certain growth conditions. Some of the toxins may require enzymatic activation.[113]

Enterotoxins

Two distinct types of enterotoxins from *Aeromonas* spp. strains have been reported:

(1) Cytotoxic enterotoxin that produces an extensive damage to epithelium.
(2) Cytotonic enterotoxin, which does not cause degeneration of epithelium.

Cytotoxic enterotoxin: (synonyms: aerolysin, cytolysin, Asao toxins and β-hemolysins) These toxin molecules have hemolytic and cytotoxin activities in addition to an enterotoxic activity.[114] However, toxin molecules with hemolytic or cytotoxic activity alone have also been isolated from aeromonads.[115] These toxin molecules are often found in diarrheal isolates. A number of different aerolysins have been cloned and sequenced from a variety of aeromonads, including *A. hydrophila* (HG1, HG2 and HG3) and *A. veronii* bv. *sobria* (HG8/10), which produces the highest titers of these toxins.[114,116–119] *A. caviae* (HG4) tend not to produce enterotoxins under the same conditions but around 50% of isolates carry the aerolysin gene.[120] Some clinical strains of *A. caviae* under different culture conditions produce cytotoxic activities.[15] The different aerolysin show heterogeneity in size (from 49 to 65 kDa), DNA and amino acid sequence and cytolytic activities. The protein is synthesized inside the cell as a preproaerolysin with a classical N-terminal signal sequence of 23 amino acids which is removed when it crosses the cytoplasmic membrane. The product is the still inactive proaerolysin activated by cleavage of 25 amino acids from the C-terminus and can now bind to the receptor glycophorin on erythrocyte cell membranes. Proaerolysin can also bind the receptor but cannot oligomerize like aerolysin to form channels in the cell membrane with around 1.5 nm exhibiting weak anion selectivity.[121]

In addition to the general biological function of aerolysins, Ferguson *et al.*[122] described the cytotoxic enterotoxin Act from *A. hydrophila* and have recently reported its implication on increasing tumor necrosis factor (TNF)-α and interleukin (IL)-1β production in macrophages and intestinal epithelial cells (IECG). This increment in the level of

TNF-α and IL-1β can be responsible for the pathogenicity seen with *Aeromonas*, or Act cloned, when infecting the small intestine.[123]

Cytotonic enterotoxin: These are like cholera toxin (CT) despite their different molecular sizes and variable reactivity with cholera antitoxin serum. They can be divided into two types: (1) Heat-labile (56°C for 10 minutes) and does not cross-react with cholera antitoxin serum. (2) Heat-stable (100°C for 30 minutes) and cross-reacted with cholera antitoxin serum.

Their mechanism of action is similar to that of CT, elevating levels of complementary adenosine monophosphate (cAMP) and prostaglandins (PgE_2) in eukaryotic cells such as the Chinese hamster ovary (CHO) cells.[124,125] The increase in cAMP results because these toxins may have adenosine diphosphate (ADP)-ribosyltransferase-like activity that activates adenylate cyclase. Both cAMP and PgE_2 can stimulate intestinal adenylate cyclase, causing fluid secretion.[124] Moreover, these toxins also produce elongation in CHO cells.

A variety of cytotonic enterotoxins from *Aeromonas* spp. strains have been described and some of them have been cloned and sequenced.[126–130] Some of these purified toxins exhibit not only one but also two or three bands of different molecular mass in SDS-PAGE.

Hemolysins

In addition to cytotoxic enterotoxins with β-hemolysis activity, aeromonads synthesize other β-hemolysins that produce holes in cell membranes by osmotic lysis and completely destroy the erythrocytes. However, they are codified by genes not homologues of previously described aerolysin genes. β-hemolysins from *Aeromonas* are heat-labile (56°C for 5 minutes) probably due to inactivation of a protease required for its activation.[1]

The second type of hemolysins in the genus is α-hemolysins that produce cytotoxic reversible effects and incomplete lyses of erythrocytes[117] (Table 2).

Lipases

Aeromonas spp. strains produce more than one enzyme with lipopolytic activity, and some of them play an important role in pathogenenesis.

Table 2 *Aeromonas* exotoxins.

	Activity	*Aeromonas* spp.	References
Enterotoxins			
Cytotoxic (or aerolysin, cytolysin, Asao toxin, β-hemolysin)	Cytotoxic Hemolytic Enterotoxic	*A. hydrophila*	114, 116–118
		A. sobria	117, 119
Cytotonic			
Heat-labile (no cross-reaction with CT)	Enterotoxins	*A. hydrophila*	127–129
Heat-stable (cross-reaction with CT)			
		A. sobria	126
Hemolysins			
Other β-hemolysins	β-hemolysis		1
α-hemolysins	Cytotoxic reversible effects Incomplete lysis of erythrocytes	*A. hydrophila*	117
		A. sobria	117

These enzymes vary in molecular size and activity. Phospholipases (PL) produced by bacteria are involved in different pathogenic processes and are often associated with intestinal damage.[131] These PL can act as hemolysins or as glycerophospholipid-cholesterol acyltransferases (GCAT). Two GCAT from *A. hydrophila* and *A. salmonicida* have been cloned and sequenced, and their implication in pathogenicity has been reported.[132,133] The properties of GCAT in *A. hydrophila* have similarities with mammalian lecithin-cholesterol acyltransferases, functioning as a lipase or phospholipase. Moreover, it can cause erythrocyte lyses by digesting their plasma membrane.[132]

Other secreted lipases have been reported from *A. hydrophila* strains, a group with high homology: PLA1, LipE, Lip and Apl-1.[134–136] Moreover, Apl-1 possesses phospholipase C (PLC) activity.[136] Although PLA1 show

high similarity with the heat-labile cytotonic enterotoxin from *A. hydrophila*, Alt,[125] it is non-hemolytic, non-cytotoxic and non-enterotoxic. PLC from *A. hydrophila* is an important virulence factor for mesophilic *Aeromonas*.[134] This PL has lecithinase activity and show homology with a hemolysin from *A. salmonicida*,[137] although PLC has only a little hemolytic activity. This enzyme is cytotoxic but non-enterotoxic.

In addition to the hemolytic or cytotoxic activity of a number of lipases, they may be important for bacterial nutrition and may also constitute virulence factors by interacting with human leukocytes or by affecting several immune system functions through free fatty acids generated by lipolytic activity.[1]

Proteases

At least three different types of proteases have been identified in *Aeromonas* spp. strains; including a thermolabile serine protease and two metalloproteases, both thermostable but EDTA-sensitive or insensitive, respectively.[138,139] Among the two major extracellular proteolytic activities of *A. hydrophila* that have been described and also cloned, there is a 38 kDa thermostable metalloprotease[140] which is very similar in molecular mass to the serine protease AspA produced by *A. salmonicida*[141] and a 68 kDa temperature-labile serine protease.[142] These two proteins are present in most *A. hydrophila* culture supernatants and these strains also show elastolytic activity, although it has not been attributed to any of the proteases described. Recent work by Cascón *et al.*[143] reported the cloning and sequencing of the metalloprotease AhpB, of molecular weight 38 kDa (in mature state), working as an elastase protein, and should be considered as a virulence factor.

In addition, some aminopeptidases may have a specific function, for example extracellular activation of the toxin aerolysin, cleavage of N-terminal methionine from newly synthesized peptide chains (methionine aminopeptidases), and the stabilization of multicopy ColE1 plasmids (aminopeptidase A). Moreover, other aminopeptidases play an important role in the catabolism of exogenously supplied peptides.[112]

Proteases can contribute to pathogenicity by causing direct tissue damage, enhancing invasiveness or with the proteolytic activation of toxins.[1] In addition, they also contribute to the establishment of infection by overcoming host defences and providing nutrients for cell proliferation.[144,145]

Other Enzymes

Other secreted enzymes such as amylase, gelatinase, nuclease or chitinase are detected in most aeromonads but their roles in virulence have not been determined. It seems clear that they may contribute significantly to the wide distribution and great adaptability to environmental changes of the genus.[112] With regards to antibiotic therapy, it is important to point out that aeromonads such as *A. hydrophila* strains are a significant reservoir of β-lactamase genes.[146]

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Chapter 5

Molecular Mechanisms of Host–Pathogen Interactions Between *Vibrio anguillarum* and Fish

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Introduction

Several bacterial species have been associated with epizootics in hatchery and fish farming operations. Examples of these diseases are vibriosis caused by *Vibrio anguillarum*, *V. ordalii* and other species of marine vibrios, furunculosis caused by *Aeromonas salmonicida* and enteric red mouth caused by *Yersinia ruckeri*.[1–3] Although vaccines against *V. anguillarum* and some of these other pathogens have been developed,[4–7] very little is known about the details of colonization and the *in vivo* expression of virulence genes in the fish. Of these pathogens, *V. anguillarum* is by far the best characterized genetically, since extensive work has been carried out to identify the genes necessary for the virulence attributes of this bacterium. Consequently, the *V. anguillarum* fish system is an excellent paradigm to assess the pathogen-host interactions leading to the colonization and subsequent mortality of salmonids.[2,8–24]

The bacterial fish pathogen *V. anguillarum*, a Gram-negative polarly flagellated comma-shaped rod (Fig. 1a), causes the fish disease vibriosis (Fig. 1b) that has become one of the economically most important diseases in marine fish culture, affecting a large number of species, including eels.[1,3,25] It is also an important disease of many wild fish

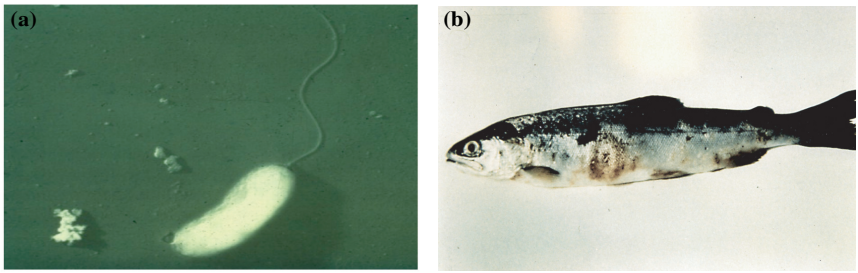

Fig. 1 Electron micrograph of *V. anguillarum* (**a**). This bacterium causes the fish disease vibriosis (**b**).

populations. Vibriosis, is a highly fatal hemorrhagic septicemic disease which shows striking similarities to the septicemic disease in humans caused by *V. vulnificus* and *V. parahaemolyticus*. Figure 1b shows that the external pathology of vibriosis caused by *V. anguillarum* includes hemorrhaging at the base of the fins, around the vent and inside the mouth.[4,26] Petechiae, necrotic lesions and diffuse hemorrhages can appear on the body surfaces. Internally, the intestine is often inflamed with petechiae present on the viscera and musculature. The intestine may be distended and filled with clear viscous fluid.[4,26] Experimentally induced infection by water-born exposure demonstrated that *V. anguillarum* likely enter the fish by penetrating the descending intestine and rectum.[4,26] In diseased rainbow trout (*Salmo gairdneri*), vibriosis results in muscle necrosis, accompanied by inter-fibrillar hemorrhages, congestion of interfibrillar vessels, and an absence of leucocytic response. In winter flounders, muscle necrosis and focal interstitial and tubular necrosis of the kidneys were reported.

Several parameters could affect infection rates and colonization by *V. anguillarum*. The occurrence of fish disease, depends on the balances among several factors, which include the pathogenic agent, the host and environmental parameters, such as temperature and salinity as well as over-crowding, and poor water quality including low oxygen levels and high suspended solids. There also appears to be a seasonal effect on the disease, outbreaks occurring mainly in spring and autumn, though this may simply reflect rapid changes of temperature and salinity.[3,4,26]

Many species of marine fish are susceptible to vibriosis and many fish can act as *V. anguillarum* carriers. Vibriosis generally occur at temperatures above 10°C, particularly when the surface of the fish is damaged; there is a high stocking density or there are other stress-related conditions present. *V. anguillarum* is considered to be part of the environmental flora, but may also be isolated as part of the normal gut microflora. Predisposition to infection can occur via skin wounds and loss of scales after grading and/or transport, infection can also occur by ingesting infected material from other fish. Furthermore, *V. anguillarum* can survive for a considerable time in the slime of uncleaned tanks and fouled nets, which thus act as a reservoir of infection. Recurring outbreaks such as those occurring with recycling furunculosis caused by *Aeromonas salmonicida* are unlikely as long as dead fish are promptly removed and husbandry standards are maintained, including prompt treatment with oxytetracycline or oxolinic acid included in the feed which usually saves those fish which are still feeding.[4,26,27]

By examining the histopathology of salmon which were sacrificed at various times following infection by immersion, Ransom,[4,26] investigated the route and spread of infection. In this experiment, bacteria were first observed in the lower intestine and colon, followed by accumulation of fluid and severe tissue damage in these areas. This fluid accumulation and damage to tissues suggests the production of toxins. Next, bacteria were observed in the surrounding tissues, and soon after in the bloodstream; after which time, the infection spread quickly throughout the fish.

Further studies have shown that this pathogen can invade the fish epithelium at multiple sites, including the skin and the intestinal tract.[16–18,22] Of course, the skin is directly exposed to water carrying the pathogen, and it has been shown that *V. anguillarum* adheres to fish mucus, invading when lesions are created experimentally or through captivity. Because marine teleosts constantly drink water, they subject the gastrointestinal tract to waterborne infection. It is now clear that the intestinal tract is a site of adhesion, colonization and proliferation,[22] during which *V. anguillarum* can utilize intestinal mucus as a nutrient source, systemic disease occurring when the bacterium is transported across the intestinal epithelium by endocytosis.[4,22,26]

The Virulence Factors of *V. anguillarum*

The following factors have been demonstrated to be associated with virulence in different strains of *V. anguillarum* causing vibriosis in fish:

(1) High virulence strains of *V. anguillarum* harbor the large plasmid pJM1 (65 kbp) shown in Fig. 2a. This plasmid encodes the anguibactin-mediated iron uptake system that enables the bacterium to obtain iron necessary for its metabolism from the iron-binding proteins of the host.[2,8–11,24,28]

(2) High virulence strains of *V. anguillarum* also resisted the bactericidal effects of normal serum and agglutinated trout erythrocytes. This trait being the consequence of several genetic determinants located in the chromosome.[15,23,29]

(3) Proteases, hemolysins, cytolysins and other extracellular toxic substances encoded by the chromosome have also been demonstrated among some *V. anguillarum* strains.[15,17,18,23,30]

(4) *V. anguillarum* possesses a single polar sheathed flagellum (Fig. 1a), which enables the bacterium to swim rapidly in a liquid environment and that plays a role in chemotactic motion and therefore in colonization.[21,22]

The first step in the pathogenesis of vibriosis is colonization, which requires chemotactic motility of the bacterium. Mutation of an open reading frame (ORF) corresponding to *cheR* results in a defect in chemotactic motility (Fig. 2b).[19,21] In experimental infections with this *cheR* mutant it was determined that this attribute was essential for virulence only when fish were immersed in water harboring the bacteria, but not when bacteria were injected intraperitoneally.[19,21] This result suggests that chemotactic motility is needed for colonization but not proliferation within the host. The importance of the flagellum in virulence of *V. anguillarum* has also been clearly demonstrated.[16,20] Flagellin-deficient mutants or mutants in the *rpoN* gene, which intervenes in the regulation of flagellar biosynthesis, were also affected in infectivity by immersion but not by the intraperitoneal route.[16,20] It is clear now that chemotactic motility is a required function of the

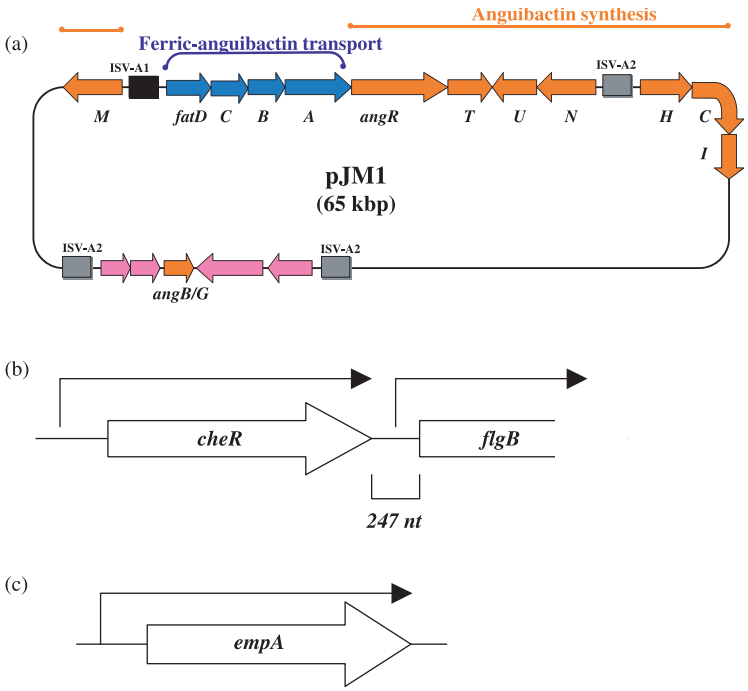

Fig. 2 Virulence determinants of *V. anguillarum*.

flagellum for the virulence of *V. anguillarum* for rainbow trout. Fish skin and intestinal epithelial surfaces are protected by a layer of mucus, therefore *V. anguillarum* must direct its passage towards and through the mucus. It is not unexpected then, that *V. anguillarum* exhibits strong chemotaxis towards fish mucus,[22] and that mutations in the *cheR* gene lead to a loss of virulence, therefore components of the mucus must act as chemo-attractants, promoting infection. Based on this, we hypothesize that the genes necessary for flagellin biosynthesis and chemotaxis will be highly expressed during the colonization phase of infection and shut off later in infection.

In infection studies with *Pseudomonas aeruginosa*, *V. cholerae* and *Helicobater pylori*, the flagellum and in particular motility were also found to contribute to the pathogenicity of these microorganisms in

their respective animal models.[29,31–35] In all cases the function of the flagellum is related to its role in conferring an increased ability to either adsorb to segments of the intestinal mucosa, like in the case of *V. cholerae* or to maintenance as it occurs with *Campylobacter jejuni*.

In the case of *V. anguillarum*, in addition to chemotactic motility, an extracellular metalloprotease has been shown to be an important factor necessary for early steps in the infection process. When the gene encoding this metalloprotease, *empA* (Fig. 2c), is mutated, the resulting strain is 1000-fold less virulent than the wild type when infection is initiated by immersion or anal intubation, but only ten-fold less virulent when fish are infected intraperitoneally.[17,18,30] It has been suggested that bacterial proteases act as virulence factors by causing massive tissue damage in the host and thereby aiding in host cell entry.

Another important virulence factor is the bacterium's ability to utilize iron complexed by the high affinity iron-binding proteins of the host, such as transferrin and lactoferrin. Iron is an essential element for nearly all microorganisms, yet in biological fluids it exists only as a complex with iron-binding proteins, making it essentially unavailable.[10,11,36] Therefore, invasive microorganisms must have the ability to utilize this complexed iron in order to grow within their hosts. The 65 kbp virulence pJM1 plasmid from the marine bacterium *V. anguillarum* encodes an iron-scavenging system, which is associated with the ability of this bacterium to cause septicemia in marine fish.[2] This system consists of a low molecular weight iron-binding compound, anguibactin; once secreted, this compound competes for bound iron within the host fish.[10,11] The iron-anguibactin complex is then internalized by an energy-dependent transport system which includes the FatA,-B,-C, and D proteins (Fig. 2a). Mutants lacking either the ability to synthesize or utilize the siderophore anguibactin show an approximate 10,000-fold decrease in virulence when tested by intraperitoneal injection, showing that this iron-scavenging system is absolutely necessary for virulence in this pathogen. We expect that components of this system are needed, and therefore expressed, during the proliferation and spread of the pathogen through the host, however it is unknown whether this system is needed during the colonization phase of infection. We are presently

investigating in which tissues expression of the different virulence factors occurs during the later stages of infection. It could be the case that either iron concentration or other factors influencing transcription, operate on the *itb* promoter at different tissue locales.

Conclusion

The long-term goal of the research in my laboratory is to gain an understanding of the timing and location of virulence gene expression. While many bacterial virulence factors have been identified, little is known regarding the *in vivo* expression of these factors in fish. Many *V. anguillarum* genes have orthologs in other vibrios, such as *V. cholerae, V. vulnificus* and *V. parahaemolyticus*, thus the *V. anguillarum* fish disease model provides an excellent opportunity for studies of the virulence mechanisms of these other pathogens.

Acknowledgments

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Chapter 6

Molecular Characterization and Pathogenicity of White Spot Syndrome Virus

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Introduction

White spot syndrome (WSS) is a viral disease that affects both wild and cultured shrimps.[1–14] The most commonly observed clinical sign of WSS in diseased shrimp is white spots in the exoskeleton and epidermis. These spots range from minute spots to discs several millimeters in diameter and they may coalesce into larger plates (Fig. 1). The causative agent, the white spot syndrome virus (WSSV), is an enveloped non-occluded rod-shaped DNA virus (Fig. 2). WSS can cause up to 100% mortality, with a correspondingly devastating economic impact, and since WSS was first recognized in 1992, it has become one of most serious problems facing the shrimp industry worldwide.[13–24] Lightner pointed out that no significant resistance to this disease had been reported for any species of shrimp, and this still remains true today.[15]

Based on studies[25–34] of individual genes and analysis of the complete genome sequence, WSSV has been erected as the type species of a new genus (*Whispovirus*) of a new virus family *Nimaviridae* (www.ncbi.nlm.nih.gov/ICTvdb/Ictv/Index.htm). In this chapter, the general properties of WSSV as well as its pathology, pathogenicity and molecular characteristics are reviewed and discussed.

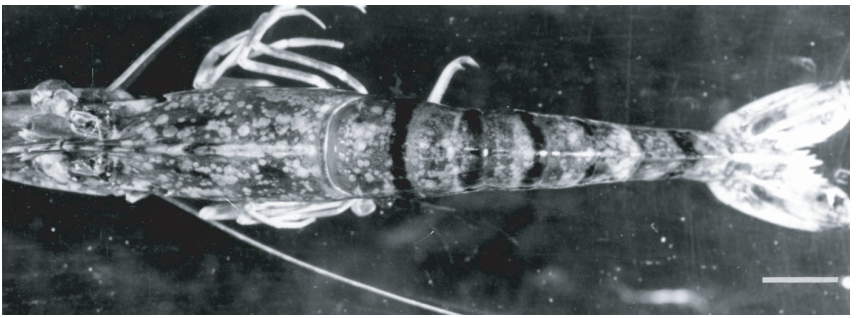

Fig. 1 Photograph of naturally WSSV-infected *P. monodon* showing the cuticular inclusions which range from minute spots to discs several millimeters in diameter (bar: 1 cm).

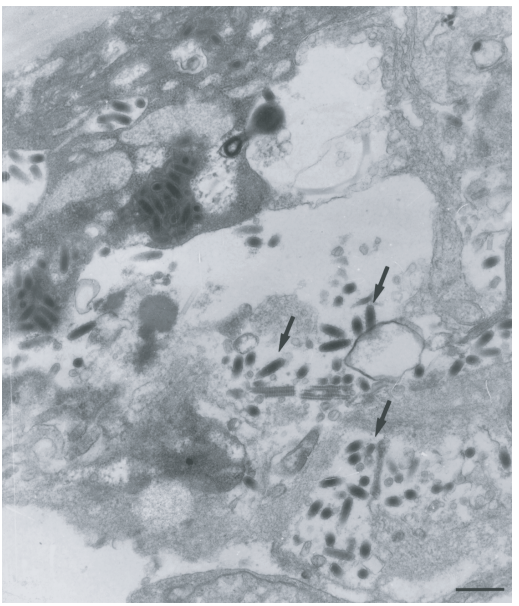

Fig. 2 TEM of thin-sectioned infected tissues underneath the cephalothoracic exoskeletal cuticle from *P. monodon* with WSS showing virus particles in the necrotic area (arrows) (bar: 0.5 μm).

General Properties of WSSV

WSSV is extremely virulent, has a wide host range[12,13,35–37] and targets various tissues.[11,35,36] WSS is notable for its rapid onset and lethality.[7,35,38] The virions are large (80–120 × 250–380 nm) and fusiform with bluntly rounded ends.[1,2,6,10,11,39]

Since no shrimp cell lines are currently available, instead of virus titer, WSSV infectivity is measured using challenge tests. In challenge tests with juvenile *Penaeus monodon*, WSSV became completely non-infectious after 60 minutes of UV irradiation (9×10^5 μW s/cm^2). WSSV was rendered non-infectious by 55°C and 70°C heat treatment within 90 minutes and 5 minutes, respectively. WSSV was also completely inactivated by high acidity (pH 1 for 10 minutes, pH 3 for 1 hour) and by high alkalinity (pH 12 for 10 minutes) at 25°C. The effective concentration for ozone to reduce WSSV's infectivity to zero was 0.5 μg/ml as a total residual oxidant for 10 minutes at 25°C. WSSV was inactivated by contact for 10 minutes at 25°C with a final concentration of 100 ppm of sodium hypochloride and providone-iodine and 75 ppm of benzalkonium chloride. On the other hand, neither high nor low concentrations of sodium chloride (0%–10%) could inactivate WSSV within 24 hours.[40] Challenge tests with juvenile *P. japonicus* showed that a stock solution of viral preparation in sea water maintained its infectivity for over 120 days at 4°C. At 25°C, the infectivity of the same preparation lasted more than 60 days but was lost by 120 days.[37] [Salt concentration may also be critical for long-term storage of the virus. In any case, for long-term storage, hemolymph from diseased shrimp in PBS (1 : 5) is a preferable medium.[41]]

Gross Clinical Signs and General Pathology

Although the white spots are the principal clinical sign, the presence of white spots does not always mean that the condition is terminal. For instance, under non-stressful conditions, infected shrimp that have white spots may survive indefinitely. However, if the shrimp also appear lethargic; or if their color changes to a pink to reddish-brown coloration;

or if they gather around the edges of ponds at the surface during the day; or if there is a rapid reduction in food consumption, then a very high mortality rate in the shrimp population can be expected within a few hours to a few days after the onset of these signs.[42] The moribund shrimp exhibit systemic destruction of tissues of ectodermal and mesodermal origin with many infected cells showing homogeneous hypertrophied nuclei (H&E staining) (Figs. 3 and 4). At the advanced stage of infection, numerous virus particles are released into the hemolymph from the lesions, causing viremia.[2,5,43] When baculovirus-infected insects die, they usually liquefy, mainly because of the effects of viral chitinase and cathepsin.[44] However, after death from WSSV infection, the shrimp carcass does not liquefy, even though the WSSV genome has homologs to chitinase and cathepsin. (Note however that

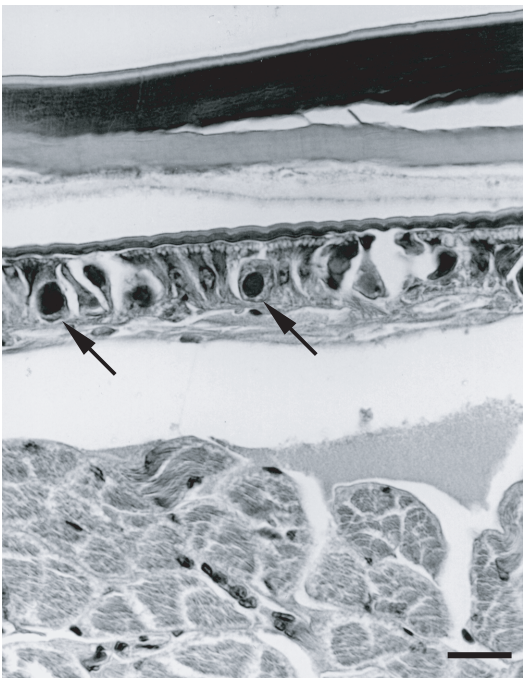

Fig. 3 Light micrograph of cuticular epidermis under the cephalothorax exoskeleton from *P. monodon* with WSS showing basophilic inclusions in hypertrophied nuclei of degenerated cells (arrows) (bar: 20 μm).

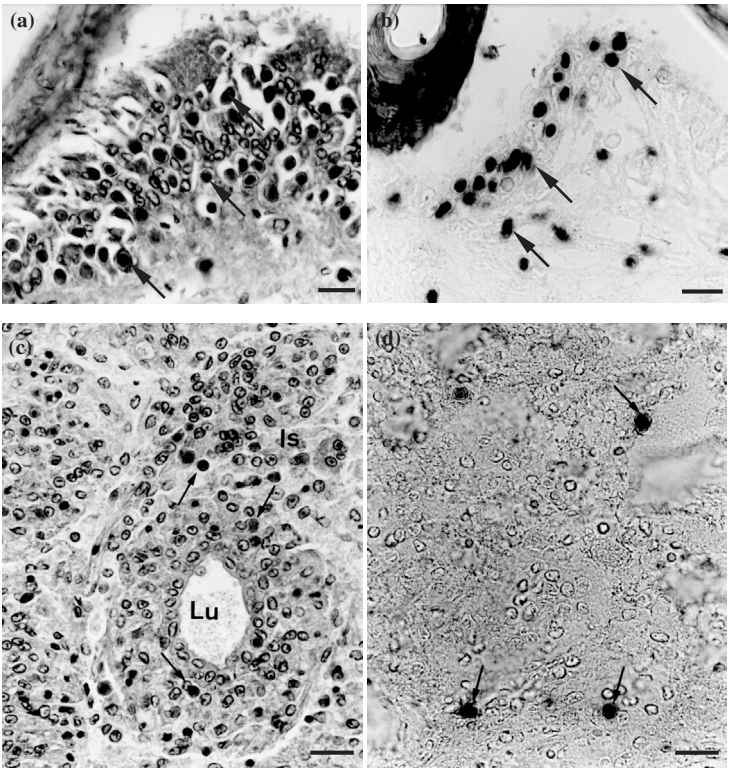

Fig. 4 Detection of WSSV in stomach **(a and b)** and lymphoid organ **(c and d)** of *P. monodon* by *in situ* hybridization (b and d) as compared with H&E stain (a and c). Arrows indicate WSSV-infected cells. Is: Interstitial sinus, Lu: lumen (bars: 20 μm).

the enzyme activity and function of these homologs remain to be determined.)

Pathogenicity

Changes in Total Hemocyte Count in WSSV-infected Shrimp

WSSV infection always leads to changes in hemolymph parameters. Several studies have shown a significant reduction of the total hemocyte count (THC) when the shrimp were infected with WSSV.[45–50] For example, in Wongprasert *et al.*[50] the average THC of shrimp before

WSSV infection was $20.9 \pm 0.7 \times 10^6$ cells/ml, but this count decreased significantly between 12 to 36 hours post-infection (hpi), after which the number stabilized at about 10% of the initial count.[50] In this study, an increase in the number of the hemocytes that underwent apoptosis was observed at 24 hpi and thereafter. In these WSSV-infected shrimp, apoptosis also occurred in hematopoietic tissue, which suggests that the decline in the number of hemocytes resulted from both the hematopoietic tissue and the hemocytes themselves being targeted by WSSV. On the other hand, hemocyte counts also decreased in the early period of viral infection, even before obvious apoptotic cells were observed. This is probably because crustacean immune systems have a mechanism which removes virus-infected hemocytes from circulation by attaching them to host tissues,[51] and it has been suggested that this mechanism applies in the case of WSSV infection.[46] van de Braak *et al.* and Wongprasert *et al.* also cite this mechanism to explain the early drop in the hemocyte count in terms of a migration of the infected hemocytes to tissues.[49,50] In any case, since hemocytes play an important role in cellular defense, a low THC will weaken shrimp defenses and reduce shrimp health. Since hemocytes are also necessary for clotting, the low hemocyte count also accounts for the well-known phenomenon that hemolymph withdrawn from WSSV-infected shrimp always has a delayed (or sometimes completely absent) clotting reaction.

Pathogenicity of WSSV to Shrimp in Different Developmental Stages

Pathogenicity of WSSV to *P. japonicus* in different developmental stages was studied by Venegas *et al.*[52] In this study, an experimental challenge by immersion was carried out at different larval (nauplius, zoea and mysis) and postlarval (PL1, 6, 9 and 11–12) stages of *P. japonicus.* Their results suggest that WSSV may not be pathogenic in the larval and early (younger than PL6) postlarval stages, and that the susceptibility of the shrimp to WSSV increases along with the growth of the host. This pattern of WSSV pathogenicity, i.e. that it does not cause the disease until the host has reached the later postlarval stages, is similar

to that of the Taura syndrome virus (TSV) in *P. vannamei*.[53] Although the reasons for this pattern are still not clearly understood, Venegas *et al.* suggested that the rapid growth (ecdysis), metabolic processes and incomplete development of the target organs or tissues during these stages may be involved.[52] Furthermore, as Venegas *et al.* also pointed out, in *P. japonicus* the branchial complex only becomes fully morphologically and functionally developed at the PL11 stage,[54] and since this complex is one of the possible entrance sites of WSSV,[35] this could account for the delayed susceptibility of shrimp to WSSV.

However, except for shrimp still in these early developmental stages (i.e. up to about PL6), WSSV can cause disease in shrimp at any growth stage. Infection trials using three different sized groups of juvenile *P. japonicus* (mean weights 0.08, 0.16 and 0.26 g, respectively) showed that WSSV inoculum was highly pathogenic to the smallest shrimp tested; all of these shrimp died within 5 days.[7] By contrast, only 35% cumulative mortality was found in the 0.16 g shrimp group after 7 days, although mortality reached 100% in 12 days, while a relatively low mortality (10%) was observed in the group of the largest shrimp (mean weight 0.26 g). Note, however, that in addition to the age of the host, several other factors, including the infective dose, and physiological and environmental conditions, also affect the progress and signs of the disease.

The Route of WSSV Entry and Progression of the Disease Caused by WSSV

In situ hybridization was used to identify the major tissues that are sites of initial viral infection as well as the preferred attack sites of WSSV in experimentally infected shrimp.[35] In this study, the experimentally challenged shrimp (0.35–0.45 g *P. monodon*) were fed with pieces of an adult shrimp that had died from a natural WSSV infection. Tissue samples were taken from the challenged shrimp at 0, 16, 22, 40, 52 and 64 hpi and sections were hybridized *in situ* with a WSSV-specific probe labeled with digoxigenin. WSSV-positive cells were first observed at 16 hpi in the stomach, gill, cuticular epidermis

and hepatopancreas. Some shrimp sampled at this time showed the virus in the stomach but not in the gills, while others sampled at the same time showed the virus in the gills but not in the stomach, which implies that WSSV infection can occur either via the oral pathway or via water to the gill or cuticular epidermis. In further confirmation of this conclusion, many studies that needed to challenge shrimps with WSSV have successfully used methods that involved either immersion or *per os.*[38,55–57] At 22 hpi, the lymphoid organ, antennal gland, muscle tissue, hematopoietic tissue, heart, midgut and hindgut were found to be WSSV-positive.[35] The nervous tissue and compound eyes did not have WSSV-positive cells until 40 hpi. Thus various tissues from the mesoderm (e.g. connective tissue) and ectoderm (e.g. epithelium) can all be infected by WSSV. By 52–64 hpi, it was found that the stomach, gill, cuticular epidermis, lymphoid organ, hematopoietic tissue and antennal gland were all heavily infected with WSSV and that these tissues had become necrotic. Visible white spots in the cuticle first appeared at 40 hpi and the shrimp began to die at 64 hpi.

The cuticular epithelium from every part of the shrimp body is one of the main target tissues for WSSV. The connective tissues of some organs are also infected by WSSV. In other tissues, such as nervous tissue, muscle tissue, lymphoid tissue and hematopoietic tissue, positive signals of WSSV DNA can also be observed. In terms of the organs, WSSV does severe damage to the stomach, gills, hematopoietic tissue, lymphoid organ, antennal gland and cuticular epidermis of the shrimp. At the late stages of infection, these organs are destroyed and many cells are lysed. Organs which are more lightly infected by WSSV include the hepatopancreas, nerve ganglia, compound eye, muscle tissue (mostly connective tissue cells), and the connective tissue of the midgut and hindgut. The degree of infection of these organs does not increase and they maintain organ integrity up to the late infection stage, although a few cells have cytopathological signs and are lysed.

Distribution of virions appears to be the primary mode by which the virus is spread to other tissues. Liu *et al.* monitored the presence of the viral DNA by polymerase chain reaction (PCR) in shrimp injected with WSSV inoculum at 2, 4, 6, 8, 18, 24, 36 and 60 hpi.[27] The viral

DNA was first detected at 2 hpi and then continued to be found through to 60 hpi. The relatively small amounts of WSSV observed at 2 hpi were almost certainly the result of some of the injected virions reaching the target cells. At 4 hpi there was an increase in the amount of WSSV DNA, which was interpreted as evidenced the replication of viral DNA. These elevated WSSV DNA levels reached a plateau at 18 hpi and were maintained through to the end of the observation period (60 hpi). Thus, in the absence of a cell line, this suggests that the WSSV replication cycle is about 18–20 hours, which is in close agreement to the estimate based on the *in situ* hybridization analysis.[35]

Another notable feature of WSSV is that its replication is easily triggered by stressful conditions. We have classified WSSV infection into three stages: the asymptomatic carrier, transition and acutely affected stages.[19] The carrier stage may persist for months, but as soon as certain triggering conditions are reached, the disease progresses to the transition and patent stages within a few hours. The virus load (copies per σg total DNA) may increase from 10^1 (carrier state) to 10^7 (patent state) within a few hours after exposure to environmental and/or spawning stress(es) (our unpublished data).

Transmission

As mentioned in the previous section, WSSV can be transmitted horizontally either orally by predation on diseased individuals, or by virus particles in the water, primarily through the gills but also via other body surfaces.[7,35,38] The virus is also transmitted vertically, that is from brooder to offspring.[36,58–60] *In situ* hybridization has detected WSSV-positive cells in reproductive organs, and with transmission electron microscopy (TEM), virus particles are readily seen in the nucleus of young oocytes. WSSV-positive cells have been found in the ovary, follicle cells, oogonia, oocytes and connective tissue,[36] but to date positive signals have never been observed in epithelial cells of the spermatophore, nor in sperm cells, perinucleolus oocytes or any other advanced developmental stages of the egg (e.g. yolk stage oocytes). The virus appears to attack only oocytes that are still young, and if

a young developing oocyte does become infected, it will die before it reaches maturation. This means that transovarial transmission (i.e. through an infected egg) is an unlikely route by which the disease might pass from brooder to offspring. The transovum transmission pathway (i.e. via egg-mass contamination; vertical transmission in broad definition), remains a possibility. However, although it can be difficult to distinguish between WSSV contamination and WSSV infection during the nauplius, zoea and mysis stages,[59] there is evidence that resistance to WSSV may be quite high during the early larval stages.[52]

Apoptosis in Virus-infected Penaeid Shrimps

Apoptosis is a cell suicide mechanism that enables metazoans to control cell numbers in tissues and to eliminate individual cells that threaten the animal's survival. Apoptosis is frequently characterized by chromatin condensation, phosphatidlyserine exposure, cytoplasmic shrinkage, membrane blebbing and caspase activation.[61,62] In the case of virus-infected cells, early cell death severely limits virus production and reduces or eliminates the spread of progeny virus in the host, and it is therefore frequently used as an antiviral defense. In consequence, most animal viruses have evolved strategies to evade or delay early apoptosis to maximize virus progeny or facilitate a persistent infection. On the other hand, a growing number of viruses are now known to induce apoptosis actively at late stages of infection, which may serve to spread virus progeny to neighboring cells while evading host immune inflammatory responses and protecting progeny virus from host enzymes and antibodies.[63,64]

Several studies have shown that apoptosis occurs in virally infected shrimp. Anggraeni and Owens used the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-nick end labeling (TUNEL) assay to study the occurrence of apoptosis in the lymphoid organ (LO) of *P. monodon* with midcrop mortality syndrome (MCMS).[65] They found a significant difference ($t = -5.533$, $df = 58$, $p < 0.05$) in the mean percentage of apoptotic spheroid cells between laboratory-held prawns without MCMS ($52 \pm 24\%$) and farmed prawns with MCMS ($80 \pm 12\%$), and concluded that apoptosis was used to eliminate the

virus-infected cells. Conversely, Khanobdee *et al.* used histological, cytochemical, ultrastructural and biochemical evidence to confirm that widespread and progressive apoptosis occurred in the hemolymph, gill and LO of *P. monodon* infected with yellow head virus (YHV), and they concluded that apoptosis was a major cause of functional failure in vital tissues and the death of the host.[66]

Apoptosis (Fig. 5) was detected in WSSV-infected *P. monodon*[50,67,68] and *P. japonicus*.[69] In *P. monodon*, TUNEL positive nuclei were first observed in the subcuticular epithelium at 6 hpi, and the number increased significantly as the infection advanced.[50] Caspase-3 activity in WSSV-infected shrimp cells was also about six-fold higher than in uninfected shrimp, which further supports the argument that apoptosis is involved in the shrimp response to the virus and that it is important in the pathophysiology of WSSV infection. Wongprasert *et al.* particularly noted that some cells undergoing apoptosis did not contain virions, while other cells that did contain WSSV virions were not apoptotic.[50] A similar phenomenon has been reported by Kaplan and Sieg in HIV-1 infection, where CD_4-T cells infected with HIV-1 did not become apoptotic although nearby uninfected "bystander" cells did.[70] In the case of HIV-1, the viral TAT protein secreted by infected cells is believed to either directly enhance apoptosis in uninfected cells or render them hypersensitive to Fas-Fas L-mediated apoptosis,[70,71] and by "committing suicide" in this way, the adjacent uninfected cells help to limit viral

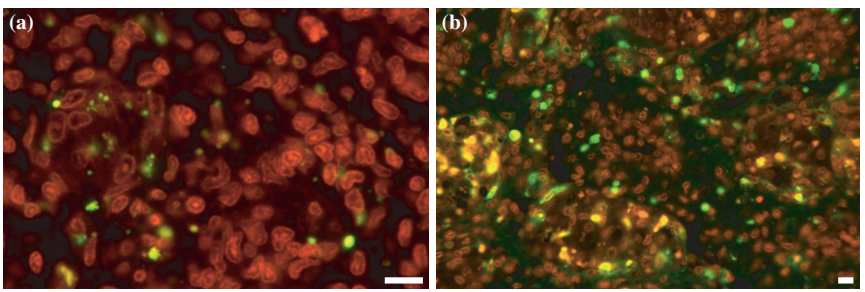

Fig. 5 Fluorescent photomicrographs of TUNEL-positive nuclei (green fluorescence) in lymphoid organs of WSSV-infected *P. monodon* at **(a)** 6 hpi and **(b)** 72 hpi (bar: 0.1 μm).

spread. Wongprasert *et al.* have argued that since a similar situation occurs in WSSV-infected shrimp, then the shrimp could be using apoptosis as a protective response to limit the spread of the viral pathogens.[50]

In *P. japonicus*,[69] the apoptotic cells were usually observed in the early stage in WSSV infection, and they were more abundant in the LO than in the stomach epithelium. However, there was no evidence of increased apoptosis in WSSV-resistant "quasi-immune" (see below) shrimp. In a study on WSSV-infected *P. japonicus* hemocytes, gene expression analysis identified several expressed sequence tags (ESTs) that represent apoptotic peptides and tumor-related proteins.[72] Expression of the programmed cell death 6-interacting protein (Alix, an apoptotic factor) was 3.6 times higher in the viral-infected library than in the normal one.[72] However, much more work needs to be done; for now the role and regulation of apoptosis in virus-infected penaeid shrimps remain obscure.

Quasi-immune Response in WSSV-exposed *P. japonicus*

Shrimp viral epizootics typically cause high mortality within 1.5 to 2 years from the first appearance of a new virus, after which the severity of the epizootics usually declines. Examples of viruses that follow this pattern include monodon baculoviral disease,[73] yellow head viral disease,[13] white spot syndrome[13] and mid-crop mortality syndrome.[74] Assuming that the shrimp are cultured under relatively favorable conditions, then after an initial period of catastrophic mortality, they are no longer so severely impacted, even though the causative viral agents remain ubiquitous and can still be detected in the shrimp. Concepts such as viral accommodation,[75] and tolerance[59,60] may partly account for this phenomenon. Selection pressure may also be a contributory factor, and there is indirect evidence for this in the declining infection rate of wild caught brooders in Taiwan, down from 80% in 1995 to 50% in 2002.[76,77] *P. japonicus* also exhibit a "quasi-immune" response to WSSV, and this is discussed at some length below.

An epizootiological investigation made in Japan in 1997 found that most of the kuruma shrimp that survived a WSS outbreak in a culture pond subsequently grew to market size even though WSSV was detected by PCR in these survivors.[78] These surviving shrimp also had a significantly higher survival rate than naive shrimp when challenged with WSSV by intramuscular (IM) injection. A similar resistance to WSSV was shown by survivors of an experimental WSSV infection when they were re-challenged one month after their first exposure to the virus by IM injection.[78] Subsequent experiments have shown that at 24°C, this "quasi-immune" response (to differentiate it from the true adaptive immune response in vertebrates) begins to appear three weeks after initial infection, develops almost fully at week 4, and persists until the end of the second month.[79]

Evidence for virus neutralizing activity in the plasma portion of the survivors' hemolymph was demonstrated by injecting plasma-treated WSSV into naive shrimp.[79] Gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of plasma from the survivors and from controls found no significant differences in the respective protein profiles, but cation exchange chromatography detected a unique substance in one of the fractions of the survivors' plasma (Wu, personal communication). The identity and possible role of this substance has yet to be elucidated.

Specificity was examined by challenging (IM) WSS survivors with *Vibrio penaeicida*, the causative bacterium of vibriosis in kuruma shrimp, and by an *in vitro* assay on the neutralization activity of the survivors' plasma against infectious hematopoietic necrosis virus (IHNV, chAb), viral hemorrhagic septicemia virus (VHSV, Obama25) and infectious pancreatic necrosis virus (IPNV, VR-299) (Wu, personal communication). Preliminary results showed that 30 days after initial exposure, the shrimp survivors were resistant to WSSV [relative percent survival (RPS): 83.3%] but not to *V. penaeicida* (RPS: 11.8%), and that the survivors' plasma showed no neutralizing activity against any of the three viruses tested. These results are consistent with WSSV-specificity in the quasi-immune response, but true WSSV-specificity remains to be confirmed by more rigorous testing.

Söderhäll and Thornqvist do not advocate trying to immunize crustaceans (or other arthropods) because their non-adaptive immune system means that vaccination can at best only enhance their immunity for a limited period of time.[80] Nevertheless, vaccinations of lobsters with injections of *Aerococcus viridans* var. *homari* have succeeded in inducing resistance to this bacterial pathogen.[81,82] More recently, experimental tests of vaccines made of inactivated WSSV with or without immunostimulants (β-1, 3-glucan or killed *V. penaeicida*) and of recombinant proteins of WSSV (rVP26, rVP28) have suggested that for kuruma shrimp challenged by injection, resistance to WSSV can be enhanced for periods extending from 10–30 days post-vaccination.[83]

Molecular Characteristics of WSSV

WSSV is the type species of the genus *Whispovirus*, family *Nimaviridae*. The family name reflects the most notable physical feature of the virus, that is a tail-like projection extending from one end of the WSSV virion (Fig. 6). Another unique feature of WSSV is that the components of the non-enveloped nucleocapsids form parallel cross-striations, so that the capsid seems to be composed of a stacked series of rings that are perpendicular to the longitudinal axis of the capsid.[10,11,84,85] The thickness of the rings is very constant, at about 20 nm. Each ring consists of two rows of 12 to 14 globular subunits, each approximately 10 nm in diameter (Fig. 6).

Complete genome sequencing has been performed on three WSSV isolates.[28–31] The WSSV genome (~300 kb) is ~30 kb smaller than the 335,593 bp genome of *Ectocarpus siliculosus* virus (EsV-1), which is the largest virus genome sequenced to date.[86] The WSSV genome has a total G + C content of 41%. Three percent of the WSSV genome is made up nine homologous regions (hrs) containing 47 repeated minifragments that include direct repeats, atypical inverted repeat sequences and imperfect palindromes, while the remaining 97% of the sequence is unique. A total of 532 putative open reading frames (ORFs) (>60 codons) were identified by sequence analysis, among which 149 ORFs have a potential polyadenylation site (AATAAA) downstream of the ORF.

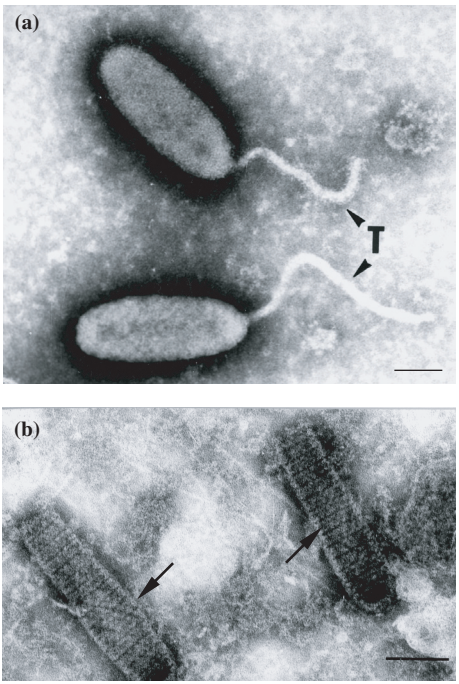

Fig. 6 TEM of negatively stained **(a)** purified virions showing a tail-like projection (T) extending from one end of the virus, and **(b)** non-enveloped nucleocapsid showing the cross-striations on the capsid formed by the ring subunits (arrows). The rings align perpendicularly to the longitudinal axis of the capsid (bar: 0.1 μm).

Because of the large size of the genome and the uniqueness of the proteins that the WSSV ORFs encode, WSSV has not yet been fully characterized. To date only a few WSSV genes have been studied beyond sequence analysis.[25–27,29–32,87–95] Below, we review what is currently known about some of the WSSV structural and non-structural proteins.

Structural Proteins

WSSV virions have a complicated SDS-PAGE protein profile (Fig. 7). By using SDS-PAGE coupled with Western blotting and/or protein N-terminal sequencing, at least six structural proteins have been

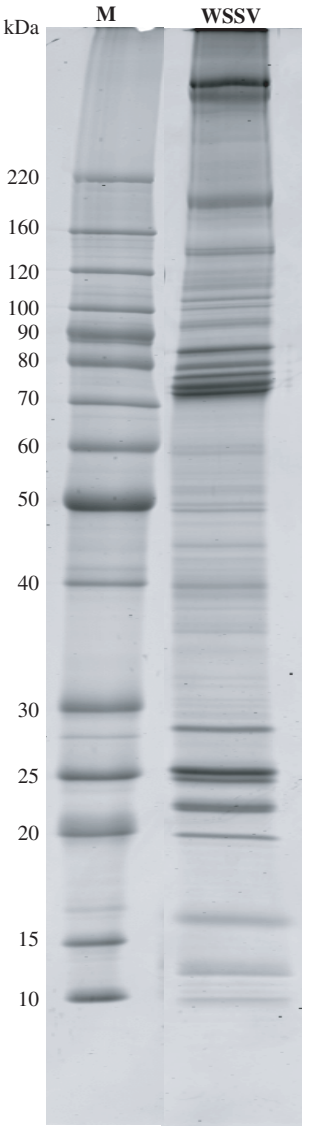

Fig. 7 Sypro Ruby stained SDS-PAGE of the structural proteins of WSSV virions purified from hemolymph of infected crayfish *Procambarus clarkii* separated by a 8%–18% gradient gel. M: BENCHMARK® Protein Ladder (Invitrogen).

identified which include VP28, VP35, VP26, VP24, VP19 and VP15.[30,84,87,89,90,92,95–105] However, there are many other structural proteins that have not yet been identified by these conventional tools.

Proteomics is defined as the large-scale analysis of the function of genes, and it is becoming a central field in functional genomics.[106] The major tool to study purified proteins in this field is mass spectrometry. Combining mass spectrometry with database searches of sequenced genomes offers a powerful means of identifying the proteins.[107] We have recently applied this technology to provide an exhaustive list of structural proteins of WSSV. Starting from the protein profile of WSSV virions purified from the hemolymph of experimentally infected crayfish *Procambarus clarkii* separated by 8%–18% gradient SDS-PAGE and stained with Sypro Ruby (Fig. 7), the visible bands were then excised from the gel. Following trypsin digestion of the reduced and alkylated WSSV proteins, nano-electrospray ionization tandem mass spectrometry (LC-nanoESI-MS/MS) for peptide sequencing was performed using a quadrupole/time-of-flight (Q-TOF) system equipped with a nanoLC source and a CapLC system (UK Micromass and UK Manchester). The resulting peptide sequence data were then compared against the NCBInr database (accessed via Mascot server) to find proteins with matching peptide sequences. The original SDS-PAGE protein bands matched at least 33 distinct WSSV ORFs, i.e. 33 WSSV structural genes were identified, including 13 WSSV structural protein genes previously identified by mass spectrometry,[92,93] N-terminal sequencing[87,99] as well as Western blot analysis.[84,89,92,95,97,98,100,103–105,108] There are also six other structural proteins that have been previously reported (four by mass spectrometry,[93] one by N-terminal sequencing[99] and one by Western bloting[30]) but which we have so far not been able to identify by using mass spectrometry on the bands excised from our SDS-PAGE gels. There must therefore be a total of at least 39 (33 + 6) WSSV structural proteins.[109]

VP28 is an important major envelope protein that is involved in the systematic infection of WSSV.[84,87–89,108] The *vp28* gene is transcribed at a late stage of infection in *P. monodon*,[27] *P. japonicus*[95] and crayfish.[32] Western blotting analysis with VP28-specific antiserum readily detected

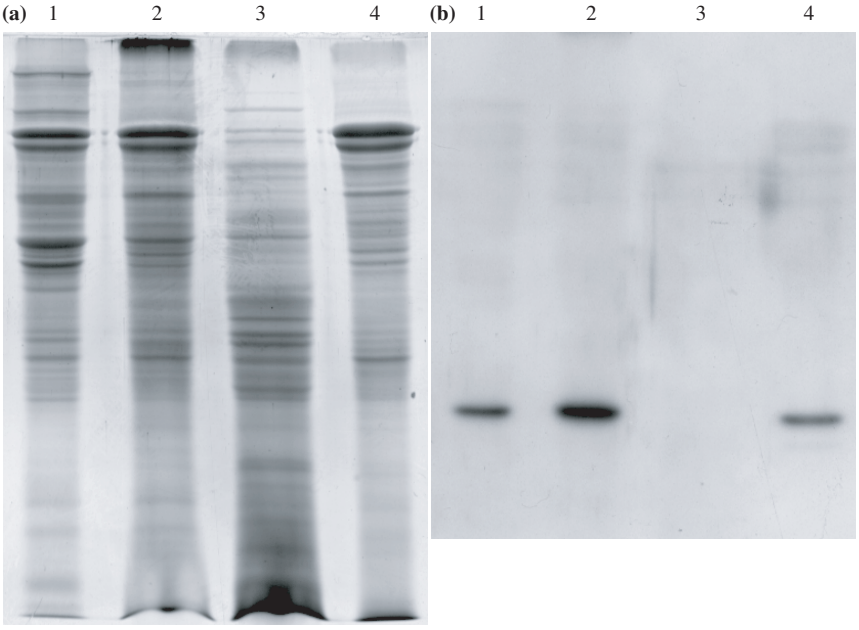

Fig. 8 (a) Coomassie brilliant blue-stained 12.5% SDS-PAGE gel with total lysates from different organs of infected *P. monodon*. **(b)** Western blots of a duplicate gel using VP28-specific antiserum and developed by an ECL chemiluminescence system using goat anti-rabbit IgG coupled to horseradish peroxidase as second antibody. VP28 was detected in the (lane 1) stomach, (lane 2) gill and (lane 4) hemocytes, but in this case not detected in the (lane 3) hepatopancreas.

VP28 in many tissues and organs of WSSV-infected shrimp at the late infection phase (48–96 hpi) (Fig. 8).

Considering WSSV's uniqueness, then ideally for the gene expression analysis of VP28, a continuous cell line should be used. However, to date no such line is available for the study of shrimp viruses. Another alternative would be to use shrimp primary cell cultures from the lymphoid organs of *P. vannamei*[110] or *P. monodon*,[67,111] but this approach is apt to produce results that are inconsistent and hard to repeat. Thus, to study VP28 in hemocytes during the infection cycle, we developed a gene expression analysis system based on a monolayer of shrimp hemocytes.[112] In this system, hemocytes collected from

experimentally WSSV-infected shrimps were attached to a coverglass to form a monolayer, immunostained so as to label the VP28 indirectly with FITC and then counterstained with Evan's blue so that in contrast to the green-fluorescing FITC, the cell bodies appeared as a diffuse red under a fluorescence microscope. In this study, at 36 hpi, green fluorescence (i.e. FITC-labeled VP28) was observed mainly in the cytoplasm of the hemocytes, and in the nuclei of only a few hemocytes. At a later phase of infection (72 hpi, Fig. 9), the intensity of the green fluorescence (at higher intensities, the green fluorescence shades to yellow) increased both in the cytoplasm and in the nuclei indicating that VP28 targeted the nuclei efficiently. This study also showed that primary hemocyte culture provides a good tool for studying gene expression at a cellular level.

Structurally, the nucleoproteins (DNA-protein core) at the core of the WSSV virion are packed within a capsid shell, which is in turn surrounded by an intermediate layer and an outer envelope. As expected, VP28 was detected only in the envelope protein fraction and not in the nucleocapsid fraction.[30,87,97] Further, in another immunogold assay

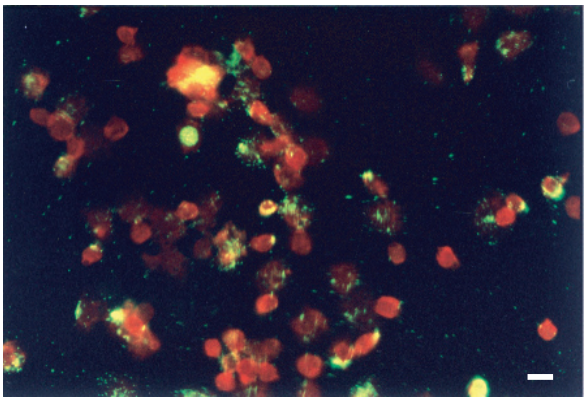

Fig. 9 Immunocytochemistry assay of VP28 (green fluorescence) in the hemocytes collected from WSSV-infected *P. monodon* at 72 hpi. Hemocytes were labeled with VP28-specific antiserum and treated with FITC-conjugated goat anti-rabbit IgG antibody. Evan's blue was used for (red fluorescent) counterstaining (bar: 10 μm).

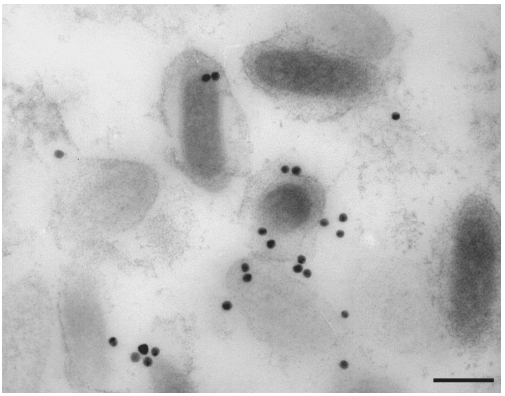

Fig. 10 Immuno-electron micrograph showing immunogold localization of WSSV VP28 in LR Gold sections of infected *P. monodon* hemocytes (60 hpi) (bar: 100 nm).

on Triton X-100 purified virions, VP28 antibody reacted only with the envelope and not with the purified capsid.[95] However, in our recent study, which used thin sections instead of purified virus, micrographs of virion sections (Fig. 10) clearly show that most of the gold particles were located not on the envelope's outer surface but on its inner surface, or in the intermediate layer between the envelope and the capsid shell.[112] From this we concluded that VP28 is indeed an integral envelope protein, but instead of protruding outwards from the outer surface of the envelope, most of its molecule extends inwards from the inner surface of the envelope and through the intermediate layer, where it interacts with capsid shell protein(s).

Non-structural Proteins

The WSSV DNA polymerase gene (*DNA pol*) defines a polypeptide of 2351 amino acids residues.[29,31] WSSV DNA polymerase shares the characteristics of the eukaryotic-type family B pols, and likewise it contains the DNA polymerase domain and exonuclease domain. Although WSSV DNA pol is much larger than the other known viral

DNA pols, this is due to the expanded spacer regions surrounding the conserved motifs, while the motifs themselves are still conserved. Northern blot analysis with a WSSV *dnapol*-specific riboprobe found a major transcript of 7.5 kb. 5' RACE revealed that the major transcription start point is located 27 nucleotides downstream of the TATA box, at the nucleotide residue A within a CAGT motif, one of the initiator motifs of arthropods. In a temporal expression analysis using differential RT-PCR, WSSV *dnapol* transcripts were detected at low levels at 2–4 hpi, increased at 6 hpi and remained fairly constant thereafter. This is similar to the transcription patterns for genes encoding ribonucleotide reductase, which is the key enzyme of nucleotide metabolism.[25,31] However, whether or not *dnapol* expression is regulated by viral transcription factors remains to be determined. Because of its potential importance in chemotherapeutics, the characterization of WSSV DNA polymerase should also be a high priority.

Other enzymes involved in nucleotide metabolism include ribonucleotide reductase (RR) subunits, thymidine-thymidylate kinase (TK-TMK), thymidylate synthetase and dUTPase. The genes for all of these enzymes seem to have been captured from eukaryotes directly or indirectly.[25,26,28,29] Two of these enzymes are key for the synthesis of the DNA precursors: RR for the *de novo* pathway and TK for the salvage pathway. The presence of the RR genes suggests that WSSV uses its own enzyme to reduce all four common ribonucleoside diphosphates in the *de novo* biosynthesis pathway of the deoxyribonucleotides.[25,87] The presence of WSSV RR allows the WSSV virus to attack cells even in the rest stage of the cell cycle (i.e. when the level of the host RR is very low).[91]

The WSSV *tk-tmk* gene has some unusual characteristics, and uniquely, it encodes a novel chimeric protein of 388 amino acid residues with significant homology to two proteins, thymidine kinase (TK) and thymidylate kinase (TMK). So far only the TK activity of WSSV TK-TMK has been demonstrated. WSSV TK activity data suggest WSSV TK is similar to eukaryotic cytosolic TKs in terms of the usage of phosphate donors, nucleoside substrate specificity and inhibitory activity of TTP. Unfortunately, this close overall similarity means that it may

be difficult to use WSSV TK as a target to develop a chemotherapeutic agent that acts specifically against WSSV.[94]

Viral TK appears to be a virulence factor of viruses *in vivo*, even though viruses that are deficient in TK can often multiply readily in cell culture (i.e. where resources are abundant).[113–115] TK and deoxycytidine kinase (dCK) are important because they phosphorylate T and dC, respectively, and if pyrimidine nucleosides such as T and dC are not phosphorylated, they could otherwise cross the cell-membrane and leak out into the surrounding medium.[116–120] WSSV does not have its own dCK, which suggests that the virus is using host cell dCK to prevent the leakage of dC. In most cell types, dCK is expressed constitutively and is thus available throughout the cell cycle; by contrast, TK activity is strictly S phase correlated.[121] Thus by expressing its own TK in infected cells, WSSV provides a mechanism for preventing wastage of the thymine-containing compounds that have been derived from the dTMP pool through dephosphorylation or DNA degradation. WSSV's inability to express dCK, on the other hand, may help to account for the tissues/cell types that WSSV targets: in mammals, some cell types (notably brain and muscle cells) do not express dCK, so if dCK is also not expressed in shrimp muscle, this may explain why WSSV does not seem to target muscle cells even though it attacks almost every other cell type.[94]

Conclusions

Considerable progress has been achieved in the identification of WSSV genes. In particular, the recent identification of virion structural proteins provides a foundation for studying the virus-host interaction. Future work can now focus on the molecular basis of how the structural proteins function. This should lead to an explanation of how WSSV enters the host cells, how WSSV proteins (PAMP; Pathogen-Associated Molecular Patterns) interact with host PRRs (Pattern Recognition Receptors), and the roles of these proteins in apoptotic responses and defense.[122] Meanwhile, it is also important to elucidate the contribution

of each WSSV structural protein to virion architecture because this may eventually help us to understand the role of WSSV structural proteins in the completion of the viral infection cycle.

Acknowledgments

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Chapter 7

Use of Functional Genomics to Identify and Characterize Virulence Factors of *Edwardsiella tarda*

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Introduction

In the world today, even as sanitation conditions and hygiene practices improve in many countries, public health remains a major issue of concern. Humans and animals by and large are still threatened by not only new infectious diseases but also re-emerging ones. Bacterial infections, one of the leading causes of diseases, are still plaguing mankind as well as animals, be they feral or domesticated. Research into bacterial diseases is thus imperative. The appearance of antibiotic-resistant strains is complicating the disease problem. Treatment can no longer be effected by sheer administration of antibiotics. Scientists must therefore seek to comprehend the pathogenic bacteria at the molecular level, finding out how their virulence genes act in concert to establish infection. It is only with this knowledge that effective preventive measures can be implemented, hence bringing humankind closer to conquering the battle against the microbes. Many new platform technologies in genomics and proteomics are accelerating the research and development in bacterial pathogenesis especially in medical microbiology. However, such technologies are not commonly used for the studies of fish pathogens. In this chapter, we are presenting the use of functional genomics to examine a less studied fish pathogen, *Edwardsiella tarda*. Using a genome-wide analysis, we have identified

more than 40 virulent genes that are important in *E. tarda* pathogenesis. Our studies will spin off the development of DNA and peptide chips for rapid and sensitive diagnosis, vaccine development, enhancement of food safety and quality control, and facilitation of the screening of new drugs against animal and human diseases.

Bacterial Disease and Aquaculture

Although a large number of bacteria are commensal flora inhabiting animals,[1] the "opportunistic" ones have the potential to cause infections under circumstances when the host is rendered susceptible, for instance when it sustains a mechanical injury or it is immunocompromised. In fact, many bacteria, such as the aeromonads, salmonellae, *Edwardsiella* and *Yersinia* species, are zoonotic affecting both humans and animals. Most investigations centered on the study of important infectious human pathogens using tissue culture and small animal infection models to gain insights into the mechanisms and principles of pathogenesis. Even though some of the infection model systems may not always reflect natural infection, the information gained is vital for the application of the appropriate treatment.

The study of human bacterial pathogen is albeit crucial, fish bacterial diseases also demands attention from the scientific community as fish, a good protein source for human sustenance, are being rapidly depleted in nature to feed the ever-increasing world population. Aquaculture is thus essential. In addition to food fish culture, the ornamental fish industry is also gaining importance as fish are being bred for aesthetic interest. In intensive fish farming systems, fish are stocked in high density, leading to problems of overcrowding and ease of disease transmission. The fish industry will be severely affected by disease outbreak and thus huge losses will be incurred. This has vast economic impact on the affected sites. Full exploitation of the potential of fish farming with minimal losses can be achieved only when there is a good general understanding of the interaction between pathogens and their fish hosts.

Platform Technologies to Study Pathogens

Various bacterial pathogens employ different strategies to overcome the host immune barriers to bring about successful colonization and infection. Dissection of the disease-causing mechanisms will facilitate the development of new vaccines and antibiotics to curb infections.

Proper methodologies must be adopted to study the strategies used by pathogenic microorganisms to cause infection. Some of the molecular and cellular techniques currently available for the identification and analyses of virulence factors include signature-tagged transposon mutagenesis (STM) for the detection of genes essential for survival in a host as each mutant carries a unique tag,[2,3] and subtractive hybridization for the comparison of complex genomes to locate genomic differences.[4–7] In addition, there is the differential fluorescence induction (DFI) procedure, essentially a promoter trapping technique in which bacteria bearing random transcriptional fusions to the promoterless green fluorescent protein (GFP) can be sorted by a fluorescence-activated cell sorter on the basis of stimulus-dependent synthesis of GFP.[8,9] In this way, genes expressed under certain conditions can be identified. Furthermore, it is necessary to identify virulent genes that are specifically induced during infections. The *in vivo* expression technology (IVET) allows this to be achieved.[10] A library of random genomic fragments with a selectable marker required for survival in the host is created. Only those bacteria harboring a fusion with an active promoter will survive passage through the host. A new recombination-based IVET (RIVERT)[11] in which fusions are made to promoterless resolvase gene such as *tnpR* from Tnγδ[12] is subsequently developed for the identification of low or transiently expressed *in vivo* induced genes.

With such platform technologies available, virulence factors of various pathogenic bacteria can be examined more closely and effectively. These technologies have been widely used in medical microbiology and have contributed to the rapid unravelling of the virulence mechanisms of many human pathogens. It is our goal to employ these cutting-edge technologies to study fish pathogens so that disease control in aquaculture can reach a greater height in the near future.

E. tarda and Its Hosts

The genus *Edwardsiella* was first reported in Japan by Sakazaki and Murata in 1962[13] and described by Ewing and coworkers in 1965.[14] It consists of three known species, namely *E. tarda*,[14,15] *E. ictaluri*[16] and *E. hoshinae*.[17] They are Gram-negative and exhibit characteristics typical of the Enterobacteriaceae family.[18]

E. tarda has a broad host range, affecting not only animals (fish,[19–22] amphibians,[23,24] reptiles,[25,26] birds[27,28] and mammals[23,29] but also infecting humans.[30–32] In fact, it is the only species in the genus that has been demonstrated to be pathogenic to man.

In humans, it mainly causes gastrointestinal diseases, but it has also been found to cause extraintestinal diseases such as myonecrosis,[33] peritonitis with sepsis,[34] bacteremia[35] and wound infections.[36,37]

E. tarda infections are usually associated with exposure to aquatic environments or exotic animals such as the amphibians and reptiles, pre-existing liver disease, conditions leading to iron overload and dietary habits like the ingestion of raw fish.[30,38] This bacterium is generally susceptible to most common antibiotics, but cases of fatal gastro and extraintestinal infections have been reported.[23,30] By seeking to learn more about this bacterium, we hope to shed light on how it brings about disease so that relevant preventive measures can be introduced.

Virulence Factors and Pathogenesis

The pathogenesis of *E. tarda* and its disease-causing mechanism remain largely unknown. However, several studies have identified a number of potential virulence factors associated with the pathogenicity of this bacterium. They include its ability to invade non-phagocytic cells such as HeLa,[39] HEp-2[40] and epithelioma papillosum of carp, *Cyprinus carpio*;[41] resistance to phagocyte-mediated killing,[42,43] and production of two hemolysins[44–46] and catalases.[47]

As compared to other infectious bacterial pathogens such as the salmonellae, *Yersinia* species and enteropathogenic *Escherichia coli*, *E. tarda*, being a relatively less common pathogen, is receiving less

attention and hence less well studied. Over the recent few years, our laboratory has devoted much time and effort to unravel the pathogenesis of this bacterium. The knowledge generated from our research is fundamental to the understanding of this pathogen not only from the viewpoint of fish diseases but it is also relevant in the human context. We thus sought to decipher its pathogenicity at the molecular level using the functional genomics approach.

Functional Genomics Approach to Study *E. tarda* Pathogenesis

Tn*phoA* Transposon Tagging

The first strategy we adopted is to use Tn*phoA* transposon tagging to identify virulence genes in *E. tarda*. This method dates back to 1985 when the Tn*phoA* transposon was described by Manoil and Beckwith.[48] Since most bacterial virulence factors are either on the cell surface or secreted for interaction with the host,[49] the use of this method allows for the preferential identification of genes encoding for outer membrane or extracellular proteins (ECPs). A library of 450,000 *E. tarda* Tn*phoA* mutants of the virulent PPD130/91 strain was created and 490 of them harbored active alkaline phosphatase gene fusions ($PhoA^+$).[43,50] Our initial screening using motility, serum resistance, production of catalase and siderophore, and stimulation of reactive oxygen intermediate in fish phagocytes have confirmed that the library of 490 $PhoA^+$ mutants were random in nature and non-redundant. We were able to isolate corresponding genes for particular phenotypes from the $PhoA^+$ library as long as a clear screening system was available and none of the mutants had identical insertions.

Subsequently, the 490 $PhoA^+$ mutants were screened using the fish infection model to identify genes involved in *E. tarda* virulence.[51] Mutants could be easily detected by their attenuation in blue gourami. Through this screening exercise, 15 mutants (encoding 14 genes) showed apparent diminution in virulence as indicated by the increased 50% lethal dose (LD_{50}) values of more than one log difference as

compared to that of the wild type. Sequence analysis showed that some mutants have disruption in genes homologous to virulence-related genes, namely, *fimA, gadB, katB, pstS, pstC* and *ssrB*, while others have insertions in genes (such as *orfA*) that are not known to be associated with pathogenesis. The inability of these attenuated mutants to multiply and disseminate and cause mortality in fish observed in *in vivo* infection kinetics studies, and the mapping of these genes to most virulent but not avirulent strains, served to augment the elucidation of the role of these genes in *E. tarda* pathogenesis.

Comparative Proteomics Analyses of *E. tarda* Proteins

In order to obtain a more comprehensive synopsis of the pathogenesis of *E. tarda*, it is important to study the bacterial pathogen using the proteomics approach as well. In this post-genomic era, genomic research alone could not suffice the understanding and knowledge of virulence. Therefore, examination of the protein complement becomes an integral aspect not to be overlooked.

As mentioned earlier, since most bacterial virulence factors are either on the surface or secreted,[49] when the proteomics approach was first employed, it was used to study the ECPs of *E. tarda*. An initial comparison made between the ECPs of virulent and avirulent *E. tarda* strains using one-dimensional polyacrylamide gel electrophoresis (1-DE) showed most virulent strains produce major protein bands, which were absent or diminished in the avirulent ones.[52] Two representative virulent (PPD130/91) and avirulent (PPD125/87) strains were selected and two-dimensional polyacrylamide gel electrophoresis (2-DE) was used to further resolve their ECPs.[52] Nine protein spots that were unique to the virulent strain, were excised, digested with trypsin and subjected to matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and nanoflow electrospray ionization (ESI) tandem mass spectrometry (MS). Based on the mass spectra pattern obtained, the nine spots were categorized into three groups. Using Edman N-terminal

sequencing, they were identified as the *sseC, sseD* and *sseB* homologs of *Salmonella*. These three proteins form the translocon structure of the type III secretion system (TTSS) encoded for by the *Salmonella* pathogenicity island 2.[53] The presence of the genes, encoding for these putative proteins in *E. tarda* was confirmed by PCR amplification and DNA sequencing.

Similarly, in another comparative proteomics study of ECPs and whole cell proteins from *E. tarda* PPD130/91 wild type and five of its attenuated Tn*phoA* mutants revealed four of the ECPs homologous to the *Salmonella* SseB, SseC and SseD proteins (EseB, EseC, EseD: ***E****. tarda* **s**ecretion system **e**ffector proteins) and the *E. ictaluri* putative 17.8 kD protein (EvpC, ***E****. tarda* **v**irulence **p**rotein), and one of the cellular protein spots homologous to the putative 19.5 kDa protein of *E. ictaluri* (EvpA)[54] to be unique to the wild type.

In both studies, TTSS proteins were identified in the ECPs. Although TTSSs are supposedly contact-dependent[55] secretion systems specialized for the delivery of virulence factors into host cells,[56] environmental cues such as temperature, growth phase and salt conditions are also able to induce the expression of the secretion apparatus and effectors in various pathogens.[57–59]

As for the Evp proteins, homology comparison of *evpA* and *evpC*, showed similar gene clusters of unknown function to be widely distributed in other pathogens such as *Rhizobium leguminosarum*, *Salmonella typhimurium* and *Yersinia pestis*.[54] ImpB and C in *R. leguminosarum*, which are EvpB and EvpC homologs respectively, are involved in temperature-dependent protein secretion system required for infection.[60]

In *E. tarda* PPD130/91, the expression of both the TTSS and the EvpA and EvpC proteins were also found to be regulated by temperature as indicated by the dramatic decrease in production level at elevated temperature (37°C).[54]

In order to establish the involvement of these two systems in *E. tarda* virulence, the gene clusters of the TTSS and *evpA-D* were sequenced, insertional mutants were created and their LD_{50} values in blue gourami were determined.[54,61] Mutants with disruptions in the

esrA and *esrB* (***E. tarda*** **s**ecretion system **r**egulator proteins) were highly attenuated while mutations in other TTSS gene members did not affect virulence significantly.[61] On the other hand, *evpA*, *evpB*, and *evpC* knockout mutants appeared to be attenuated with two log decreases in virulence.[54] These results are indicative of the probable roles of the TTSS and the *evp* operon in *E. tarda* pathogenesis.

The proteomics approach as well as transposon tagging employed in our studies as described above permitted the identification of more than 40 putative virulence-associated proteins in *E. tarda* and some of the genes are showed in Table 1. Since this bacterium is heavily lacking in genomic information, the protein identities obtained through the comparative analyses of proteomes provided a useful stepping stone for us to move into *E. tarda* genomics. The identification of a TTSS and Evp gene cluster in *E. tarda* for the first time certainly offers greater insight into the pathogenesis of this bacterium. Since TTSSs play important roles in virulence in many pathogenic bacteria,[62] there is a need to establish the function of this similar system in *E. tarda*.

Table 1 Virulence associated genes of *E. tarda* PPD130/91 identified and characterized using functional genomic approach.

Virulence gene	Putative or known function	LD_{50}
pstC	Peripheral membrane protein C	$>10^8$
pstB	ATP binding protein B	$>10^8$
pstS	Phosphate binding protein	$>10^8$
orfA	No homology	$10^{7.7}$
fimA	Fimbrial protein precursor	$10^{6.3}$
katB	Catalase precursor	$10^{6.6}$
evpA	*E. tarda* virulence protein A	$10^{6.9}$
evpB	*E. tarda* virulence protein B	$10^{6.9}$
evpC	*E. tarda* virulence protein C	$10^{6.8}$
eseB	*E. tarda* secretory system effector B	$10^{6.0}$
eseC	*E. tarda* secretory system effector C	$10^{6.1}$
eseD	*E. tarda* secretory system effector D	$10^{6.1}$
esrA	*E. tarda* secretory system regulator A	$10^{7.6}$
esrB	*E. tarda* secretory system regulator B	$10^{8.2}$

Overview of *E. tarda* Infection

Our earlier research attempt to map the portal of entry of *E. tarda* into the fish host using GFP-tagged bacteria in an immersion challenge model, followed by histological and infection kinetics studies, revealed the gastrointestinal tract, gills and the body surface to be the sites of entry.[63] This information, coupled with the more recent experimental data acquired using the functional genomics approach, allowed us to map the *E. tarda* infection pathway more comprehensively by assigning gene(s) that are likely to be responsible at each stage of the infection process.

To initiate an infection, the bacterium must first "bind" itself to the host surface. One of the presumed binding factors used by the bacterium to adhere to the host could be the fimbrial protein (*fimA*).[51] Upon binding and internalization, the bacterium will find itself faced with the hostile environment of host defences such as serum and phagocytes. Hence, genes required for neutralizing these effects (*gadB* and *katB*) must come into action. Some of these genes are regulated by temperature, for example *esrB* and *evp*.[54,61] When the putative TTSS, presumably required for the survival in phagocytes and virulence, is turned on at the appropriate environmental signal (at the lower temperature within the fish host) by the *esrA* and *esrB* two-component regulatory system, the bacterium can effectively overcome the host immune killing action and exert its virulence effect. As infection progresses, the bacterium needs to acquire nutrients within the host for growth and proliferation. Both the *pst* genes and *astA* can thus serve the function of phosphate and iron uptake respectively within the host. The *pstSCAB-phoU* operon belonging to the pho regulon[64] and the *esrB* may in addition play more sophisticated regulatory roles on other virulence genes, allowing the bacterium to successfully colonize the host organism, disseminate and bring about infection, finally killing the fish host.

Conclusions and Future Perspectives

We have presented here a simplified overall infection pathway tracing the probable route of *E. tarda* entry to its establishment within the

host, leading to disease and eventual death of the organism. The functional genomics approach, encompassing both the genomics and proteomics aspects, adopted to dissect the pathogenesis of *E. tarda* has undoubtedly provided much insight into this relatively less well-studied bacterium. Since many of the genes identified are specific to the pathogenic strains, they can be used for diagnostic purposes.

Further and more-in-depth study of the function of each of these virulence genes will facilitate the elucidation of the virulence strategy of this bacterium and its interaction with the host organism. This knowledge will pave the way for the development of new therapeutics as well as immunoprophylactic measures to prevent infection and hence disease outbreaks.

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Chapter 8

Use of Genomics and Proteomics to Study White Spot Syndrome Virus

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Introduction

In the last two decades, large-scale shrimp farming has arisen sharply due to huge consumer demands.[1] In the year 2000 alone, the world's shrimp production totalled 4,168,400 tons and more than 30 species were cultured. However, shrimp diseases, especially those caused by viruses, present the biggest threat to the shrimp farming industry.

Since *Baculovirus penaei* was first discovered in 1974, approximately 20 shrimp viruses have been reported in cultured penaeid shrimp. Among them, white spot syndrome virus (WSSV), considered to be a new virus,[2] is the causative agent of a major pandemic that has led to severe mortalities of cultured shrimps worldwide.[3] The virus, first appearing in the early 1990s in Taiwan, has spread rapidly resulting in huge economic losses. Most organs and tissues of shrimp, except for hepatopancreatocytes and epithelial cells of the midgut, can be infected by WSSV, which is obviously manifested as white spots on the shrimp's cuticle.[4] The virus has a broad host range, including other invertebrate aquatic organisms, such as crab and crayfish.[5] Therefore, the virus is not only a major threat to the shrimp industry but also to the marine environment at large.

Many polymerase chain reaction (PCR), *in situ* hybridization and immunoassay-based methods were established for the early detection of WSSV.[6,7] In 1997, WSSV genomic DNA was first successfully purified from *Penaeus japonicus* shrimp.[8] The WSSV genome contains a 305 kb

double-stranded circular DNA, which has the capacity to encode 181 presumptive open reading frames (ORFs) of 50 amino acids or more.[9] However, in contrast with the best-studied insect baculoviruses, only a few WSSV genes have been reported.[10] The elucidation of the WSSV genome facilitates the proteomic analysis of the virus to rapidly and efficiently reveal the coding capacities of all the predicted ORFs. Several reports also focused on shrimp immune responses against WSSV infection. This chapter will review the studies on WSSV.

Pathology, Morphology and Detection of WSSV

Pathology of WSSV

White spot syndrome disease of penaeid shrimp is a serious ongoing epizootic in the shrimp growing countries.[11] The disease is caused by WSSV, the infection of which can reach a cumulative mortality of up to 100% within three to ten days in cultured shrimp.[11] The name of white spot syndrome virus or white spot baculovirus (WSBV) was first given because of the distinctive feature of white spots about 0.5 to several mm in diameter in the cuticle (which are abnormal deposits of calcium) of the acutely infected shrimp.[4,12] The formation of the white and cuticular lesions appeared to be related to the disruption of exudate transfer from epithelial cells to the cuticle via cuticular pore canals.[4] In the early reports on WSSV, other names such as systemic ectodermal and mesodermal baculovirus (SEMBV), rod-shaped virus of *Penaeus japonicus* (RV-PJ), penaeid rod-shaped DNA virus (PRDV) and hypodermal and hematopoietic necrosis baculo-like virus of *P. chinensis* (HHNBV) also appeared.[11]

Besides the characteristic white spots in the cuticle, histopathological features revealed by light and electron microscopy have also been extensively described for WSSV-infected shrimp.[13,14] WSSV, circulating ubiquitously in the hemolymph of infected shrimp, can infect most organs and tissues, except for hepatopancreatocytes and epithelial cells of the midgut, which are regarded as refractory tissues.[4] Upon infection by the virus, the infected cells are observed first in the stomach, gill

and cuticular epidermis of the shrimp, and subsequently in other tissues of mesodermal and ectodermal origins.[15] WSSV has an extremely broad host range. Almost all the species of penaeid shrimp are susceptible to its infection. Moreover the virus can infect other freshwater and marine crustaceans, including crabs, spiny lobsters and crayfish.[11]

Morphology of WSSV

The WSSV virion, non-occluded and elliptical to bacilliform in shape with double envelopes, consists of nucleocapsid, typically measuring 70–130 nm in diameter and 300–350 nm in length[2,14,16] (Fig. 1). A multifilament appendage is often seen attached at the narrow end in the purified virion,[16] which is excluded in the intact virion from the

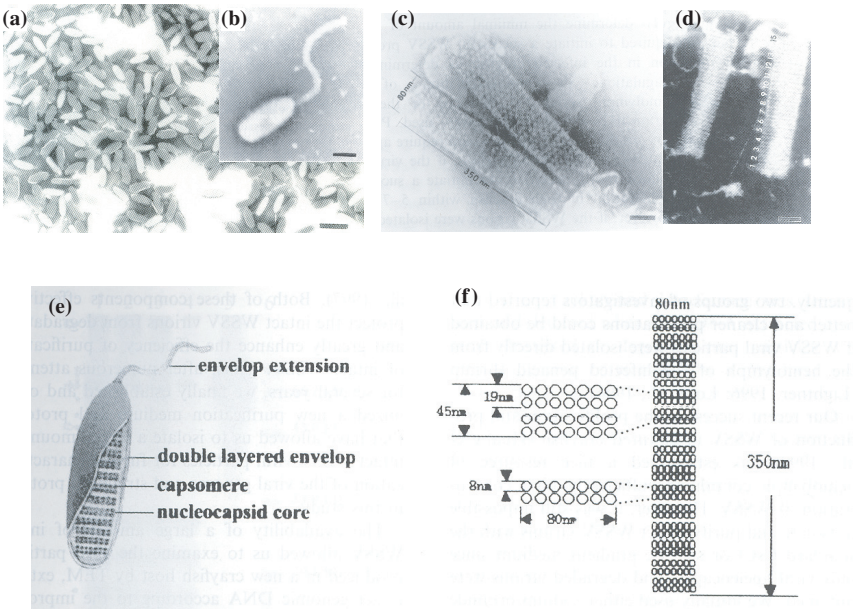

Fig. 1 Electron micrographs of purified intact WSSV virions (**a**, scale bar = 416 nm and **b**, scale bar = 104 nm) and nucleocapsids (**c**, scale bar = 50 nm, and **d**, scale bar = 54 nm), and the proposed WSSV particle structures (**e** and **f**).[16,46]

direct negative staining of WSSV-infected shrimp hemolymph.[13] The nucleocapsid, which contains a DNA-protein core bounded by a distinctive capsid layer, is wrapped often singly into an envelope to shape the virion.[14] The DNA replication and *de novo* envelope formation of WSSV take place in the nucleus.

Based on the analyses of WSSV-specific sequences and its structural proteins, it can be concluded that there is a genetic variation among WSSV isolates from different geographic locations.[11] The virion morphology, nuclear localization and morphogenesis of WSSV are reminiscent of the baculoviruses in insects.[14] However, the distinct biological properties highlight its uniqueness.[13] Inspection of the WSSV genome sequence further suggests that it is a new virus with unknown family classification.[17]

Detection of WSSV

In order to curtail the infection of this virus, highly sensitive, specific and efficient diagnostic tools are essential to detect WSSV in the early stage of shrimp. So far, diagnoses of WSSV are done by bioassays using indicator hosts, examination of clinical sign, histopathological assay, molecular biology method including gene probe and PCR and immunoassay. The latter two are most commonly used.

As one of the molecular biological approaches for diagnostic detection of WSSV, many WSSV-specific gene probes have been generated and used in dot blot,[18] *in situ* hybridization[18] and miniarray.[7] By *in situ* hybridization, the WSSV DNA-specific probe labeled with digoxigenin hybridizes with viral DNA located in nuclei of WSSV-infected tissue sections. *In situ* hybridization can identify the target organs of WSSV infection. This cannot be achieved by other molecular methods, for example, PCR detection. The miniarray method, similar to dot blot, was newly developed using DNA array technology.[7] The WSSV DNA fragments are amplified by PCR and arrayed on a nylon membrane. After hybridization with DNA-contained sample, the detection is performed by a colorimetric reaction using enzyme-linked antibodies, which produces a dark-blue precipitate with a substrate.

PCR, one of the most sensitive and efficient diagnostic tools for virus detection, was first successfully applied to the WSSV detection. So far, numerous PCR techniques for WSSV detection have been established. These include the conventional amplification with a single sense/antisense primer set,[18] nested amplification,[12] PCR combined with molecular beacon probe,[19] reverse transcription-polymerase chain reaction (RT-PCR),[20] competitive PCR,[21] real-time PCR[22] and quantitative PCR.[23] Nested PCR provides an increased level of sensitivity compared with conventional single primer-pair PCR. The lowest detection level is estimated to be 5 fg of WSSV DNA equivalent to approximately 20 viral particles on the basis of only a single copy of the target sequence for the primers.[12] The PCR combined with molecular beacon probe contains a fluorescein-labeled probe which possesses a stem and loop structure.[19] The amplified WSSV DNA is detected by its hybridization with the probe through fluorescence resonance energy transfer. The competitive PCR can be used for WSSV detection and quantification.[21] But the method is not suitable for detection of very low viral loads. A highly sensitive real-time PCR method was developed using SYBR Green as a fluorescence dye, which could detect a single copy of WSSV.[22] Upon binding to the minor groove of double-stranded DNA (dsDNA), the SYBR Green exhibits fluorescence enhancement that is proportional to the initial concentration of the template DNA. However, a higher background is often encountered. The quantitative PCR is the best tool to quantify WSSV copy number by comparing the amplified products with the internal control plasmid.[23] At present, many commercial products for PCR detection of WSSV are available.

The quantification of WSSV is critical for the development of a specific pathogen-free shrimp-breeding program, the screening of broodstock held in quarantine facilities, and the detection of viral pathogens in imported or exported shrimp. However, due to no suitable cell culture system available for shrimp viruses, the quantification of WSSV is now achieved by real-time PCR, competitive PCR or quantitative PCR.

In addition to gene probe and PCR diagnosis, immuno-detections have been developed to detect WSSV. The enzyme-linked

immunosorbent assay (ELISA),[6] dot-blot enzyme immunoassay[24,25] and Western blot[26] were successfully used for the WSSV detection. The primary antibody was raised against purified virions.[24–26] However, in general, immunization of purified virions generates high titer antibodies, but at a lower specificity. Several strategies were performed to increase the specificity, such as the monoclonal antibody (MAb),[24–26] the antibody against WSSV-specific expressed protein,[6] and the Fab fragment of IgG obtained by pepsin digestion.[6]

Genomics of WSSV

General Characteristics of WSSV Genome

The outbreak of WSSV infection in cultured shrimp has prompted the demand for elucidating the entire nucleotide sequence of the WSSV genome to further study the taxonomic position of WSSV, to allow a detailed understanding of the pathology of this virus in shrimp, and to understand the molecular basis of viral replication and infection. In 1997, the WSSV genomic DNA was first successfully purified from *Penaeus japonicus* shrimps in China and the estimated genome size was more than 290 kb.[8] Based on the genomic DNA libraries of WSSV, the entire nucleotide sequence was revealed by several groups.[17,27] A more detailed information of WSSV genome, such as gene transcriptions, is available from Yang *et al.*[17] Sequencing of WSSV genomic DNA reveals that it contains a 305 kb double-stranded circular DNA[17] (Fig. 2). This is different from 293 kb determined from a Thailand isolate of WSSV[27] (AF369029) and 307 kb from a Taiwan isolate of WSSV[28] (GenBank accession number AF440570). WSSV is the largest animal DNA virus sequenced to date.

The complete DNA sequence of the WSSV genome is assembled into a circular sequence of 305 kb in size (Fig. 2). The genome has a G + C content of 41% uniformly distributed over the genome. Three percent of the WSSV genome consists of nine homologous regions (*hrs*) dispersed throughout the genome with different sizes, while the remaining 97% of the sequences are unique.[17,27] The WSSV genome

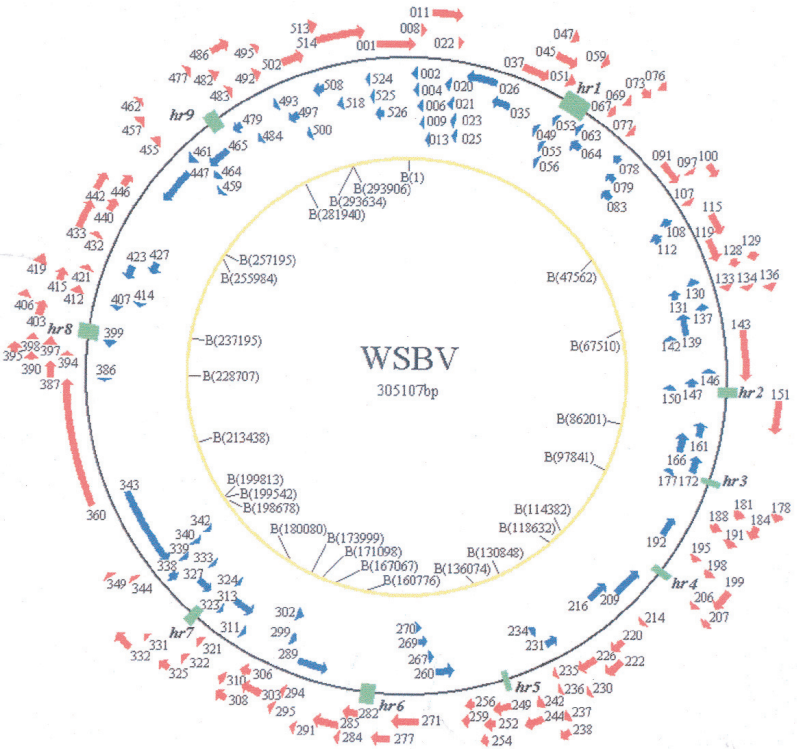

Fig. 2 The WSSV genome.[17] Arrows, positions of the 181 ORFs. Rectangles, 9 *hrs*. B, sites of BamHI restriction enzymes (their positions are in parentheses).

has the capacity to encode 181 presumptive ORFs of 50 amino acids or more, ranging from 61 to 6077 amino acids.[17] Since the origin of replication is unknown, a guanine residue from the beginning of the largest BamHI fragment is designated as the starting point of the physical map of the WSSV genome.[17] The ORFs are present on both strands in almost equal proportions (54% forward, 46% reverse). A TATA box sequence is found in the promoter regions of 46% of the WSSV ORFs. Consensus poly(A) signal sequence can be found for 80% of the ORFs.

ORFs with Potential Functions

Only ~30% of the WSSV ORFs have putative homologues to any known proteins or motifs, representing genes encoding enzymes for nucleotide metabolism, DNA replication and protein modification[17,27] (Table 1). The remaining ORFs are unassigned.

Many of the large DNA viruses encode a set of genes involved in nucleotide metabolism, DNA replication and repair. Genes coding for ribonucleotide reductase (WSV172 and WSV188), thymidine kinase (WSV395), thymidylate kinase (WSV395), thymidylate synthase (WSV067), dUTPase (WSV112), DNA polymerase (WSV514), DNA helicase (WSV447) and DNA-binding proteins (WSV214) are found in WSSV after homology searches (Table 1). The large and the small subunits of ribonucleotide reductase, which reduces ribonucleotides into deoxyribonucleotides as immediate precursors of DNA, were identified previously.[29] The two subunits, early transcribed, are located in proximity on the WSSV genome, separated by 5941 bp. A chimeric protein (early transcribed) consisting of a thymidine kinase (TK) and thymidylate kinase (TMK)[30] is a unique feature of WSSV, as these genes are normally encoded by separate ORFs in other large DNA viruses. The WSSV genome contains a highly conserved gene for thymidylate synthase, which catalyzes the methylation of dUMP to the nucleotide precursor dTMP, thus representing an important part of the *de novo* pathway of pyrimidine biosynthesis. A putative non-specific nuclease (WSV191) and three potential protein kinases (WSV083, WSV289 and WSV423) are also revealed. An ORF (WSV001), coding for a collagen-like protein with a typical repeat of Gly-X-Y (X is mostly proline and Y can be any amino acid), is found in the WSSV genome.[17] This is the first report of an intact collagen gene in a virus genome. Because of the homology with the class I cytokine receptors, an ORF-encoded protein (WSV220) is possibly involved in signal transduction related to the defense response system in shrimp. An ORF (WSV045) has 43% similarity in a 220-amino acid-long overlap with an *sno* gene of *Drosophila melanogaster*.[27] The *sno* product is part of a complex which negatively regulates transforming growth factor-b (TGF-b) signaling. This process is

Table 1 WSSV ORFs (modified from Yang *et al.* 2001).[17]

ORF	Position in the WSSV genome		Size		pI†	Characteristics of deduced proteins‡	Confirmed by the following methods§	Reference No.
	Start codon	Stop codon	aa*	Mass (kDa)				
WSV001	300501	445	1,684	186	9.4	Collagen, TM	MS, RT-PCR	
WSV002	1118	495	208	23	8.7	Nucleocapsid protein VP24, TM, SP	MS, RT-PCR	27
WSV004	1511	1200	104	12	9.6		cDNA	
WSV008	1749	2360	204	21	10	TM	RT-PCR	
WSV009	2672	2388	95	11	4.6		MS, RT-PCR	
WSV011	3051	6953	1,301	144	5.5	TM, SP	RT-PCR	
WSV026	13936	9332	1,535	172	6.2	TM	MS, RT-PCR	
WSV035	16983	14068	972	108	7.0	Cell attachment sequence, TM, SP	RT-PCR	27
WSV051	23710	24297	196	23	4.9		cDNA	
WSV056	25878	25201	226	26	4.5	Cys2/His2-type zinc finger	RT-PCR	
WSV059	26631	27254	208	25	8.8		RT-PCR	
WSV067	31092	31958	289	33	7.1	Thymidylate synthase	RT-PCR	
WSV069	32125	32796	224	25	4.8	Cys2/His2-type zinc finger	cDNA	
WSV073	32948	34213	422	47	4.8	TM, SP	RT-PCR	
WSV077	35074	35964	297	33	4.7	Cell attachment sequence, TM	RT-PCR	27
WSV078	37245	36052	398	45	9.6		RT-PCR	
WSV079	38917	37385	511	57	4.2	EF-hand calcium-binding motif	RT-PCR	
WSV091	42054	45488	1,145	126	4.8	Cell attachment sequence, TM	RT-PCR	
WSV100	45951	47822	624	69	5.1	Cys2/Cys2-type zinc finger, TM	RT-PCR	

WSV108	50300	49083	406	44	8.9	Membrane-associated protein, TM	RT-PCR	
WSV119	55055	58186	1,044	118	6.4		cDNA	
WSV129	58956	60026	357	39	10.3		MS, RT-PCR	
WSV130	60581	60132	150	17	8.0		cDNA	
WSV137	65042	64014	337	38	7.5	TM	MS, cDNA	
WSV139	68659	65036	1,208	138	6.0		RT-PCR	
WSV143	69265	76203	2,313	289	5.1	TM	cDNA	
WSV147	77653	76277	459	52	7.7		RT-PCR	
WSV151	79065	83372	1,436	161	5.4	TM	RT-PCR	
WSV161	85707	83431	759	85	5.6		RT-PCR	
WSV166	88980	85765	1,072	122	5.5	Cys2/Cys2-type zinc finger, TM	RT-PCR	
WSV172	91607	89064	848	96	7.8	Ribonucleotide reductase large subunit, TM	RT-PCR Northern blot	29
WSV178	93229	94134	302	35	4.5	TM, SP	cDNA	
WSV188	97548	98786	413	48	4.8	Ribonucleotide reductase small subunit, TM	RT-PCR Northern blot	29
WSV191	98854	99786	311	36	8.8	Nuclease, TM, SP	RT-PCR	
WSV192	102885	99829	1,019	117	8.1	TM	RT-PCR	
WSV199	104760	107327	856	98	8.2	Ring-H2 finger motif, TM	MS, RT-PCR	
WSV206	108550	109161	204	23	9.6		RT-PCR	
WSV207	109261	110085	275	32	7.9	TM	cDNA	
WSV214	115053	115292	80	9	12.6	DNA-binding protein	cDNA	41
WSV216	118987	115406	1,194	132	5.8	Protein-splicing signature, TM	RT-PCR	
WSV220	119057	121078	674	76	5.6	Class I cytokine receptor	MS, RT-PCR	27
WSV222	121100	123631	844	97	6.3	Ring-H2 finger motif, ATP/GTP-binding motif, TM	MS, cDNA	
WSV230	126755	127000	82	9	4.0		cDNA	
WSV231	129006	127162	615	71	7.1	TM	RT-PCR	

(continued)

Table 1 (*continued*).

ORF	Position in the WSSV genome		Size		pI†	Characteristics of deduced proteins‡	Confirmed by the following methods§	Reference No.
	Start codon	Stop codon	aa*	Mass (kDa)				
WSV234	130290	129409	294	34	5.5		RT-PCR	
WSV237	130566	131441	292	33	4.5	Envelope protein	MS, RT-PCR	Unpublished data
WSV238	131481	132938	486	51	4.6	TM, SP	RT-PCR	
WSV242	132994	133893	300	34	5.8	TM	MS, RT-PCR	
WSV244	133969	136341	791	90	8.9	TM	cDNA	
WSV249	137589	139937	783	89	7.2	Ring-H2 finger motif	MS, RT-PCR	
WSV252	140111	141613	501	56	5.2		cDNA	
WSV254	141696	142538	281	32	4.6	Envelope protein, cell attachment sequence	MS, RT-PCR	42, 27
WSV256	142545	143696	384	43	4.6	TM, SP	MS, RT-PCR	
WSV259	143760	144686	309	35	4.6		cDNA	
WSV260	147517	144752	922	103	4.4	TM	MS, cDNA	
WSV267	148612	147770	281	31	6.1		RT-PCR	
WSV277	154557	156929	791	87	6.3	TM	RT-PCR	
WSV282	159352	161253	634	69	5.5	SP	RT-PCR	
WSV285	161718	165017	1,100	123	5.1	ATP/GTP-binding motif, cell attachment sequence, TM	cDNA	27
WSV289	169814	165120	1,565	174	6.6	Protein kinase, TM, SP	RT-PCR	

WSV295	170832	171458	209	22	4.0	TM	cDNA	
WSV299	172439	171513	309	34	8.6	TM, SP	cDNA	
WSV303	173178	175850	891	100	9.2	Cys2/Cys2-type zinc finger, TM	MS, RT-PCR	
WSV306	175840	177096	419	47	5.5	TM	RT-PCR	
WSV308	177124	178521	466	52	7.2	Envelope protein	MS, RT-PCR	39
WSV311	180036	179425	204	22	9.3	Envelope protein VP26/P22, TM, SP	MS, cDNA	46
WSV323	185082	184819	88	10	4.2		cDNA	
WSV327	190743	188176	856	96	9.0	Cell attachment sequence, TM	cDNA	27
WSV332	190876	193233	786	88	6.4		cDNA	
WSV338	194629	193331	433	48	4.6	TM, SP	cDNA	
WSV343	209342	196803	4,180	467	6.0	TM	cDNA	
WSV360	209616	227846	6,077	664	6.7	Cell attachment sequence, leucine-zipper motif, TM	cDNA	27
WSV386	228196	227993	68	7	8.2	Envelope protein, TM, SP	MS, RT-PCR	Unpublished data
WSV395	231603	232796	398	43	6.3	Chimeric thymidine kinase, thymidylate kinase, ATP/GTP-binding motif	RT-PCR, Northern blot cDNA	30, 45
WSV403	236679	238601	641	74	6.7	Ring-H2 finger motif, SP	RT-PCR	
WSV406	238659	239435	259	30	6.6	TM, SP	RT-PCR	
WSV414	241637	241275	121	13	4.2	Envelope protein VP19, TM, SP	MS, cDNA	27
WSV415	241775	243406	544	62	7.1	TM	MS, RT-PCR	
WSV421	244242	244853	204	23	4.6	Envelope protein VP28, TM, SP	MS, cDNA	10
WSV423	247143	244954	730	82	9.3	Protein kinase, TM	cDNA	
WSV442	255075	257474	800	89	6.5	ATP/GTP-binding motif, TM	MS, RT-PCR	
WSV446	257552	259129	526	59	6.0	ATP/GTP-binding motif, TM, SP	RT-PCR	
WSV447	264975	259168	1,936	216	7.0	Helicase, ATP/GTP-binding motif Asp-protease motif, TM	RT-PCR	

(continued)

Table 1 (*continued*).

ORF	Position in the WSSV genome		Size		pI†	Characteristics of deduced proteins‡	Confirmed by the following methods§	Reference No.
	Start codon	Stop codon	aa*	Mass (kDa)				
WSV457	265606	266400	265	30	5.0	TM, SP	RT-PCR	
WSV465	272423	268695	1,243	138	6.0	Cys2/Cys2-type zinc finger, TM	RT-PCR	
WSV477	274527	275150	208	24	6.2	Cys2/Cys2-type zinc finger, ATP/GTP-binding motif	RT-PCR	
WSV479	276736	275210	509	58	5.5	TM	cDNA	
WSV482	277035	277571	179	19	5.9	TM	cDNA	
WSV484	278423	277776	216	25	9.0	TM	RT-PCR	
WSV489	281865	281131	245	28	5.2		RT-PCR	
WSV493	283360	282677	228	26	4.5	Nucleus-targeting protein	cDNA	28
WSV497	285773	284079	565	65	8.6	TM	cDNA	
WSV500	286706	286080	209	25	7.6	Cys2/Cys2-type zinc finger, ATP/GTP-binding motif	RT-PCR	
WSV502	286606	289632	1,009	113	8.2	Cys2/His2, Cys2/Cys2-type zinc finger, ATP/GTP-binding motif, TM, SP	MS, RT-PCR	
WSV508	291298	289685	538	62	8.3	TM	cDNA	
WSV514	292190	298774	2,195	245	6.9	DNA polymerase, TM	cDNA	
WSV526	300432	299089	448	50	5.2	TM	MS, RT-PCR	
WSV006	2425	1541	295	33	4.2	TM, SP		
WSV013	3955	3716	80	8.6	8.6			

WSV020	6604	6254	117	12	6.6	
WSV021	7645	7046	200	23	9.4	TM
WSV022	7250	7432	61	6.7	9.4	
WSV023	8502	7645	286	31	4.8	
WSV025	9248	8556	231	26	9.1	TM
WSV037	17000	20839	1,280	144	5.2	
WSV045	20784	23726	981	109	5.7	ATP/GTP-binding motif, TM
WSV047	21688	22047	120	14	11.7	
WSV049	22759	22145	205	22	9.6	TM, SP
WSV053	24906	24664	81	9	9.8	
WSV055	25153	24965	63	7	9.8	SP
WSV063	29077	28334	248	28	5.6	Cys2/Cys2-type zinc finger
WSV064	30861	29080	594	68	7.8	TM
WSV076	34218	35045	276	32	8.6	
WSV083	40718	38976	581	66	6.9	Protein kinase, TM
WSV097	45175	45471	99	11	11.6	
WSV107	48635	48943	103	11	4.6	TM, SP
WSV112	51809	50427	461	52	5.4	dUTPase
WSV115	52007	54910	968	108	6.3	TM
WSV128	58948	60057	370	42	10.7	
WSV131	62127	60676	484	53	4.8	
WSV133	62204	63016	271	31	4.8	TM
WSV134	62991	63656	222	25	6.5	
WSV136	63666	64049	128	15	9.0	TM, SP
WSV142	69118	68708	137	16	8.0	
WSV146	75119	74922	66	7	9.0	
WSV150	78365	77451	305	36	9.5	
WSV177	92964	92647	106	12	8.4	

(*continued*)

Table 1 (*continued*).

ORF	Position in the WSSV genome		Size		pI†	Characteristics of deduced proteins‡	Confirmed by the following by methods§	Reference No.
	Start codon	Stop codon	aa*	Mass (kDa)				
WSV181	94624	95739	372	43	7.1			
WSV184	95744	97366	541	63	9.0	Cys2/Cys2-type zinc finger, TM		
WSV195	103071	103841	257	29	6.7	TM, SP		
WSV198	103844	104677	278	31	6.7			
WSV209	114953	110136	1,606	174	6.3	TM, SP		
WSV226	123758	126547	930	108	7.4	TM		
WSV235	129611	129811	67	7.6	11.9			
WSV236	130076	130306	77	8.7	9.2	TM		
WSV269	150145	148679	489	56	8.8	TM		
WSV270	150675	150166	170	20	9.1			
WSV271	150688	154341	1,218	135	7.8	Cell attachment sequence, TM		
WSV284	161263	161562	100	11	4.8	TM, SP		
WSV291	167278	167532	85	9.3	4.5	TM, SP		
WSV294	170113	170730	206	23	5.5			
WSV302	173075	172509	189	21	5.1			
WSV310	178530	179345	272	31	7.8	TM		
WSV313	183817	180279	1,180	132	4.2	TM		
WSV321	184132	184482	117	13	9.4	TM, SP		
WSV322	184499	185179	227	26	8.8	TM		
WSV324	185434	185189	82	9	9.2			

WSV325	185433	186827	465	51	8.5	TM, SP	
WSV331	190094	190306	71	7.8	9.2		
WSV333	191135	190932	68	7.7	8.9	TM, SP	
WSV339	195503	194655	283	32	4.9		
WSV340	196292	195510	261	30	6.9	Cell attachment sequence	27
WSV342	196697	196398	100	12	11.3		
WSV344	197221	197517	99	11	8.9	TM	
WSV349	199510	199779	90	10	4.5	TM, SP	
WSV387	228375	230561	729	85	8.3		
WSV390	230617	231579	321	36	5.0		
WSV394	231422	231724	101	12	9.9		
WSV397	232819	233331	171	20	4.9		
WSV398	233383	233763	127	14	9.1	TM, SP	
WSV399	234330	233782	183	22	9.1		
WSV407	240139	239459	227	26	6.2		
WSV412	240713	241189	159	19	5.3		
WSV419	243217	243795	193	23	9.2		
WSV427	249230	247362	623	70	7.5	EF-hand calcium-binding motif, TM	
WSV432	249151	249456	102	11	5.8		
WSV433	249426	253208	1,261	142	9.0	TM	
WSV440	253297	255117	607	67	7.6		
WSV455	265079	265597	173	19	11.9	TM, SP	
WSV459	266838	266446	131	13	11.2	TM	
WSV461	267400	266930	157	18	7.8		
WSV462	267399	267647	83	9.8	10.4		
WSV464	268584	267721	288	33	6.6		
WSV483	277705	278076	124	15	4.7		

(*continued*)

Table 1 (*continued*).

ORF	Position in the WSSV genome		Size		pI†	Characteristics of deduced proteins‡	Confirmed by the following by methods§	Reference No.
	Start codon	Stop codon	aa*	Mass (kDa)				
WSV486	278637	280973	779	91	9.5	TM		
WSV492	282176	282583	136	16	5.8			
WSV495	283754	284011	86	10	5.2			
WSV513	291720	292202	161	18	8.5			
WSV518	293724	293275	150	17	10.9	SP		
WSV524	298729	298526	68	7.8	10.1			
WSV525	299033	298821	71	8	4.7	TM, SP		

*aa: Amino acids.

†pI: Predicted isoelectric point.

‡TM: Transmembrane domain, SP: signal peptide.

§The encoding capacities of ORFs are confirmed by RT-PCR, Northern blot, cDNA sequencing and (or) mass spectrometry.

important in mediating inflammatory and cytotoxic reactions. As not much is known about the shrimp immune system, the presence of a putative *sno* gene in the WSSV genome cannot be fully explained, but might be involved in abrogating the host defense response. After analyses for the presence of putative transmembrane domain (TM) and signal peptide (SP) sequences, one or more TMs are found in 95 ORFs and SPs located in 38 ORFs (Table 1). Two ORFs (WSV079 and WSV 427) contain an EF-hand calcium-binding domain, suggesting that their products may belong to the class of the calcium-binding proteins. However, most of the above-mentioned characteristics of ORFs are not yet confirmed by functional assays.

Forty-nine percent of 181 ORFs are transcribed and (or) expressed as proteins through the detection by RT-PCR, Northern blot, cDNA sequencing and mass spectrometry (Table 1), while the coding fidelities of the remaining ORFs are not known. On the basis of sequencing of WSSV cDNA library,[27] a bicistron, the only one in the WSSV cDNAs sequenced to date, was found in the transcript of an envelope-encoding gene (vp28) preceded by an in-frame minicistron.[10] Minicistrons, also found in the leading sequences of genes from other viruses, had effects on the expressions of the downstream genes.[31] However, their functions are not clear.

Microheterogeneity in WSSV Isolates

WSSV genome has been reported for different virus isolates: 305107 bp,[17] 292967 bp[27] and 307287 bp,[28] respectively. These size differences are mostly due to several small insertions and one large (~12 kb) deletion.[28,32] This indicates a certain degree of genetic instability of the WSSV genome. By examining the sequence of WSSV genome DNA in the shrimp *Penaeus japonicus, P. vannamei, P. monodon, P. chinensis, Metapenaeus ensis* and crayfish using successive PCR amplification of the DNA fragments in the whole genome, a sequence deletion hotspot ranging 277,566–285,714 bp in the genome is found.[32] This deletion region, usually related to reduced viral infectivity, low replication activity, reduced cell fusion activity and reduced virulence,

contains five ORFs, one of which is predicted to encode a nucleus-targeting protein.[28] The presence of AT-rich sequences at the deletion junctions may facilitate the recombination.[32]

Proteomics of WSSV

With the completion of the WSSV genomic sequence, it is natural that the research at the molecular level is now focused on the biological properties of the gene products. Essential to this functional analysis is to identify the WSSV proteins by the proteomic approach. To this end, mass spectrometry (MS) has proven to be the most effective technology for the identification of proteins because of its high throughput and sensitivity.[33,34]

Proteome/Proteomics

In 1995, a new term "proteomics" was first coined by Marc Wilkins who derived it from *prote*in complement of a gen*ome*.[35] Proteomics is the study of the entire protein complements expressed by a cell or tissue or a whole organism. The study includes not only the identification and quantification of proteins, but also the determination of their subcellular localization, modifications, interactions, and ultimately, their functions.[36] At present, proteomics, usually combining protein gel electrophoresis with a high throughput mass spectrometer coupled with bioinformatics, has provided a powerful means to identify proteins and has become an indispensable tool for large-scale and high-throughput protein analyses in the post-genome era.

Two-dimensional gel electrophoresis (2-DE), one of the methods used for protein separation, can resolve complex protein mixtures first by isoelectric point and then by size.[37] The powerful resolution of 2-DE makes it a useful technique to identify post-translationally modified proteins. However, low-abundance, hydrophobic, highly insoluble, very basic, as well as very small and very large, proteins are difficult to be detected by this method.[33]

The demand to characterize gel-separated proteins and the limited sensitivity of automated Edman sequencers have fostered an increasing use of MS for protein characterization. Now, MS has been widely recognized as a cornerstone of proteomic research. The most commonly used MS approaches are peptide mass mapping and tandem MS of a proteolytic digest of proteins.[37] Typically, after the in-gel tryptic digestion of interest protein,[38] the masses of the resulting peptides are measured by matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) MS. These masses, the so-called peptide mass fingerprint (PMF), can be compared to peptide mass sets obtained from theoretical protein or genomic databases to help characterize proteins. MALDI-TOF MS is relatively simple to use, less time consuming, high mass accuracy and reasonably tolerant of contaminants and solvent.[33] Alternatively, nano-electrospray ionization tandem mass spectrometry (nano-ESI-MS/MS) utilizing a quadrupole time-of-light (Q-TOF) mass spectrometer can be performed. In the tandem MS, the digested peptide mixtures are first scanned and particular peptides are fragmented during a second step to generate amino acid sequence information ("sequence tag"), which provides a highly specific probe for sequence database searches.[36,37] MALDI-TOF MS and nano-ESI-MS/MS are two complementary MS methods used for proteomic analysis. Each of these MS methods can be used independently, but when the high-throughput MALDI-TOF MS analysis is combined with the sequence specificity of nano-ESI-MS/MS analysis, identification of unknown proteins from database searching is greatly facilitated. MALDI-TOF MS is an ideal first pass analysis due to its high throughput and the easier sample preparation and data process.

Proteomic Analysis of WSSV

Based on the separation of the proteins from the purified WSSV virions by SDS-PAGE, more than 20 bands ranging from 5–190 kDa were visible with Coomassie blue staining (Fig. 3a).[39] Following trypsin digestion of the gel-excised proteins, the peptides were analyzed by MALDI-TOF

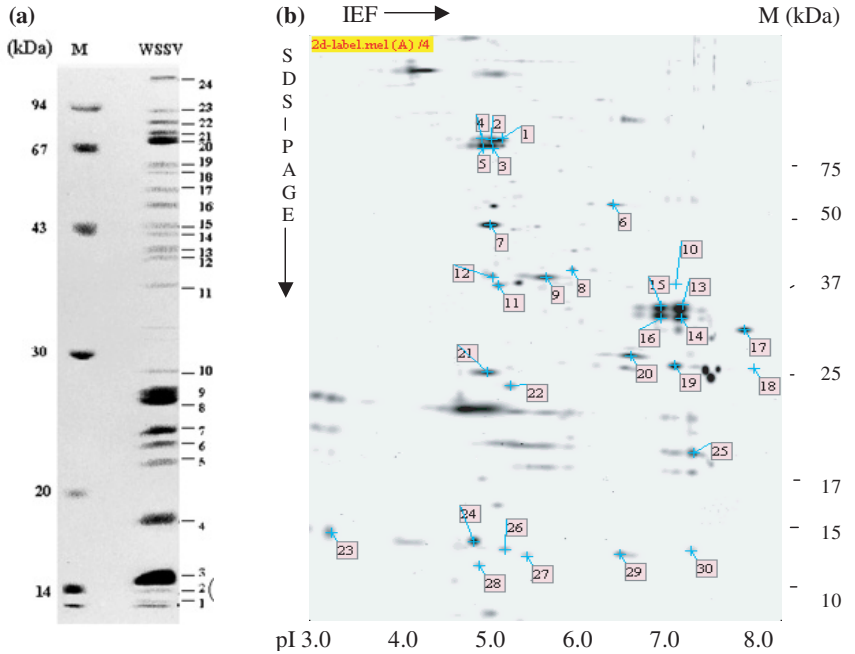

Fig. 3 (**a**) SDS-PAGE of proteins from purified WSSV virions (Coomassie blue staining). Numbers indicate excised bands for mass spectral analyses. (**b**) 2-DE of proteins from purified WSSV virions. The protein samples were separated in the first dimension by IEF, and subsequently in the second dimension of 12% SDS-PAGE, followed by silver staining. The basic side is on the left, and the acidic side on the right (M: protein marker).

MS and nano-ESI-MS/MS, respectively. Searches against the theoretical WSSV ORF database identified a total of 18 proteins of WSSV (Table 2a). This study also shows a gene-protein band pattern of WSSV based on SDS-PAGE. Due to post-translational modifications or protein interactions, the same protein could be found in different bands for several proteins. On the other hand, different WSSV proteins were revealed in the same band. In an attempt to further separate the proteins from the purified WSSV virions, the proteins were separated by 2-DE in our subsequent experiment (unpublished data). More than 60 polypeptide spots could be detected by silver staining (Fig. 3b). After tryptic in-gel digestion and MS analyses (MALDI-TOF MS and nano-ESI MS/MS), nine

2-DE spots were identified from the WSSV ORF database (Table 2b). In total, 25 WSSV proteins were identified by combining SDS-PAGE and 2-DE with mass spectrometry. The results show that the proteomic approach is one of the most efficient and sensitive ways for discovering the viral proteins and their corresponding genes.

The transcripts of all 25 viral genes, newly retrieved by MS, were detected at different stages post-infection with WSSV by RT-PCR (Table 2). This further confirms the coding fidelity of the 25 WSSV ORFs. The viral proteins are traditionally divided into three temporal classes: the early proteins (synthesized before five to six hours post-infection), the intermediate proteins and the late proteins (synthesized from five to six hours post-infection onwards).[40] Based on the temporal transcription analysis, all the 25 genes were transcribed after six hours post-infection, suggesting that the genes are expressed in the late course of WSSV infection (Table 2). Because the proteins used for the proteomic analysis in our studies were from the purified WSSV virions, principally comprised of the structural proteins encoded by the late genes, the early regulative proteins and the secretory proteins as well as the low-abundant proteins were not detectable by Coomassie blue or silver staining. Thus a general observation we make is that the proteins from the purified WSSV are mainly the late proteins.

Characterizations of WSSV Genes

Many WSSV genes have been revealed by DNA and cDNA sequencing, protein N-terminal sequencing and proteomic approach.[17,27,29,41,42] However, the subsequent gene characterizations, at present, are handicapped by the lack of suitable cell line to culture WSSV. Some reports showed that the lymphoid cells remained viable for more than one week in the primary cell cultures from the lymphoid organ of *Penaeus monodon* and the consequent cytopathic effects were documented by light and electron microscopy after inoculation with WSSV.[43] But this cell culture is inadequate for the functional analyses of WSSV genes.

Table 2 WSSV genes identified by mass spectrometry.

(**a**) Proteins of WSSV separated by SDS-PAGE.

Band no.	Gene	Position in WSSV genome		GenBank accession number	Characteristics of deduced proteins	Gene transcription p.i. (h)	Sequence coverage of MS*(%)	
		Start codon	Stop codon				MALDI	ESI
1	vp68	228196	227993	AF411464	Envelope protein	>6		19
2	vp95	2672	2388	AF402996	Not known	>6	72	
4	vp121	241637	241275	AF402997	Not known	>6	28	19
5	vp184	173178	173729	AF402998	Not known	>6	7	
6	vp24	1118	495	AF402999	Capsid protein	>6	61	33
7	vp26/p22	180036	179425	AF227911	Envelope protein	>6	44	48
8	vp28	244242	244853	AF308164	Envelope protein	>6	42	45
9	vp28						42	36
10	vp28							19
11	vp281	141696	142538	AF411634	Envelope protein	>6	27	7
12	vp28							12
13	vp300	132994	133893	AF403003	Not known	>6	24	
	vp292	130566	131441	AF411636	Envelope protein	>6	24	5
14	vp26							4
	vp357	58956	60026	AF403004	Not known	>6	20	
16	vp466	177124	178521	AF395545	Not known	>6	24	11
	vp384	142545	143696	AF411635	Not known	>6	12	8
18	vp448	300432	299089	AY048543	Not known	>6	9	

19	vp544	241775	243406	AY044842	Not known	>6	8	8
21	vp674	119057	121078	AY048545	Class I cytokine receptor	>6	9	
23	vp800	255075	257474	AY044843	ATP/GTP-binding motif	>6	18	3
24	vp1684	300501	445	AY048547	Collagen	>6	22	

(**b**). Proteins of WSSV separated by 2-DE.

Spot no.	Gene	Position in WSSV genome		GenBank accession number	Characteristics of deduced proteins	Gene transcription p.i. (h)	Sequence coverage of MS*(%)	
		Start codon	Stop codon				MALDI	ESI
2	vp844	121100	123631	AF493144	Not known	>6	7	
6	vp544	241775	243406	AY044842	Not known	>6	8	
7	vp507	10854	9332	AF493146	Not known	>6	7	
8	vp362	288547	289632	AF493149	Not known	>6	17	
9	vp387	145914	144752	AF493147	Not known	>6	13	
10	vp337	65042	64014	AF493148	Not known	>6	19	
11	vp281	141696	142538	AF411634	Envelope protein	>6	27	7
17	vp320	106368	107327	AF493150	Not known	>6	12	
18	vp208	1118	495	AF402999	capsid protein	>6		33
21	vp28	244242	244853	AF308164	Envelope protein	>6		19
25	vp216	139290	139937	AF493151	Not known	>6	20	
30	vp68	228196	227993	AF411464	Envelope protein	>6		19

*MALDI, MALDI-TOF MS; ESI, nano-ESI-MS/MS.

To date, only 11 WSSV genes have been characterized by bioassays. A basic peptide encoded by p6.8 gene (WSV214) has the capacity to bind DNA, indicating that it may be involved in WSSV DNA packaging.[41] The basic peptide is highly homologous to the DNA-binding proteins of insect baculoviruses rich in arginine and lysine. A protein encoded by vp35 (WSV493), a nucleocapsid protein with two potential nuclear localization signals, targets the nucleus of cell.[28] This protein may play a role in mediating the import of WSSV DNA into the nuclei of infected cells. Two subunits of ribonucleotide reductase (WSV172 and WSV188), identified previously,[29] are further characterized by enzyme activity assay.[44] In WSSV-infected hemocytes of shrimp, both of the subunit proteins (RR1 and RR2) are concentrated mainly around the nuclei, suggesting that WSSV ribonucleotide reductase is functionally involved during WSSV infection. An ORF (WSV395) probably encodes a unique chimeric protein of cellular-type thymidine kinase (TK) and cellular-type thymidylate kinase (TMK) as revealed before.[30] The recombinant TK-TMK protein catalyzes the phosphorylation of thymidine to thymidine monophosphate (TMP), but no evidence for further catalyzing the phosphorylation of TMP to thymidine diphosphate (or thymidine triphosphate).[45] Based on the immunoelectron microscopy, six genes [vp28 (WSV421), p22/vp26 (WSV311), vp68 (WSV386), vp281 (WSV254), vp292 (WSV237) and vp466 (WSV308)] were characterized to encode envelope proteins of WSSV in our laboratory.[10,39,42,46] Of the viral structural proteins, the envelope proteins play very important roles in virus infection, such as recognition and attachment to receptors in the host cell surface, as well as fusion with the host cell membrane during the virus assembly.

Shrimp Immunity

The intensification of shrimp farming has been accompanied by the occurrence of infectious diseases especially from viral origins.[47] WSSV is at present a major scourge to worldwide shrimp industry. In this context, the control of viral disease is important to ensure the long-term survival

of shrimp aquaculture. It is expected that basic research on immune processes will lead to a better understanding to provide improved strategies for disease prevention, including genetic selection of disease-resistant animals from domesticated stocks. However, in contrast to extensive studies on the morphology and genome structure of the virus, little work has been done on the defence mechanism of the host after WSSV infection.

Shrimp, like other invertebrates, lack a true adaptive immune response system.[48] However, living in an aquatic environment rich in microorganisms, shrimp have developed an effective non-specific innate immune response for detecting and eliminating noxious microorganisms. The cellular and humoral responses of shrimp, as known so far, concern hemocytes and non-specific molecules including phenoloxidase, bactericidins and lectins.

The defense systems of shrimp are largely based on the activities of hemocytes, including hemolymph coagulation, a rapid and powerful system that prevents blood loss upon wounding and participates in the engulfment of invading microorganisms.[49,50] A significant decline in free circulating hemocytes and higher numbers of granular hemocytes at tissue sites with many virus-infected cells have been observed after WSSV infection. Upon infected by virus, shrimp hemocytes leave the circulation and migrate to tissues where many virus-infected cells are present, suggesting that hemocytes are activated by WSSV infection. Hemocyte aggregation at infected sites is probably a general defence response. A decrease in total hemocyte count has often been described in crustaceans as a reaction after fungal and bacterial infection or after injection of foreign materials. However, the function of this hemocyte aggregation is unknown.

The documented non-specific molecules, to date, are phenoloxidase, bactericidins and lectins.[47,50,51,53] Several proteins, for example, antimicrobial peptides (penaeidins)[50] prophenoloxidas,[54] hemolymph-clotting protein,[55] syntenin-like protein[56] and lipopolysaccharide and glucan-binding protein,[53] have been characterized. The antimicrobial and antifungal peptides, generated from the C-terminus of hemocyanin, have been isolated from shrimp.[47,51] This showed that hemocyanin might be a non-specific molecule against invading microbes.

Summary

WSSV, bacilliform in shape with double envelopes, is the most economically important viral pathogen of farmed penaeid shrimp. Its 305 kb dsDNA genome has the capacity to encode 181 presumptive ORFs. To date, the transcriptions and expressions of 49% of WSSV ORFs are revealed. However, most of the presumptive proteins are still not identified and characterized due to the lack of suitable cell line to culture shrimp virus. Fortunately, many new methodologies are now available for WSSV studies. The high throughput strategies in analyzing gene transcription (DNA microarray) and protein activity (proteomics and protein chips) have provided the platform technologies in analyzing gene functions and screening drugs and biomarkers. The specificity and potency of RNA-mediated interference (RNAi), successfully used in the functional genomic analysis of nematode, makes it ideal for investigating WSSV gene functions and potential anti-virus strategies. Structural genomics, a new concept, will elucidate the function of the presumptive proteins based on the determined structures, provide many insight mechanisms of host-pathogen interaction, the maturation and assembly of the virus particles, and also provide the information necessary for drug targeting and design. To control the shrimp disease, the interaction between virus and host is crucial. However, in contrast to extensive studies on WSSV itself, little work has been done on the defence mechanism of the host against the virus. At present, except for the use of non-specific immunostimulants and low-density culture conditions, there is no effective control protocol for WSSV infection. In this context, the development of transgenic anti-virus shrimp promises to be another strategy to curtail the disease outbreak.

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Chapter 9

Virus–Host Interactions of White Spot Syndrome Virus

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Introduction

White spot syndrome (WSS), a most serious and devastating disease of cultured shrimp in the world, emerged in Taiwan in 1992 and rapidly spread to most major shrimp farming areas and wild shrimp populations in Asia, Central and South America and the southern states of the USA.[1–4] Due to intensive shrimp cultivation, inadequate sanitation and worldwide trade, WSS has quickly developed into an epizootic disease, causing large economic losses to the shrimp farming industry.[2,3] The broad host range of WSS is not only a major threat to shrimp culture but also to worldwide marine ecology.[3] In penaeid shrimp, WSS can reach 100% mortality within three to ten days after initial infection,[2] but in other crustaceans (e.g. lobster, crayfish and crab) the infection is not lethal, and therefore these species may serve as reservoirs and carriers of the disease.[5,6]

WSS is caused by a virus and in the past ten years or so, probably the same viral agent causing serious mortality among various populations of penaeid shrimp species was noted in the literature by several different names: white spot baculovirus (WSBV) from Taiwan in 1992,[7,8] hypodermal and hematopoietic necrosis baculovirus (HHNBV) and

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Chinese baculovirus (CBV) from China in 1993,[9,10] rod-shaped virus of *Penaeus japonicus* (RV-PJ) or penaeid rod-shaped DNA virus (PRDV) from Japan in 1993,[11,12] and systemic ectodermal and mesodermal baculovirus (SEMBV) from Thailand in 1993.[13] Currently, the majority of research groups now use the name white spot syndrome virus (WSSV). Originally, WSSV was classified as an unassigned member of the Baculoviridae.[14] However, based on its unique morphological and genetic features, WSSV was reassigned to a new virus family *Nimaviridae* (Nima = thread), consisting of a single genus (Whispovirus), and as of today white spot syndrome virus I as its sole species.[15]

WSSV Virion Morphology

Electron microscopy studies on thin sections and viral suspensions obtained from infected shrimp revealed that the virion of WSSV is an ellipsoid to bacilliform shaped, enveloped, non-occluded particle, which measures about 270 × 120 nm. Most notable feature is a tail-like polar extension at one end of the virus particle (Fig. 1). The nucleocapsid is cylindrical, about 300 × 65 nm in size and is formed by stacks of rings (about 14 in total), which are in turn arranged in two parallel rows of 8 nm in diameter of regular spaced globular subunits.[10,16,17]

WSSV replication occurs in the nucleus (Fig. 2) and early signs of infection are characterized by the appearance of hypertrophied nuclei and chromatin margination.[2,13,18] Virus morphogenesis is initiated by the formation of fibrillar, viral envelopes (about 7 nm thick) which are synthesized *de novo* in the nucleoplasm. This envelope has the structure of a trilaminar unit membrane. The formation of nucleocapsids begins with extended, empty, long tubules, which break up into fragments of 12 to 14 rings to form empty nucleocapsid shells. Subsequently, the empty capsids are surrounded by a loosely-fitted envelope leaving at one end an open extremity. The nucleoprotein, which has a filamentous appearance, enters the empty capsid through its open end. Mature virions are obtained after narrowing of the open end and formation of a tail-like extension of the envelope.[17–19] So far, the function and

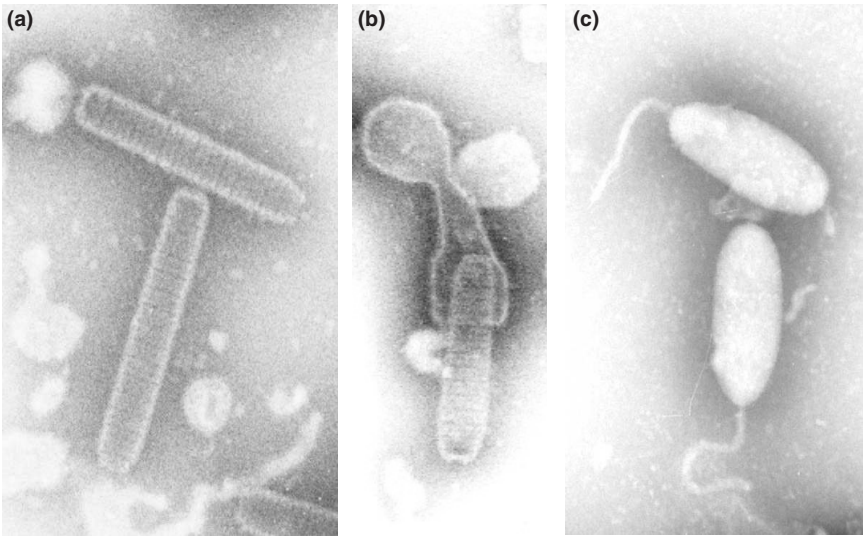

Fig. 1 Electron micrograph pictures of WSSV nucleocapsid (**a**), nucleocapsid with residual envelope (**b**) and virion with its characteristic tail-like extension (**c**).

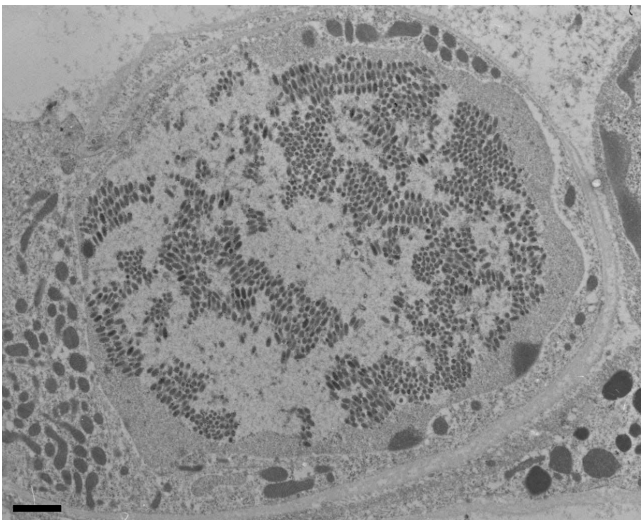

Fig. 2 Electron micrograph of hypertrophied shrimp cell nucleus containing WSSV virions (bar represents 1 μm).

composition of this tail-like extension is unknown. How the virions are released from the nucleus and infected cell, either by disruption or budding, is still an open question.

WSSV Virion Proteins

One-dimensional proteomic analysis,[20–24] using mass spectrometry, identified 18 proteins present in purified virions of WSSV (Table 1). The WSSV particle consists of five major structural proteins (Fig. 3), which have been named according to their sizes in SDS-PAGE. Viral protein (VP) 28 and VP19 are located in the virion envelope, while VP26, VP24 and VP15 are nucleocapsids proteins.[22] However, Zhang *et al.*[23] reported that VP26 (denoted as *p22* gene) is an envelope protein. It is possible that VP26 is a tegument protein, filling the space between envelope and nucleocapsids. Minor proteins VP644[22] and VP281[25] are also located in the virion envelope.[22] VP281 has a RGD motif, which is implicated as a host cell attachment domain[26] and has been shown to be critical for virus entry, e.g. foot-and-mouth disease virus and human parechovirus I.[27,28] It is speculated that VP50 has a specific role in assembling of the viral envelope. It is noted that a collagen-like protein (ORF30; Table 1) might be a minor structural protein of WSSV virions.[22] However, the reliability of these protein assignments depends on purity of the WSSV preparation used.

None of the five major structural proteins (VP28, VP26, VP24, VP19 and VP15), appeared to be glycosylated, which is an unusual feature among enveloped animal viruses.[22] VP15, a highly basic protein, has a high homology to histone proteins and therefore presumably bind to WSSV DNA, forming the nucleoprotein core.[22,29] VP28 is probably involved in the systemic infection process of shrimp, as a VP28 antiserum is able to neutralize the infection of WSSV.[30] Furthermore, Chen *et al.*[31] report the identification of another nucleocapsid protein (VP35), which includes a putative nuclear targeting signal (KRKR). It is hypothesized that VP35 is involved in the import of the viral DNA into the nucleus. However, the function of this protein is not essential

Table 1 WSSV virion proteins.

ORF[a]	Protein[b]	Size[c] (aa)	Size[d] (SDS-PAGE)	Location[e]	Motifs[f]	Transcription[g] (hours p.i.)
168	vp68	68	7	n.d.	TM, SP	18–36
109	n.dt (VP15)	80	n.dt. (15)	Nuc	TM	n.dt. (16)
34	vp95	95	11	n.d.		18
182	vp121 (VP19)	121	17 (19)	Env	TM, SP	6–24 (24)
149	vp184	184	22	n.d.	TM, TBP, Zn	30
31	vp208 (VP24)	208	24 (24)	Nuc	TM, SP	18–30 (24)
153	p22 (VP26)	204	25 (26)	Teg	TM, SP	18–36 (24)
1	p204 (VP28)	204	27.5 (28)	Env	TM, SP	18–36 (24)
127	vp281	281	35	n.d.	RGD	30
120	vp300	300	38	n.d.	TM	18
118	vp292	292	38	n.d.		24–48
75	vp357	357	41	n.d.	TM, Repeat region	36
151	vp466	466	50	n.d.	TM	24–30
128	vp384	384	50	n.d.	SP	24
29	vp448	448	55	n.d.	TM	24
183	vp544	544	60	n.d.	TM, MIP	36
112	vp674	674	76	n.d.	Cytokine receptor	24
6	vp800	800	90	n.d.	TM, ATP/GTP	36
30	vp1684	1684	180	n.d.	TM, Collagen	30

[a]ORF numbers, according to Ref. 33; [b]protein designation according to Ref. 23, in parenthesis according to Ref. 22, not detected (n.dt.); [c]protein size in amino acids (aa); [d]estimated molecular mass from SDS-PAGE, according to Ref. 23, in parenthesis, according to Ref. 22; [e]location of protein in virion, Nuc = nucleocapsid, Env = envelope, Teg = tegument, n.d. = not determined; [f]computer predicted motifs are indicated, transmembrane domain (TM), signal peptide (SP), TATA-box binding protein (TBP), Zinc finger (Zn), cell attachment sequence (RGD), MIP family signature (MIP), ATP/GTP-binding motif (ATP/GTP); [g]gene transcription in hours post-infection in *P. monodon* and in crayfish (in parenthesis).

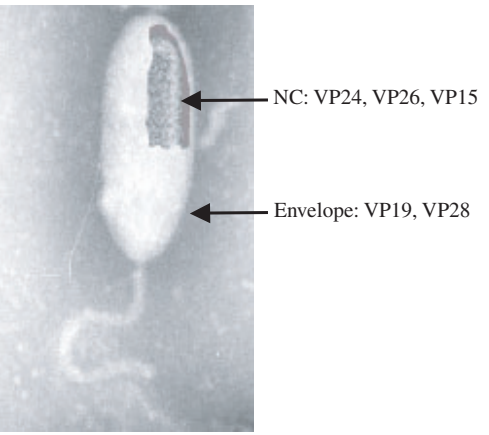

Fig. 3 Schematic representation of a WSSV virion, opened to show the nucleocapsid. Indicated are the locations of the five major structural proteins of which VP24, VP26 and VP15 are located in the nucleocapsid, and VP19 and VP28 in the envelope.

for virus replication, since the gene encoding this protein is absent in the WSSV-Thailand genome.[32]

WSSV Genomics

The virions of WSSV contain a singe molecule of circular, supercoiled, double-stranded DNA. After its first discovery, WSSV was taxonomically classified within the family of Baculoviridae, in the genus non-occluded rod-shaped viruses. This classification was based on virion morphology, morphogenesis and nuclear replication of WSSV. However, many characteristics of WSSV and its pathogenesis are quite distinct compared to the baculoviruses. Complete genome sequences of three WSSV isolates, WSSV originating from Thailand (WSSV-Th), China (WSSV-Ch) and Taiwan (WSSV-Tw), revealed a genome size of 293–307 kb (Genbank accession no. AF440570).[33,34] The genome contains nine homologous regions (*hrs*), dispersed along the genome, each containing a variable number of 250 bp tandem repeats.[33] *Hrs* of baculoviruses have been implicated in DNA replication[35,36] and as enhancers of viral

gene transcription.[37] Based on these observations and the fact that all large DNA viruses with circular genomes (i.e. baculoviruses, ascoviruses and WSSV) contain *hrs* dispersed in their genome sequences, it is tempting to speculate that the *hrs* of WSSV have a similar function in replication and transcription regulation.[33]

Computer-assisted analysis of WSSV-Th isolate identified 184 putative open reading frames (ORFs) of 50 amino acids and larger (Fig. 4). Only 12 of these ORFs show homologies to known genes in public databases, two protein kinases (*pk*),[38,39] DNA polymerase (*dnapol*),[33,40] collagen, thymidylate synthase, dUTPase, large (*rr1*) and small (*rr2*) ribonucleotide reductase,[41,42] endonuclease,[43] class I cytokine receptor, TATA box-binding protein, and chimeric thymidine-thymidylate kinase (*tk-tmk*).[44] These ORFs encode mainly enzymes involved in nucleotide metabolism, RNA biogenesis, DNA replication or protein modification. Eighteen ORFs do encode structural virion proteins (see section on "WSSV Virion Proteins"), whereas the remaining 155 ORFs are unassigned, since no homologies to known genes could be found. Unique features of WSSV are a giant ORF of 18.234 bp encoding a putative protein of 6077 amino acids, as of today with unknown function and an ORF encoding a collagen, also with unknown function.[33]

The three complete WSSV genome sequences available so far are quite similar with a nucleotide identity of over 99%. The major difference among the isolates is a 13 kb deletion (WSSV-Th) and 1 kb deletion (WSSV-Ch), which has occurred in the same genomic region compared to WSSV-Tw.[32] Also the total number of tandem repeats within the *hrs* varied between the isolates, whereas the location and sequences of these repeats were conserved. Furthermore, Marks *et al.*[32] identified variable repeat regions were almost exclusively located in ORFs, of which ORF75, ORF94 and ORF125 seem to be most suitable for PCR-based classification of WSSV isolates in epidemiological studies. The 54 bp repeats found in ORF94 have already been successfully used to study WSSV outbreaks in Thailand in 2000 and 2002.[45] Overall, the high uniformity in nucleotide sequences that was observed in the three isolates may suggest that a single WSSV species is responsible for its worldwide outbreak.[32,45] The *hrs*, variable repeats in ORFs, 13 kb

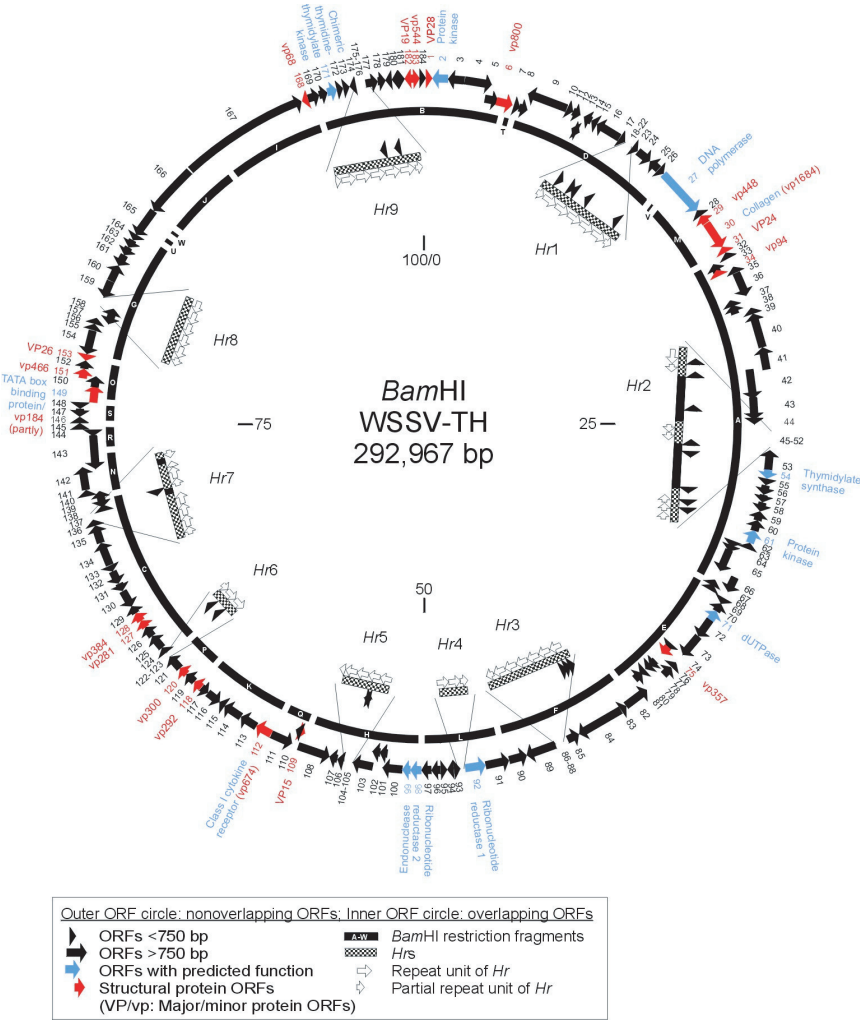

Fig. 4 Schematic representation of WSSV genomic organization. Shown are the ORFs larger than 150 bp and their direction (black arrows). ORFs with a predicted function, including the five major structural proteins identified, are indicated with blue and red arrows, respectively. Also the homologous repeat regions (dashed blocks) with their repeat units (white arrows) are indicated. The center circle shows the fragments found when the genome is digested with restriction enzyme *Bam*HI.

unstable region and one specific genomic region seem to be the genomic positions, which can be used for epidemiological, evolutionary and ecological studies.

The expression of the WSSV genes and viral DNA replication probably occurs in an ordered cascade of events, as is the case with most large double-stranded DNA (dsDNA) viruses. Gene expression is divided into two phases, an early phase that precedes viral DNA replication and a late phase that occurs as or after viral DNA replication initiates. Most eukaryotic DNA viruses rely on host RNA polymerase II for transcription of their genes, although baculoviruses, poxviruses and African swine fever virus encode a DNA-dependent RNA polymerase.[46] Viral gene expression is primarily regulated within the first 100 nucleotides or so upstream from its protein initiation codon (AUG). Transcription studies have been performed on WSSV early genes in *Penaeus monodon*, e.g. *rr1*, *rr2*,[42] *pk*,[38] *tk-tmk*[44] and *dnapol*,[40] and on WSSV late genes in grayfish gill tissue, *viz.* the major structural virion proteins, *vp28*, *vp26*, *vp24* and *vp19*.[47] *Vp15* was also included this study, however this protein seemed to be expressed earlier than the other structural virion proteins. Early genes were detected as early as two to four hours post-WSSV infection, whereas the late genes were observed at 18 to 24 hours and later post-infection. All identified 3'-termination sites of WSSV mRNA are in line with the polyadenylation sites appearing in eukaryotic mRNAs, which are typically located 15 to 25 nucleotides (nt) downstream of a poly-A signal, AAUAAA.[48] The transcription of the early genes (*rr, pk, tk-tmk and dnapol*) and *vp15* is initiated 20–28 nt downstream of a conserved TATA-box motif, suggesting a functional role for this sequence during early transcription. However, transcriptional analysis on the late genes did not reveal a consensus motif where transcription was initiated, except that all transcripts started about 25 nt downstream of an A/T rich sequence. The absence of a consensus motif for late gene transcription is quite distinct from gene regulation of many other eukaryotic DNA viruses, which suggests a unique regulation of late WSSV transcription. The lack of a convenient susceptible and reliable shrimp cell culture, however, impedes WSSV promoter as well as transcriptional *cis*- and *trans*-activation studies.

Pathology

Histopathological studies on WSSV-infected penaeid shrimp species have shown consistently that tissues of ectodermal (cuticular epidermis, fore- and hindgut, gills and nervous tissue) and mesodermal (lymphoid organ, antennal gland, connective tissue and hematopoietic tissue) origin are the prime targets for viral replication.[8,13,49] Tissues of endodermal origin (hepatopancreas and midgut) are not affected by the virus. Early in infection, the stomach, gill, cuticular epidermis and connective tissue of the hepatopancreas are WSSV positive. At later stages of infection, the lymphoid organ, antennal gland, muscle tissue, hematopoietic tissue, heart, stomach and hindgut also become positive for WSSV infection. The stomach, gill, cuticular epidermis, lymphoid organ, hematopoietic tissue and antennal gland are all heavily infected with WSSV at the late stage of infection and become necrotic.[50,51] Diseased shrimps are lethargic, have a lack of appetite and a reddish to pink body discoloration. WSSV-infected shrimp can be recognized by the appearance of white spots on the exoskeleton.[7] No penaeid shrimp species to date are known to be resistant to WSSV infection.[52]

Shrimp Defense Mechanisms

Penaeid shrimp possess a primitive open vascular system — the hemocoel. Their body fluid is known as hemolymph, since there is no separation between a lymphatic and circulatory or blood system. Hemolymph travels from the heart through a series of valved vessels to the organs, including lymphoid organ, and from there to the body cavities or interstitial spaces. After passing the gills, the hemolymph returns to the heart for distribution. The blood cells are known as hemocytes.[53,54] Three morphologically distinct types of hemocytes, i.e. hyalinocytes, granulocytes and semigranulocytes have been identified, based upon the quantity and sizes of their granules.[55–57] Van de Braak *et al.*[58] reported that upon WSSV infection of shrimp, the amount of free circulating hemocytes dropped and that they migrated to virus-infected tissues. The present

knowledge of the immune system of penaeid shrimp is very limited.

Shrimp possess an immune system that is quite different from vertebrate systems. Adaptive or acquired immunity is assumed to be absent in crustaceans, since they lack lymphocytes and specific antibodies. However, recent research suggests that the crustacean defense system may be capable of specific memory.[59,60] Shrimps also possess an innate immune system, which can be found in all multicellular organisms, and consists of cellular and humoral elements. Most research performed on the shrimp immune system has focused on bacterial defense reactions. The hemocytes play a central role in the non-specific immune response of shrimp, which rely mainly on phagocytosis, melanization, encapsulation, cytotoxity and clotting.[61] Humoral defense factors, such as clotting proteins, agglutinins, hydrolytic enzymes and antimicrobial peptides are released upon lysis of the hemocytes, which is induced by endotoxins and β-glucans.[62] All these factors are stored in the granules of the hemocytes and upon activation, they facilitate killing of the microbial invaders prior to phagocytosis or encapsulation. Once immobilized, the pathogen will be routed for ejection through the process of melanization. Melanized substances accumulate in or just under the cuticle.[63]

It has been suggested that upon viral infection, apoptosis may be another trait of a non-specific defense mechanism in shrimp.[64,65] Apoptosis or programmed cell death is a genetically regulated cell suicide mechanism that plays a critical role in development, tissue homeostasis and removal of diseased tissues of multicellular organisms. It is mediated by sequential activation of caspases (cysteine proteases), which finally lead to the death of cells. Many components of the apoptotic machinery are remarkably conserved in vertebrates as well as invertebrates. Apoptosis is characterized by membrane blebbing, cell shrinkage, chromatin condensation, DNA fragmentation and formation of apoptotic bodies. It has been proposed as the main antiviral mechanism in invertebrates.[66] Several reports showed the occurrence of classical signs of apoptosis in WSSV-infected shrimp, i.e. nuclear disassembly and increased caspase-3 activity in various infected tissues, e.g. lymphoid

organs, subcuticular epithelium and hematopoietic tissue and gills.[19,67–69] However, it is still not clear to what extent apoptosis contributes to shrimp mortality. Viruses often have a strategy in place to bypass or compromise the apoptotic response by expressing anti-apoptotic molecules.[70] More research is needed to understand the underlying mechanism of the shrimp host response in triggering apoptosis upon infection with WSSV.

Vaccination

The fast growth of the shrimp culture worldwide and intensive cultivation aggravated disease incidence and dissemination. WSSV is currently still the most serious threat of the shrimp industry, so, control of this viral disease is of vital importance for the future of shrimp farming. Although the presence and nature of a clear adaptive immune response in shrimp remains unclear, recent reports have shown that vaccination might be used to combat bacterial[71,72] and viral diseases.[60,73–75]

Non-specific antiviral substances are reported to circulate in the shrimp hemolymph[76] and it has been reported that some of these antiviral factors, i.e. defensive proteins, are upregulated upon WSSV infection of shrimp.[77,78] Venegas *et al.*[73] demonstrated the existence of a quasi-immune response in kuruma prawn *Penaeus japonicus* infected natural or experimentally with WSSV. A neutralizing activity was detected in hemolymph for up to 17 days after *in vivo* challenging, however, survivors of WSSV infection were still able to resist a WSSV rechallenging even four months after initial infection. Virus neutralizing activity in the plasma of *P. japonicus* surviving an experimental WSSV infection was examined.[74] The onset of "resistance" against WSSV developed about three weeks after the initial exposure to the virus and it lasted for another four weeks.

Shrimp vaccinated by intramuscular injection with purified envelope proteins showed protection against WSSV for at least 25 days after vaccination.[60] *P. monodon* vaccinated orally with subunit vaccines consisting of the WSSV envelope proteins have higher survival rates in

subsequent rechallenging experiments. Use of the envelope protein VP28 as a subunit vaccine gave a clear positive effect on shrimp survival (Fig. 5), whereas vaccinations with VP19 had no effect at all on shrimp survival after WSSV challenge *in vivo*.[75] Previous experiments already indicated that the envelope protein VP28 plays a key role in the systemic infection of shrimp, as it is possible to neutralize WSSV with antibodies directed against VP28.[30] The onset and duration of VP28 vaccination was also determined. A high survival rate was already observed as early as three days post-vaccination, and protection persisted up to 21 days after vaccination.[75] These experiments clearly indicate that penaeid shrimp are able to specifically recognize WSSV proteins and therefore may have some form of adaptive immunity. These results are in line with those recently obtained by Kurtz and Frantz[59] who demonstrated the existence of a specific memory in copepod. Since adhesion molecules (Ig super family) are present in invertebrates[79] it is possible that these

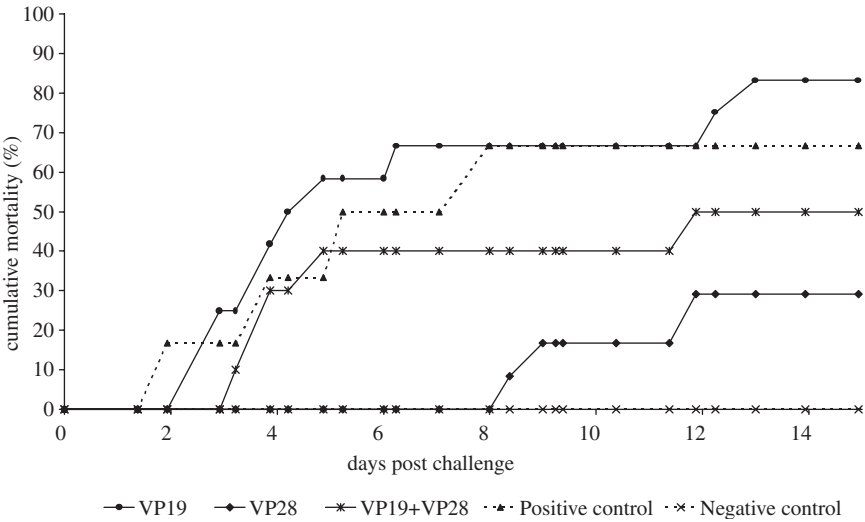

Fig. 5 Time-mortality relationship of a typical oral vaccination experiment. Cumulative mortality rates of shrimp from the experimental groups VP19 (□), VP28 (□) and VP19 + VP28 (□), Positive control (□) and negative controls (□) are plotted against the days after challenge.

molecules mediate invertebrate adaptive immunity.[80] However, none of these immunoglobulin molecules show signs of clonal rearrangements like the immunoglobulins of vertebrates.[81]

Perspectives

Although research into the genomics and proteomics of WSSV slowly progresses, some invaluable tools to study the mechanisms of the viral infection cycle still need to be developed. Detailed RNA and protein expression and gene transactivation studies, time-course analysis and generation of WSSV mutants all await the availability of a suitable, continuously growing shrimp cell line, which despite all efforts is not readily available. A plethora of proteins are present in the virus particle. It is important to understand their role in virion structure, their possible involvement in viral assembly and in the infection process of the host. More detailed information is needed about the entry and spread of WSSV into a host to design novel intervention strategies including development of vaccines. Identification of the host target cell for WSSV entry and subsequently identification of the required receptor molecules will lead to the design of new and presumably very effective ways to control viral infection. The observation that the shrimp immune system is able to specifically recognize WSSV envelope proteins can lead to new strategies to control WSSV and other invertebrate pathogens. From a host perspective, much will be learned from the host response to virus infection at the molecular level. Subtraction cDNA libraries in combination with micro-array technology will lead to the identification of host genes responding to viral infection. These can be used to monitor host responses under a variety of conditions and to aid, for example, in the selection of specific-pathogen resistant shrimp. Further information may become available, when a shrimp sequencing project is initiated and analogies with other virus-host systems can be derived. WSSV will remain a major threat of shrimp and other crustaceans for years to come. Studies on the ecology and evolution of WSSV are still in their infancy, but will have major impact when the effects of global warming on more temperate

ecosystems become clear. Studies on virus–host interaction will remain on the research agenda in the years to come.

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Chapter 10

Molecular Immunity in Fish–Pathogen Interactions

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Introduction

When a pathogen such as a virus or parasite invades a fish host, it elicits two major types of biodefense response: an innate immune response and an adaptive or acquired immune response.[1] Innate immunity serves as a first line of defense but lacks the ability to recognize certain pathogens and to provide specific protective immunity to prevent reinfection. Innate immunity of fish consists of mechanical barriers (e.g. scales and skin), humoral reaction (e.g. antibacterial peptides), antiproteases, complement, C-reactive proteins, lectins, lysozyme and proteases, transferrins, interferons, myxovirus resistance (Mx) protein, cell defense reaction (e.g. inflammation phagocytosis), and antiviral cytotoxic cells.[2] Acquired immunity, on the other hand, is relatively inactive until it is stimulated by a particular stimulant. Agents that stimulate acquired immunity are recognized as foreign by the immune system and are called immunogens or antigens. The acquired system is capable of exquisitely distinguishing among different microorganisms and significantly alters its intensity and response time upon re-exposure. Acquired immunity is further divided into humoral and cellular immunity. The acquired immune response is regulated by major histocompatibility complex (MHC) molecules at the surface of antigen-presenting cells (APC), surface immunoglobulin (Ig) molecules of B-cells and the T cell receptors (TCRs) of T-cells. MHC classes I and II, TCRs, Ig, TCR co-receptors CD4 and CD8 are all similar in

structure and are described as belonging to the "immunoglobulin superfamily." Expansion of effector cell populations of antibodies or cytokines, and development of long-term immunological memory are additional factors affecting acquired immunity.

MHC molecules are highly polymorphic in most vertebrate species.[3–5] The selection mechanism favoring extensive polymorphism is not yet clearly understood, but the most common view is that this phenomenon is related to the effect of allelic MHC molecules on the immune response and therefore on resistance to pathogens.[6–9]

The genes coding for immunoglobulin molecules are generated by gene rearrangement.[10–13] In addition, somatic mutation induces a further antibody sequence diversity allowing for an increasing affinity during the immune response. Numerous variable region (V) genes and gene families are involved in the multiepitope antigen response, although these responses may be genetically restricted in some instances.[14] In general, the assembly of DNA segments by recombination or gene conversion to form functional genes generates an enormous variety of different antigen receptors.[15]

TCRs are also generated by gene rearrangement and they recognize antigens via the TCR-CD3 complex.[16–20] T cells occur in two types, cytotoxic T cells (Tc, CD8+) and helper T cells (Th, CD4+).[21,22] Th cell responses (Th1 and Th2 types) are classified by measuring the type of cytokines that Th cells produce. When Th cells are involved in inflammation and helping Tc cells destroy a pathogen, they make a Th1-type response.[23–25] This involves producing one or more cytokines such as interleukin (IL)-1, IL-2, IL-6, IL-12, tumor necrosis factor (TNF)-α, TNF-β, and interferon (IFN)-γ, which stimulates Tc and more Th cells and could promote added inflammation and encourage an ever-increasing potent reaction against a pathogen.[26,27]

While there is substantial information on the different mechanisms involved in fish responses to invading pathogens, as well as on fish–pathogen interactions, little is known about the fish immune defense mechanism at the molecular level. In this chapter, we describe the different biodefense- and immune-related genes of Japanese flounder (*Paralichthys olivaceus*) that were identified using the expressed sequence tag (EST) approach. We also elucidated the expression patterns of these genes

employing quantitative "real-time" polymerase chain reaction (PCR) and an EST-based cDNA microarray analysis.

EST Analysis of Japanese Flounder

Expressed sequence tag (EST) analysis is a method for identifying new genes and isolating known, homologous ones in a short period of time.[28–30] We selected leukocytes for the EST analysis because of their roles in both specific and non-specific immunity.

We constructed a cDNA library of leukocytes from a cloned population of Japanese flounder infected with hirame rhabdovirus (HRV) to analyze the genes that are induced and expressed by the immune system after viral infection.[31] The cDNAs that were upregulated in hirame rhabdovirus (HRV)-infected Ig+ leukocyte cells of Japanese flounder were identified by a differential hybridization, using subtracted and unsubtracted cDNA probes.[32] We also screened EST clones from a subtractive cDNA library of Japanese flounder leukocytes which were induced by concanavalin A (Con A) and phorbol myristate acetate (PMA) and lipopolysaccharide (LPS), and from liver, spleen, skin and kidney of a cloned population of Japanese flounder.[33–35] Numerous kinds of biodefence-related peptides and proteins, including specific and non-specific antimicrobial agents, plus activators and regulators of the immune response were obtained.[36–42]

The biodefense- and immune-related genes isolated from Japanese flounder through ESTs are classified into six groups: cytokines/cytokine receptor group, plasma proteins, cell surface molecules, signal pathway molecules, apoptosis-related molecules and transcription factors (Table 1). The cytokine consist of ILs (IL-1β and IL-8), CC chemokine, Fas ligand, tumor growth factor (TGF)-β and TNF, while the cytokine receptor genes include CC chemokine receptor, IL receptors, TNF receptors, and others. Plasma proteins include complement, lysozyme, Mx protein, NK-lysin, perforin, etc. The cell surface molecules include cluster of differentiation (CD), Ig and TCRs, and other proteins. Signal pathway molecules, apoptosis-related molecules and transcription factors

Table 1 Cloned homologues of defense- and immune-related genes of Japanese flounder.

Cytokines	Plasma proteins	Cell surface molecules	Signal pathway molecules
B-cell activation protein BL34	Complement C1	CD3-γ/δ	Caspase-10
CC chemokine	Complement C3	CD3-ϵ	JAK3 tyrosine kinase
CXC chemokine	Complement C7	CD8-α	MAP kinase interacting kinase-1
Fas ligand	Complement C8-β	CD11-β	SAP90A
G-CSF	Complement C9	CD18	SH3P2
IL-1β	CRP proteins	CD20	TNFR2-TRAF SCP
IL-8	Lysozyme c-type	CD22	**Apoptosis-related molecules**
MIP-1α	Lysozyme g-type	CD49-ϵ	ANA, BTG-3 protein
MIII-1β	Mx protein	CD50	Apoptosis regulator NR-13
NK cell enhancing factor	NK-lysine	CD53	Apoptosis inhibitor RIAP-3
T-cell immune regulator-1	Perforin	CD63	**Transcription factors**
TGF-β	Thymosin-β4	CD83	BCL-3 (NF-kB)
TNF-α	Thymosin-β10	Fc-γ-1/γ-2 receptor	bZIP transcription factor Maf-A
TNF superfamily protein	Transferrin	IgD	C/EBP-β
Cytokine receptors		IgM	C/EBP-ε
CC chemokine receptor		Ig light chain κ	Early growth response protein-1
CXC chemokine receptor		Ig light chain-λ	Early growth response protein-2
G-CSF receptor		Kupffer cell receptor	ICSBP
IL-1 receptor, type II		Leukotriene β4 receptor	IRF-1
IL-6 receptor-b		Polymeric Ig receptor	IRF-4
IL-8 receptor		TCR-α	NF-IL6-β protein
TNF receptor-1		TCR-β	Immediate early gene
TNF receptor-2		TCR-γ	p55-C-FOS proto-oncogene
		TCR-δ	Transcription factor AP-1
		Toll-like receptor-2	Transcription factor JUN-B
		Toll-like receptor-3	

were also identified. Together, these results indicate that EST analysis is an effective tool for identifying and characterizing fish biodefense genes.[43,44]

Immune-related Genes of Japanese Flounder

Antigen Recognition and Presenting Molecules

T cell receptors

T cells exhibit multiple regulatory functions in the vertebrate immune response, such as effectors and as long-term memory cells.[45,46] Although T cells do not express surface Ig, they do express a variety of different cell surface receptors including the TCR for antigen recognition.[47] In mammals, T cells are classified into subsets by their functional differences, by the type of TCR chains present (α/β or γ/δ heterodimers) and by specific T cell coreceptors (CD4 or CD8).[21,22,48,49] The signal transduction pathway between the APC and the T cell — CD4 with Th cells and CD8 with Tc cells — are stabilizing factors in the TCR-MHC-peptide interaction. These coreceptors bind to the same MHC molecules as the TCR — CD4 binds to MHC class II molecules and CD8 binds to MHC class I molecules.[50]

All four TCR (α, β, γ and δ) cDNAs and their genes were identified in Japanese flounder.[34,51] This is the first and only report of all four TCRs in a non-mammalian organism. The Japanese flounder TCR-α and -δ genes are located at the same locus as the mammalian genes (Fig. 1). However, we found an isotype Cδ2 from a cDNA analysis and demonstrated its different gene locus by a BAC genomic DNA analysis. The flounder Cδ1 and Cδ2 show significant identity at the Ig-C domain, TM and CYT, but have different molecular sizes as a result of the presence of a connecting peptide region in Cδ2. Interestingly, the flounder Cδ1 and Cδ2 are located in different gene loci. Cδ1 shares the locus with Cα while Cδ2 is on the Cγ locus. This result is inconsistent with the genomic organizations of other vertebrates and may have significant implications regarding the evolution of antigen

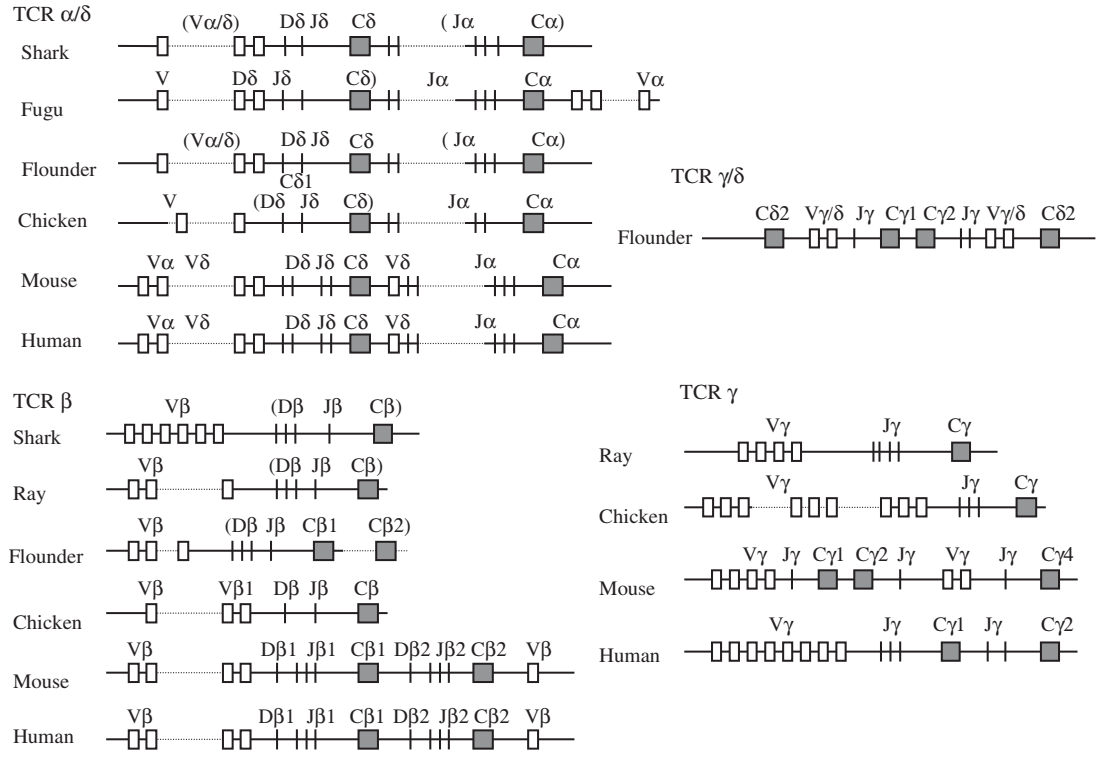


Fig. 1 Comparison of gene structures of T cell receptors (TCRs).

receptors of vertebrates. Similarly, TCR-β possesses two isotypes, both of which exist on the same locus, as is the case with other vertebrates. The TCR-α and TCR-β genes or TCR-γ and TCR-δ genes are expressed in the same cell. The existence of all four types of TCRs and the expression pattern of these genes indicates that fish, like mammals, have several types of T-lymphocytes. However, the proportions of αβ T cells and γδ T cells in peripheral blood leukocytes (PBLs) are very different from those in humans, in which αβ T cells are the major type. The proportional difference between flounder and human T cells occurring in PBLs might be due to the difference in the organism's living circumstances.

Immunoglobulins

Immunoglobulins (antibodies) are produced on the surface of B lymphocytes (B cells) in response to foreign materials.[52] Antibody-mediated responses of host cells to combat invading pathogens are either immediate, at the site of pathogen entry, or late, when the infection has already spread to the blood or organs.[53] If the host animal possesses antibodies against a particular invading pathogen, the antibodies bind directly to the pathogens and prevent further infection.

Immunoglobulins (Ig) are the major heterodimeric glycoproteins involved in the humoral immune response. They consist of two heavy chains and two light chains linked together by disulfide bonds.[54] In eutherian mammals, five main immunoglobulin classes, IgM, IgD, IgG, IgE and IgA, have been identified and are defined by the heavy chain constant region depicted by Cμ, Cδ, Cγ, Cε and Cα, respectively.[55] However, only two types of immunoglobulin heavy chain, IgM and IgD, are present in fish. The cDNAs and genes have been cloned from several different fish species.[56–59]

As expected, Japanese flounder has both IgM and IgD genes and these genes are closely related to previously reported fish immunoglobulin genes[35] (Fig. 2). The Cμ constant region gene of the IgM of this flounder is about 4.5 kb and contains four constant region-encoding exons (Cμ1-Cμ4) and two transmembrane domain

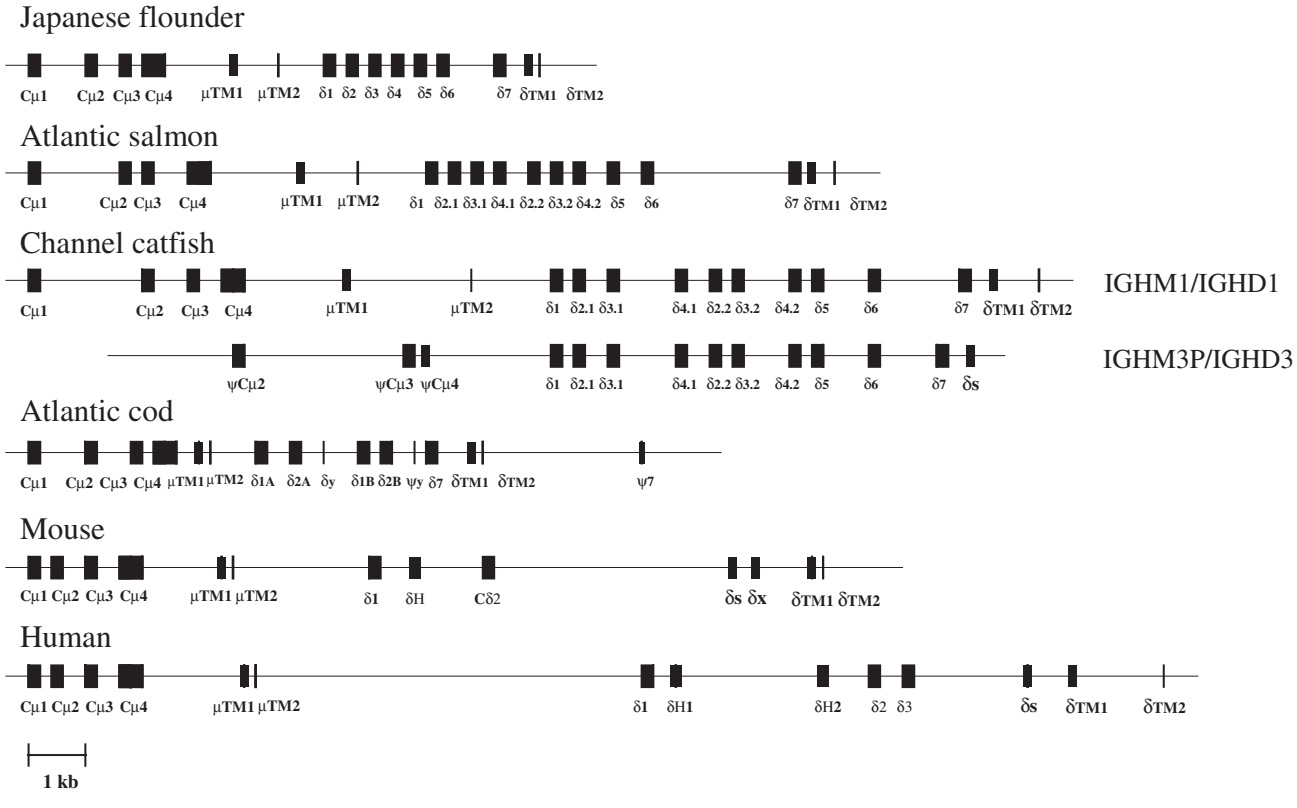


Fig. 2 Genomic organization of μ-δ regions of Japanese flounder IgH locus compared with Atlantic salmon, channel catfish, Atlantic cod, mouse and human μ-δ exons. Coding regions are indicated by black rectangles. Schematics are drawn to scale.

exons (Cμ5-Cμ6) that are responsible for translating the IgM secreted and membrane receptor forms, respectively. We also found the Japanese flounder Cδ gene, which is homologous to other fish IgD heavy chain genes, located 0.9 kb immediately downstream of the IgM heavy chain gene. This gene encodes seven extracellular constant domains (Cδ1-Cδ7) and two transmembrane domains (δTM1 and δTM2). The Cδ gene of Japanese flounder has no gene duplications of δ2-δ3-δ4 like those in channel catfish, Atlantic salmon, and no δ1-δ2 duplication, like that found in Atlantic cod. Comparison of the μ and δ organization of vertebrates revealed that the μ-δ region of Japanese flounder is very compact with a size of only about 10 kb, while the μ-δ regions in other vertebrates such as Atlantic salmon, Atlantic cod, channel catfish, mouse and human, have sizes of 15, 11, 18, 14 and 19 kb, respectively.

The phylogenetic relationship of Japanese flounder μ and δ to other vertebrate IgH isotypes revealed that the δ of Japanese flounder and teleosts clearly made a separate branch with mammalian δ sequences and the Japanese flounder μ was placed in the group of neopterygian μ (Fig. 3). The presence of the δ gene immediately downstream of the μ gene suggested that it occurred as a duplication product of genes. This possibility was tested by constructing phylogenetic trees of μ and δ. δ5 and δ7 of teleosts were closely related to human δ2 and mammalian δ3, respectively. A slight relationship of μ and δ was found. We suggest that the teleost δ2-δ3 and δ5-δ6 are duplicates of ancestral origin, and δ4 and δ7 could be the result of another duplication early in the evolution of IgD. However, in our study, all teleost δ5 genes clearly clustered with human δ2, and δ7 branched together with mammalian δ3 genes suggesting that these domains share a common ancestor. In addition, fish δ4 could be grouped with mammalian Cμ2 and fish δ6 clustered with mammalian Cμ4 because of 24% to 30% sequence identities.

Japanese flounder IgM mRNA was detected in similar amounts using reverse transcriptase-polymerase chain reaction (RT-PCR) in PBLs, head kidney and spleen, and in lesser amounts in the trunk kidney. In contrast, the IgD gene was mainly detected in PBLs and small amounts were detected in the spleen, head and trunk kidney. The expression level of

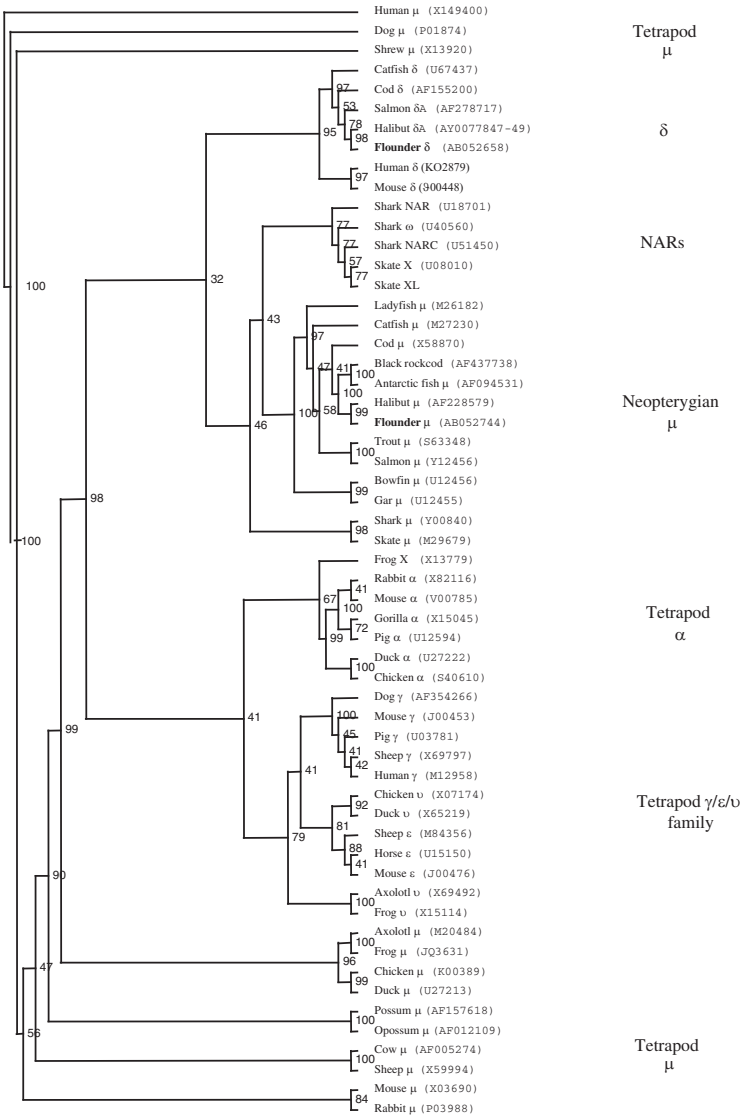


Fig. 3 Phylogenetic tree of the different isotypes of vertebrate immunoglobulin heavy chain constant regions. The tree was generated from amino acid sequences alignment using CLUSTALX. Bootstrap probabilities (%) on interior branches are for the neighbor-joining tree. GenBank accession numbers of each species and Swiss-Prot accession number for dog and rabbit μ are shown following vertebrate species common name.

IgM was higher than that of IgD in all PCR-positive organs. Among the PBLs, IgM-positive cells were detected in 24 of 295 cells (8.1%). In humans, lymphocytes account for 20% to 45% of all PBLs, and B-lymphocytes account for 15% to 30% of the lymphocytes. The proportion of Japanese flounder IgM-positive cells that are thought to be B-lymphocytes was found to be similar to that of humans.

Major histocompatibility complex

The major histocompatibility complex (MHC) is a region that is closely associated with immune function in all vertebrates since it contains genes important to both the adaptive and innate immune systems.[60] Mammals have two classes of MHC molecules. Class I consists of classical Ia molecules and non-classical Ib molecules, and Class II molecules are composed of the α and β chains. Class I molecules, which includes an α chain and β2-microglobulin (β2-m), deliver the peptides derived from endogenously synthesized proteins to CD8+ T cells. Class II molecules, on the other hand, are heterodimers consisting of both α and β chains, mainly presenting exogenously derived peptides to CD4+ T cells. The MHC genes are highly polymorphic, with high variability in the peptide-binding regions. At present, all MHC Class I and II cDNAs and genes have been cloned from a number of different fish species, the gene and domain structure of which are nearly identical to those of mammals except for the organization of the gene locus[60–64] (Fig. 4). The MHC class I and II molecules of mammals are located on the same locus while in bony fish they exist on a different locus in the chromosomes.[65–68]

Cytokines

Cytokines are a diverse group of soluble proteins and peptides which act as humoral regulators either under normal or pathological conditions, modulating the functional activities of individual cells and tissues. Three cytokines, TNF-α, IL-1 and chemokines have been isolated from

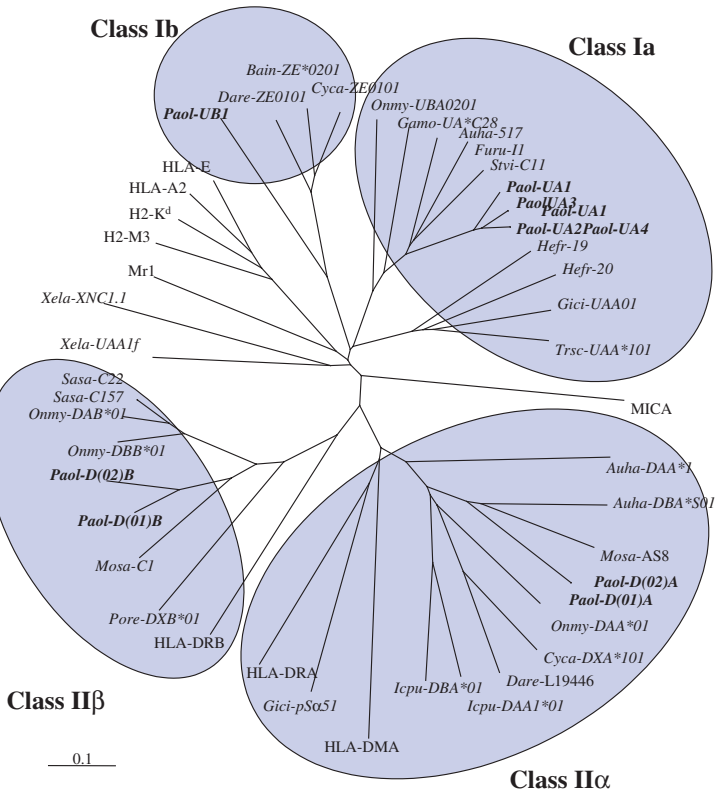


Fig. 4 Phylogenetic tree of two classes of MHC genes in Japanese flounder, *Paralichthys olivaceus* (*Paol-*) and other vertebrates: (human: HAL-A*0201 (K02883), HLA-E (M20022), MICA (A55739), Mr1 (U22963), HLA-DRA (K01171), HLA-DRB (M11161), HLA-DMA (X62744); mouse: H-2 Kd (J00402), H-2 M3 (U18797); frog: Xela-UAA1 (L20733), *Xela-XNC 1.1*; leopard shark: *Trsc-UAA*101* (AF034316); horned shark: *Hefr-19* (AF028558), *Hefr-20* (AF028559); shark: *Gici-UAA01* (AF220063), *Gici-pS? a5-1* (M89951); rainbow trout: *Onmy-UBA*201* (AF287485), *Onmy-DAA*01* (AJ251431), *Onmy-DAB*01* (U20943), *Onmy-DBB*01* (AF115529); Atlantic cod: *Gamr-UA*C28* (AJ132511); cichlid fish: *Auha-517* (AF038550), *Auha-DAA*01* (AF091557), *Auha-DBA*S01* (AF212849); walleye: *Stvi-C1-1* (AY057455); striped bass: *Mosa-AS8* (L35063), *Mosa-C1*(L33962); common carp: *Cyca-DXA1*01* (X95432), *Cyca-ZE*0101* (AJ420951); zebrafish: *Dare* (L19446), *Dare-ZE*0101* (AJ420953); channel catfish: *Icpu-DAA1*01* (AF103002), *Icpu-DBA1*01* (AF103055); guppy: *Pore-DXB*01* (AF080585); large barbus: *Bain-ZE*0201* (AJ420277); Atlantic salmon: *Sasa-C157* (X70166), *Sasa-C22* (X70167).

Japanese flounder. They play important roles in accelerating inflammation reactions either directly or by their ability to induce the synthesis of cellular adhesion molecules or other cytokines in certain cell types and some, like chemokines, induce chemotaxis.

Tumor necrosis factor-α

Tumor necrosis factor-α (TNF-α) is synthesized by different cell types upon stimulation with endotoxin, inflammatory mediators, or cytokines such as IL-1 and upon stimulation with TNF itself in an autocrine manner.[69–72] Aside from TNF-α, there are also several TNF-like proteins in mammals, which together are referred to as the TNF ligand superfamily.[73] The members of the TNF ligand superfamily share common biological activities, but some properties are shared by only some ligands, while others are unique. TNF superfamily genes are thought to be duplicated from TNF-α.[74] At present, 16 genes under the TNF superfamily have been reported in humans, including TNF-α, lymphotoxin (LT)-α and -β, and they are also members of the cytokine family.[75–77]

The non-mammalian TNF superfamily gene was first isolated from Japanese flounder which was eventually classified as TNF-α.[37] After this discovery, several fish TNF genes have been isolated by PCR using its amino acid or DNA sequence (Fig. 5).[78–82] The number of exons and introns of fish TNF-α and the positions of its exon-intron junctions are similar to those in humans. Its mode of transcription is also found to be similar to that in humans, in that it is induced by viruses — LPS and PMA. Its amino acid sequences including some potential NF-κB̄-binding motifs found in the 5' upstream region, are approximately 30% identical to those in humans.

Interleukin-1

In mammals, interleukin-1 (IL-1) serves as a starting point for a number of immunological cascade reactions facilitating CD4+ T lymphocyte proliferation, as well as B-lymphocyte growth and differentiation.[83] In

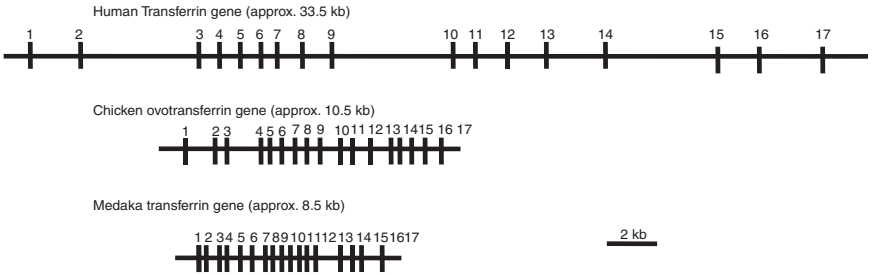


Fig. 5 Position of exons and introns of transferrin genes in human, chicken and fish.

addition, IL-1 of mammals is involved in both endogenous Th1 and exogenous Th2 pathways.[84] In fish, it was only recently that IL-1 cDNAs and genes have been successfully cloned.[85] The amino acid sequence identities of IL-1 between fish and other vertebrates including mammals, birds and *Xenopus* are approximately 30%.

An IL-1 cDNA has also been cloned and subsequently characterized for its expression in Japanese flounder. Among all the fish IL-1βs in the databases, the one with the highest identity to Japanese flounder was that of sea bass, with a 62% identity. The expression of Japanese flounder IL-1β as induced by ConA/PMA and LPS treatment is similar to that of other fish IL-1 genes.

Chemokines

Chemokines, the name of which is derived from a combination of chemoattractants and cytokines, direct leukocytes from blood vessels to inflammatory sites.[86–88] They also act on angiogenesis/angiostasis, lymphoid organ development, cell recruitment, Th1/Th2 development and metastasis. The majority of chemokines were identified from molecular cloning efforts in contrast to cytokines, which were discovered through observation of their bioactivities. This methodology accounts for the rapid identification of chemokine genes and eventually expanded the chemokine family. Chemokines are small secreted proteins ranging in size from 6–14 kDa. They show close structural similarity within a

particular group having sequence homologies ranging from 20% to 90%. The main structural domain is based on the configuration of four conserved cysteine residues near the N-terminus, which forms intramolecular disulfide bonds between the first and third cysteines and the second and fourth cysteines, resulting in a stably folded molecule. Their classification into subfamilies (CXC, CC, CX_3C and C) is defined by these four invariant cysteine residues that form disulfide bonds. In CXC chemokines, one random amino acid interrupts between the first and second cysteines, whereas the first and second cysteines are adjacent in CC chemokines. Three amino acids interrupt the first and second cysteines forming a mucin-like stalk and both transmembrane and cytoplasmic domains in CX_3C chemokines. The remaining subfamily of chemokines, C chemokines, lack the second cysteine yet they are functional.

More than 50 chemokines have been identified in mammals, both by sequences and functional assays. In fish however, chemokines have been described only in terms of their sequences and only three subfamilies, CC, CXC and C chemokines, have been identified thus far.[89–92] A cytokine in the CX3C chemokine subfamily has not previously been reported in fish.

Four different CC chemokines have been cloned from Japanese flounder, designated as JFCCL1, JFCCL2, JFCCL3 and JFCCL4.[93–95] The JFCCL1 gene is similar to mammalian CC chemokine, consisting of three exons and two introns and exists as a multicopy gene. JFCCL2 is also a multicopy gene having two forms, JFCCL2-1 and JFCCL2-2, with sizes of 1.9 kb and 1.8 kb, respectively. JFCCL2-1 consists of three exons and two introns while JFCCL2-2 consists of two exons and one intron. Because of the uncommon properties and expression pattern of the JFCCL2 gene, we have suggested that it is a pseudogene. The genomic sequence of JFCCL3 showed two isoforms, designated as JFCCL3-1 and JFCCL3-2 with sizes of 1.8 and 1.2 kb, respectively. Both isoforms contain three introns and four exons. Lastly, JFCCL4 is approximately 750 bp and is composed of four exons and three introns. The overall organization of chemokine genes of Japanese flounder is different from that in mammals. A phylogenetic analysis did

not reveal any clear evidence of orthology of fish and human chemokines, which implies that the chemokine subfamilies diverged before the segregation among fishes and mammals and the divergence within the subfamilies took place separately in the two vertebrate groups. However, the expression patterns and functional analyses of fish chemokines are similar to those of mammalian chemokines. Recombinant JFCCL1, JFCCL3 and JFCCL4 are able to attract Japanese flounder PBLs in a microchemotaxis chamber (Fig. 6).

Antimicrobial Proteins

Lysozyme

Lysozyme is a widely distributed enzyme, located in the serum, mucus and many other tissues of higher vertebrates. It catalyzes the hydrolysis of bacterial cell walls and act as a non-specific innate immunity molecule against invading bacterial pathogens.[96–97] Lysozymes are classified into five types: chicken-type lysozyme (c-type), which includes stomach lysozyme and calcium-binding lysozyme; goose-type lysozyme (g-type); plant lysozyme; bacterial lysozyme; and T4 phage lysozyme (phage-type). Only the c- and g-types have been reported in vertebrates, although in insects c-type has also been isolated.[96,98–103] Even though lysozyme is believed to play an important role in defense against infectious diseases in fish, only a few studies have investigated fish lysozymes.

The c-type lysozyme cDNA has only been so far isolated from two fish species, rainbow trout and Japanese flounder (Fig. 7).[104–106] The amino acid sequence identity of the lysozyme between these two species was 72.9%, which includes highly conserved cysteines, catalytic residues (Glu 35 and Asp 52) and glycosylation sites. The size of Japanese flounder c-type lysozyme is about 3.7 kb, inclusive of four exons and three introns.[106] Structural comparisons of the c-type lysozyme genes from other vertebrates indicated a correspondence in the positions of the exons. It is expressed in the head kidney, posterior kidney, spleen, brain and ovary, whereas the rainbow trout c-type lysozyme gene is

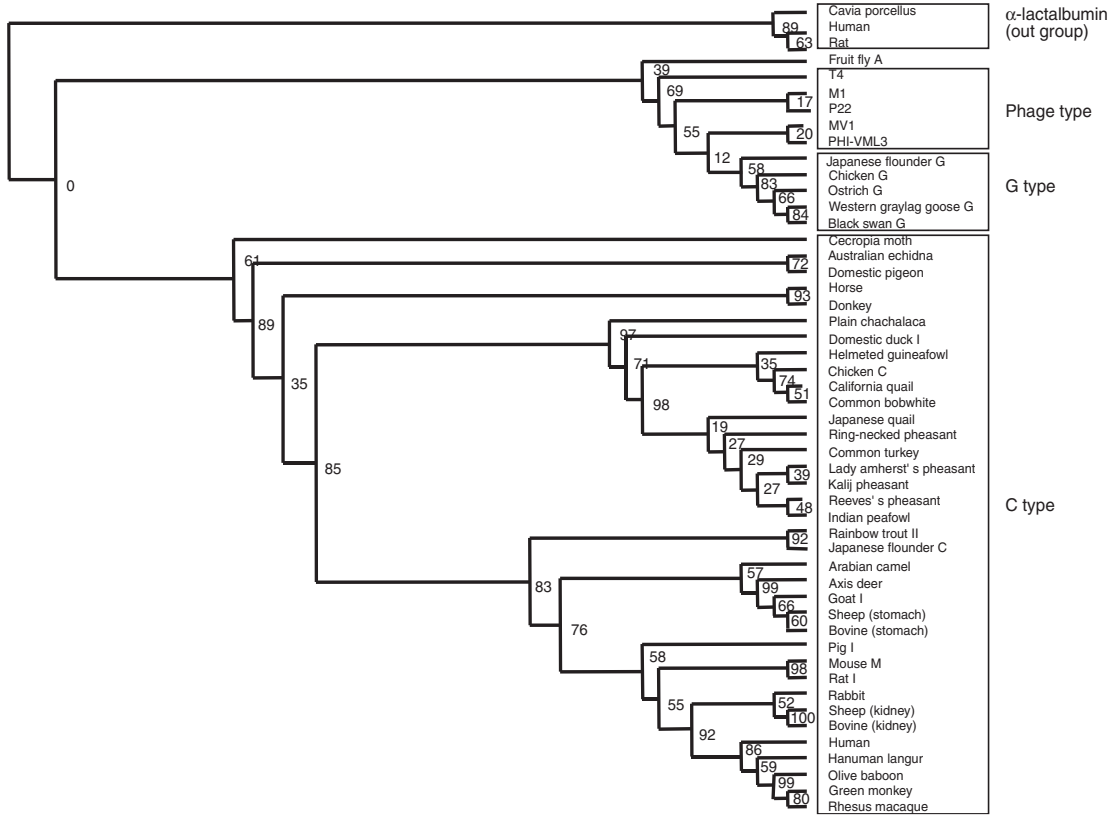


Fig. 6 Phylogenetic tree of reported lysozyme and lactalbumin amino acid sequences.

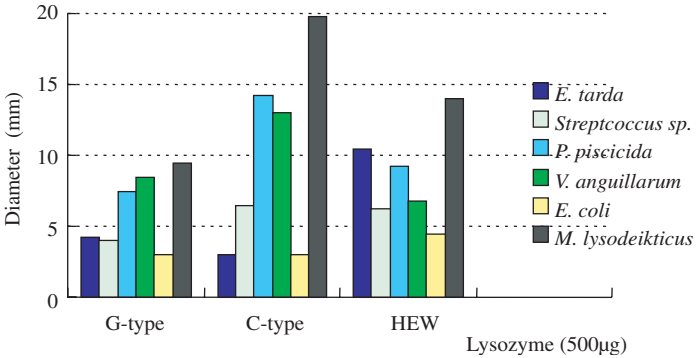


Fig. 7 Comparison of lysozyme activities against fish pathogenic bacteria.

expressed in the liver and kidney only. Interestingly, the gene expression of Japanese flounder c-type lysozyme gene is induced after *Edwardsiella tarda* infection (Fig. 8).[105,106]

The g-type lysozyme gene has likewise been isolated from Japanese flounder. This is the first report of a g-type lysozyme from a non-avian organism[42] (Fig. 7). The Japanese flounder g-type lysozyme gene has five exons and four introns, whereas chicken g-type lysozyme has six exons and five introns. As a result, the Japanese flounder gene is about 2.3 kb shorter than the chicken gene. In addition, there is a structural difference in exon 1 and intron 1 of the flounder g-type lysozyme relative to the chicken. The amino acid sequence deduced from the Japanese flounder gene possessed none of the conserved cysteine residues seen in g-type lysozyme amino acid sequences of the four avian species previously studied. The amino acid sequences of the phage-type lysozymes, and some of the bacterial lysozymes, also do not contain conserved cysteine residues, although these lysozymes have been shown to possess biological activities. The disulfide bond formed by the cysteine residues might not be necessary for lysozyme activity. Furthermore, three predicted catalytic residues and the adjacent residues of the flounder g-type lysozyme amino acid sequence are conserved with respect to the avian amino acid sequences. The Japanese flounder g-type gene is expressed ubiquitously with increased expression in the heart, intestine and whole blood after

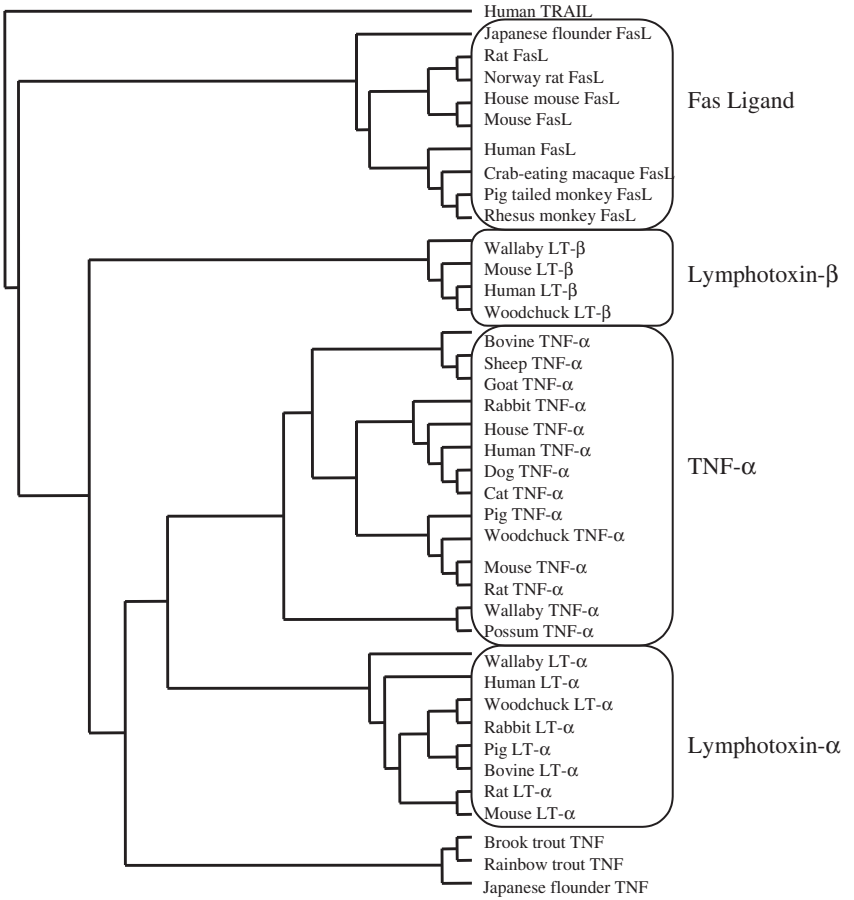


Fig. 8 Phylogenetic analysis of TNF-α, lymphotoxin-α, lymphotoxin-β and Fas ligand.

E. tarda infection. In contrast, the chicken g-type gene is expressed only in the bone marrow and lung.

The recombinant c- and g-type lysozymes surprisingly showed lytic activity against non-flounder bacterial pathogens *Photobacterium damselae* subsp. *piscicida* and *Vibrio* spp., and a non-lytic action against flounder pathogens *E. tarda* and β-type *Streptococcus* spp.[42,107] (Fig. 8). In addition, the optimum temperature and pH were different in both lysozyme activity. These findings suggest that the further elucidation

of lysozyme activity against bacteria in the presence of different gradients is important for better understanding of host–pathogen interaction and immunity.

Transferrin

Transferrin (Tf) is the major iron-binding protein in vertebrate serum. It is a monomeric glycoprotein with a molecular mass of approximately 80 kDa composed of two lobes, each possessing the capacity to bind reversibly one ferric iron.[108,109] The biological functions proposed for Tf include protection against microbial infection, iron transport, enhanced intestinal iron absorption, and modulation of inflammatory responses.[108,110,111] Tf is considered to play an important role in defense mechanisms in fishes. Genetic variations in fish Tf have been correlated with infectious-disease resistance.[112,113]

Tf cDNA of medaka *Oryzias latipes*, Japanese flounder *Paralichthys olivaceus*, Atlantic cod *Gadus morhua*, and nine species in three genera of salmonids (*Oncorhynchus nerka, O. rhyodurus, O. masou, O. kisutch, O. mykiss, Salvelinus pluvius, S. fontinalis, S. namaycush*, and *Salmo trutta*) have been cloned and characterized.[114–120] Medaka Tf cDNA is about 2.2–2.4 kb and encodes 685–691 amino acids. The predicted amino acid sequences of these fish Tfs share 56.1% to 99.0% identities. The alignment and comparison of the predicted amino acid sequences with those previously reported from the Tf family showed that fish Tf also demonstrates the duplicated structure, conserved iron binding amino acid residues and the position of cysteine residues which are characteristic of the Tf family. The relationship among these three genera of salmonids based on a phylogenetic tree of amino acid sequences of Tf cDNAs is well correlated with that derived from classical morphological and genetic analyses. Restriction fragment length polymorphism (RFLP) analysis of the rainbow trout Tf gene suggested that there are at least three different alleles.[121]

The fish Tf genes, like mammalian Tf genes, were found to be transcribed mainly in the liver.[115] The medaka Tf gene has a full length of about 8.5 kb, organized into 17 exons separated by

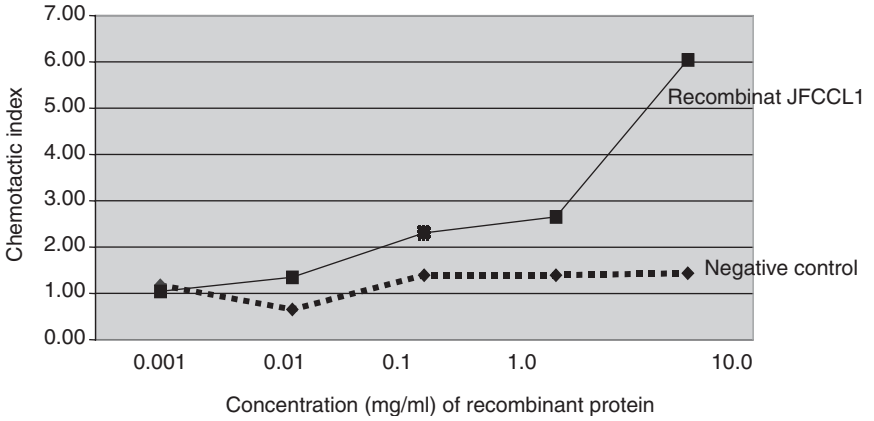


Fig. 9 Comparison of chemotactic activities of recombinant proteins of Japanese flounder CC chemokine.

16 introns (Fig. 9).[119] The exons are similar in size to those in the genes for human Tf, chicken ovoTf, and mouse and bovine lactoferrin.[119,122,123] However, the introns are smaller than these previously reported in the Tf gene family. We found several transcription factor binding sites in the 5' upstream region of medaka Tf gene.

Mx protein

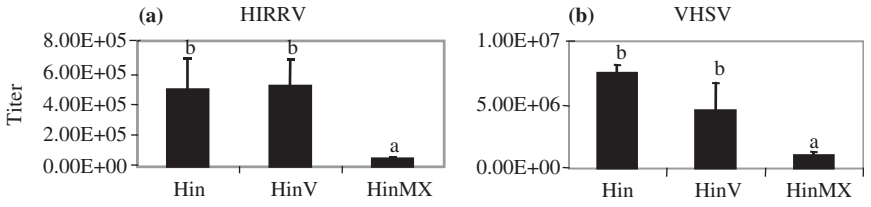
Myxovirus resistance or Mx constitutes one of the important components of the host's innate response during virus infection. It is found in a diverse number of organisms[124] and it belongs to the dynamin superfamily of high molecular weight GTPases[125,126] with sizes ranging from 70 to 80 kDa.[127] Antiviral activity of Mx has been demonstrated in mammalian cells,[124] and in chicken.[128] Mx effectively inhibits the replication of enveloped negative single-stranded RNA viruses[125,129] and some positive-stranded RNA viruses.[130–132]

In fish, Mx was first described in perch, *Perca fluviatilis*.[133] Since then, this gene has been cloned in rainbow trout (*Oncorhynchus mykiss*),[134] Atlantic salmon (*Salmo salar*),[135] Japanese flounder

(*Paralichthys olivaceus*),[136] Atlantic halibut (*Hippoglossus hippoglossus*),[137] pufferfish (*Takifugu rubripes*),[138] gilthead seabream (*Sparus aurata*),[139] channel catfish (*Ictalurus punctatus*),[140] and zebrafish (*Danio rerio*).[141]

The Japanese flounder Mx contains 2,385 bp, encoding 620 amino acids, and shares 51% and 78% amino acid identities with human and trout Mx, respectively.[136] Apparently, healthy fish constitutively express Mx but an increased expression was observed upon infection with hirame rhabdovirus, reaching a peak level at 72 hours post-infection. Constitutive expression of Mx has also been observed in other fish species[138,139,142] and this might be attributed to the presence of low levels of circulating interferons in the system that could trigger Mx expression or it may have a non-immune role. Mx expression was enhanced by stimulation with poly I:C,[135,141] virus infections,[139,140] and vaccination with plasmid DNA encoding the G-protein gene of rhabdovirus.[143]

There is some evidence that fish Mx has an antiviral role. Trout Mx proteins failed to inhibit the replication of infectious hematopoietic necrosis virus (IHNV).[127] A clear correlation between Mx expression and protection against infectious pancreatic necrosis virus (IPNV) induced by IFN in Chinook salmon embryo (CHSE-214) was observed, but there is no clear evidence that Mx is involved in the inhibition of IPNV.[144] Recently, an antiviral role of Japanese flounder Mx has been demonstrated *in vitro* (Fig. 10). Fish rhabdoviruses, the hirame rhabdovirus (HIRRV) and viral hemorrhagic septicemia virus (VHSV) replicated less in a homologous cell line stably expressing Japanese flounder Mx that was cloned in an eukaryotic expression vector.[145] Further, rhabdovirus subgenomic transcription but not primary transcription was significantly inhibited by Japanese flounder Mx.[146] Inhibition of this important step in rhabdovirus replication resulted in the synthesis of fewer viral particles, thereby causing decreased cell lysis during progressive infection. These results are not surprising because other Mx members have been shown to inhibit negative single-stranded RNA viruses, to which rhabdovirus belongs. Our results clearly show that at least one fish Mx has antiviral activity. Further studies are needed to establish the antiviral role of other fish Mxs.



% Virus Inhibition

	HIRRV	VHSV
Mx vs Vector	90.5	75.6
Mx vs Non-transfected	90.1	85.3

Fig. 10 Rhabdovirus yields (expressed as virus titer/ml) in the different cell types after infection. **(a)** HIRRV and **(b)** VHSV titers in non-tranfected HINAE (Hin), empty vector-transfected (HinV) and in JFMx-transfected HINAE (HinMX). Percentage virus inhibition was calculated based on the virus titer in non- and empty vector-tranfected cells relative to the JFMx-transfected cells. Column bars with different letters are significantly different at $p < 0.05$, $n = 3$. Data taken from Caipang *et al.*[145]

Methods for Determining Gene Expression

Quantitative "Real-time" PCR

Analyses of immune and biodefense genes are needed to characterize disease-related inflammatory pathways and to identify functional properties of immune cell subpopulations. Accurate quantification of mRNA expression is also needed to assess differential gene expression. "Real-time" PCR is a highly sensitive and reliable method for quantifying mRNA expression,[147] and has recently been used to monitor cytokine transcription.

Quantitative "real-time" PCR technology has been used for the detection of immune and biodefense gene expression (including IL-1β, IL-1 receptor, TNF-α, TNF receptor-1, TNF receptor-2, MHC class I-α, MHC class II-α, MHC class II-β, IgM, IgD, TCR-α, TCR-β1, TCR-β2 and TCR-δ) in Japanese flounder leukocytes stimulated with Con A/PMA or LPS. Comparison of stimulated and non-stimulated leukocytes using copy number profiles has shown clear and conclusive results (Fig. 11). This indicated that quantitative

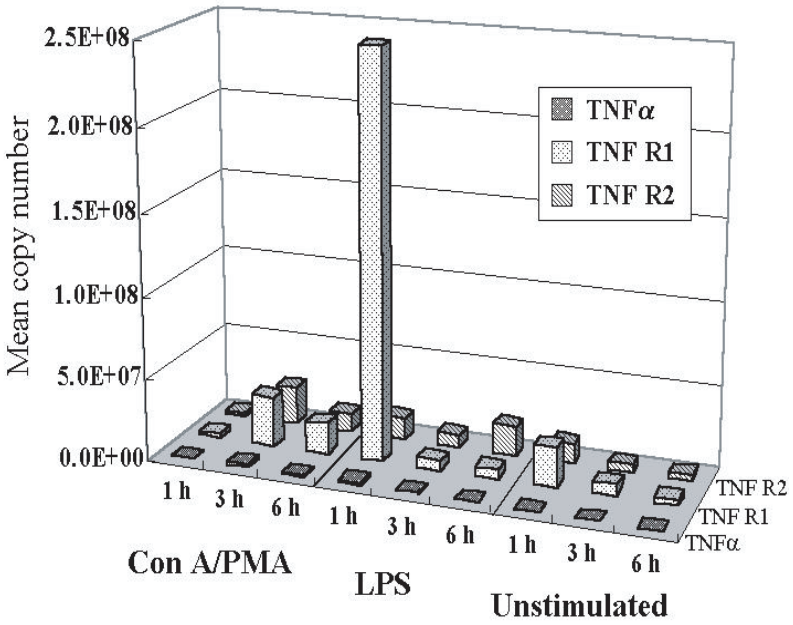


Fig. 11 Quantitative "real-time" PCR analysis of TNF-α, TNF receptor-1 and TNF receptor-2 expression in Japanese flounder leukocytes following stimulation with Con A/PMA or LPS at one, three and six hours. Quantitative TNF-α, TNF receptor-1 and TNF receptor-2 mRNA levels were determined by real-time PCR and standardized according to respective β-actin mRNA levels (cytokine mRNA expression/10^6 β-actin mRNA copies).

"real-time" PCR is an accurate method for quantification of mRNA expression which is needed to assess differential gene expression of immune network systems in fish. While a number of techniques, such as northern analysis, semi-quantitative RT-PCR, and *in situ* hybridization, are available to measure the level of mRNA expression, they have some limitations. For example, they are not very sensitive or accurate when used to quantify mRNA that is expressed in low abundance.

cDNA Microarray

Microarray is a new technology that consists of hundreds to thousands of genes robotically arrayed on specially treated glass slides.[148] By

labeling two different RNA samples with different fluorescent dyes (Cy3 and Cy5), microarrays can identify which genes are up- or down-regulated between the two conditions tested.[148] A cDNA microarray analysis has been carried out to characterize gene expression profiling of viral infection responses and/or bacterial endotoxin LPS response genes in Japanese flounder (Fig. 12).

The Japanese flounder cDNA chip consists of 871 different cDNA clones spotted on a slide glass consisting of 500 known genes and 371 functionally unknown genes. The cDNAs were synthesized from mRNA of kidney cells infected with $10TCID_{50}$ of hirame rhabdovirus (HIRV) or treated with LPS. Under LPS stimulation, some genes belong to the family of apoptosis regulators, cell cycle regulators and inflammation-related genes were upregulated, the latter being significantly induced (Table 2). Particularly, IL-1β and its receptor, monocyte chemotactic protein (MCP) and collagenase were upregulated. These genes, which have been well studied in mammals, have important roles in activating phagocytes or chemotaxis in infected areas. IL-1β is a member of the cytokine family and is mainly secreted by monocytes.[149] IL-1β is strongly

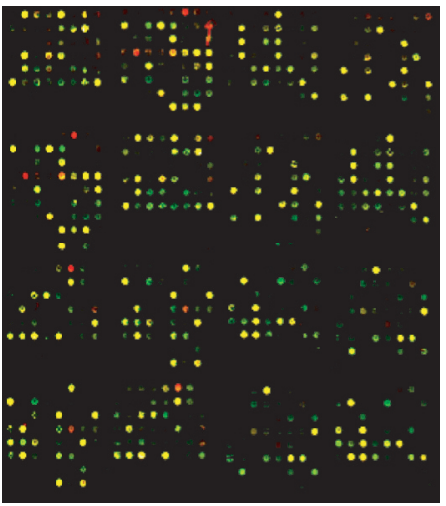


Fig. 12 Scanning image of Japanese flounder cDNA microarray.

Table 2 Gene expression profile for Japanese flounder immune-related genes.

Function	Gene name	Clone no.	Kidney cells			
			LPS1[a]	LPS6	HRV3[b]	HRV6
Apoptosis regulator	IAP-1	WE7(1)	0.8	**3.4**	1.4	1.4
Apoptosis regulator	Apoptosis regulator NR-13	B988	0.8	**2.0**	1.9	1.2
Apoptosis regulator	Cytochrome-c	B888	1.0	**2.0**	1.9	0.7
Apoptosis regulator	Cytochrome-b	B296	1.4	**2.6**	**2.4**	0.7
Apoptosis regulator	HSC 70	WF12(1)	0.7	N.D.	**2.3**	0.8
Apoptosis regulator	HSP 70	B675	1.1	1.2	**3.4**	0.6
Apoptosis regulator	HSP 90-α	B938	1.0	1.2	**4.2**	1.2
Apoptosis regulator	NGF-induced protein IB	B178	1.0	N.D.	**4.5**	1.3
Apoptosis regulator	Transcription factor BTF3	B954	1.2	1.6	**2.4**	0.8
Cell cycle regulator	Ferritin heavy subunit	LC5(10)	1.7	**7.1**	1.8	0.8
Cell cycle regulator	CCAAT/Enhancer binding protein β	WG10(20)	0.9	**2.6**	0.9	0.9
Cell growth regulator	CD83 antigen precursor	B879	1.4	N.D.	**2.6**	0.9
Cell growth regulator	Transcription factor JUN-B	B726	N.D.	N.D.	**3.1**	1.6
Cell growth regulator	c-fos	WC5(6)	1.3	1.9	**6.0**	**2.0**
Cell growth regulator, Inflammation	NF-IL6β protein	WB2(3)	0.9	1.8	**5.0**	0.7
Growth factor	Thioredoxin interacting protein	WF5(21)	1.4	1.9	0.7	0.8
Immune response regulator	Transforming growth factor-β	B901	N.D.	N.D.	**2.7**	1.1
Inflammation	CD49e (integrin-α)	WF11(9)	**2.1**	N.D.[c]	0.9	N.D.
Inflammation	Collagenase	WD5(3)	1.4	**3.3**	1.3	1.0
Inflammation	IL-1 receptor type II	B892	N.D.	**33**	N.D.	1.6
Inflammation	IL-1β	B79	**3.8**	**21**	N.D.	1.2

(*continued*)

Table 2 (*continued*).

Function	Gene name	Clone no.	Kidney cells			
			LPS1[a]	LPS6	HRV3[b]	HRV6
Inflammation	Monocytechemotactic protein-1	B247	0.8[d]	**2.4**	1.5	0.7
Inflammation	Lactate dehydrogenase	B132	**2.0**	**3.3**	**2.4**	1.0
Inflammation	Chemokine receptor	B882	**2.9**	0.8	**2.4**	1.2
Innate immunity	Transferrin	LA8(1)	1.8[c]	1.9	0.8	0.9
Innate immunity	MHC class II	B203	1.2	N.D.	**4.7**	0.7
Innate immunity	NK cell-enhancing factor	B105	0.9	N.D.	**2.8**	1.0
Innate immunity	Lysozyme II	WB2(15)	1.0	1.2	**3.1**	1.1
Signal transduction	Interferon regulatory factor	LH6(3)	1.9	**5.9**[d]	1.7	1.4
Signal transduction	Inositol 1,3,4 -triphosphate 5/6 kinase	B733	N.D.	N.D.	**2.1**	0.6
Signal transduction	MAP kinase-interacting kinase-1	B63	1.1	1.0	**3.1**	1.3
Signal transduction	Serine/threonine-protein kinase pim-1	B219	N.D.	N.D.	**2.0**	0.9
Unclassified	Synaptobrevin-like protein	WA11(21)	1.1	1.8	0.8	1.0

[a]Stimulated with lipopolysaccharide (LPS) for 1 hour.
[b]Infected with hirame rhabdovirus (HRV) for 3 hours.
[c]Scores indicate signal ratio.
[d]Upregulated genes are indicated by bold (scores are higher than 2.0).
[e]N.D. means non-detected.
[f]Downregulated genes are indicated by normal (scores are lower than 0.5).

induced by LPS treatment and has various functions related to the immune response, such as induction of proliferation, antibody secretion and activation of cytotoxic cells.[150–153] IL-1 receptor type II works as a decoy and regulates the activity of IL-1β.[154,155] MCP is a member of the chemokine family which attracts phagocytes to the inflammation site and can also induce the migration of macrophage and T-cells.[156,157] Collagenase resolves collagen as its substrate and the resolved peptides from collagen strongly induces chemotactic activity for fibroblasts.

HRV infection has also induced a number of genes that regulate apoptosis, cell growth or innate immunity (Table 2). Apoptosis regulators such as heat shock protein (HSP) 70 and 90, or NGF-induced protein IB were shown to be upregulated. These proteins regulate the protein complex that controls apoptosis, and work as activator or inhibitor, respectively.[158] Cell cycle regulators such as CD83 antigen and c-fos were also upregulated. CD83 has been used as a marker for mature dendritic cells, but recently it was found to inhibit T-cell proliferation. Thus, homeostasis regulator genes such as apoptosis inducer or growth factor were upregulated. C-fos, on the contrary, is a nuclear protein and in combination with c-jun, forms a complex, which acts as a transcription factor called AP-1. Normally, AP-1 induces cell growth and differentiation during lymphocyte development.[159–161] In Japanese flounder, as in mammals, various genes that regulate homeostasis and that have important roles in the prevention of viral infection in the primary immune response were upregulated.

A significant difference of expression pattern was observed between LPS stimulation and HRV infection. By LPS stimulation, significant upregulation was observed in inflammation-related genes, while apoptosis and cell growth regulation genes were induced under the HRV infection. We succeeded in acquiring much information about fish immune system responses against bacterial and viral infections. We also confirmed the utility of DNA chips for vaccine evaluation. This method makes it possible to select low efficiency vaccines for improvement.

It is expected that microarray technology will facilitate a better understanding of the fish immune system and bring enormous benefits to aquaculture.

Acknowledgments

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Chapter 11

Use of Molecular Diagnostic Tests in Disease Control: Making the Leap from Laboratory Development to Field Application

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Introduction

In this chapter, the term "molecular diagnosis" is defined as the use of nucleic acid-based technologies to the detection of disease agents. New tests to reveal the presence or identity of viral and bacterial pathogens continue to be published at what seems to be an ever-increasing rate. The whole discipline has been heralded as the greatest advancement in fish health study, research and management for many years. However, the diagnosis of fish and shellfish infection has not become dominated by these methods. Indeed, the adoption of these techniques has been much slower than had been expected when the necessary methodology or technology began to become available to workers in fish health. Instead, we find that innovations that have potentially enormous benefits have been subjected to more extensive and intense scrutiny than any previous significant development in systems for diagnosing disease. This chapter will not provide an exhaustive review of methods that are available for detecting pathogenic agents. Such a review would quickly be overtaken by developments that are ongoing at the time of writing, and thus be outdated and perhaps obsolete before it could be published in chapter form. Rather than review individual methods in detail, this chapter aims to take a broader view

of the field of molecular diagnostics. Areas where molecular tests have been successful will be highlighted, some of the potential reasons they have not yet been adopted as widely as expected will be discussed and some future developments in this area will be reviewed. Due to the author's experience, this will be a Euro-centric view of these situations, and there are likely to be regional differences in the relative contribution of different factors towards the adoption or rejection of molecular methodologies. Nevertheless, some of the arguments for and against the use of molecular diagnostics are familiar across the world. Although the examples used concentrate on bacterial and viral pathogens, the same techniques and arguments are valid for parasitic and fungal diseases too. It is hoped that further airing of optimistic opinions in favor of wider adoption of molecular diagnostics may promote the advancement of this field beyond the present, rather stagnant, position.

Methods

Molecular diagnostics encompasses a wide range of methodologies, not all of which can be covered in a chapter such as this. At present, certain techniques predominate in the detection and identification of fish and shellfish pathogens and these are summarized here.

Probes

A consequence of the structure of nucleic acids that was noted in the first description of DNA[1] is the pairing of nucleotide bases. This enables the use of fragments of DNA that will hybridize to DNA or RNA of a complementary sequence. Labeling the probes allows their detection, where colorimetric, fluorescent and chemiluminescent visualization have replaced the radioactive methods that were once common. Probes were commonly applied to genomic DNA that had been digested with a restriction enzyme, separated by gel electrophoresis and Southern blotted.[2,3] However, this requires large amounts of DNA, usually obtained from culture, and is less suited to detection of pathogens

directly from fish tissue. Probes are now more usually employed to confirm the identity of amplified nucleic acid.

PCR

Polymerase chain reaction (PCR), developed in the 1980s,[4,5] has been the single most significant development for molecular diagnosis. The *in vitro* amplification of DNA also enabled magnification of Complementary DNA (cDNA) generated by reverse transcription of RNA, which had been possible several years previously.[6] Most frequently, short stretches of nucleic acid that are unique, or contain sequences that are unique to the target organism, are amplified. The presence of an amplification product can by itself be considered sufficient evidence that the target organism is present. Alternatively, the product can be subjected to further analysis to yield additional detail or confirmation of specificity. Other amplification methods such as nucleic acid sequence-based amplification (NASBA)[7,8] may have advantages over conventional PCR but have not yet been widely applied in diagnosis of fish disease.

Random amplified polymorphic DNA (RAPD)[9,10] is a modification of PCR that can potentially scan the whole genome to reveal variation, rather than targeting a small portion for examination. When developing diagnostic tests for fish pathogens, RAPD is probably best used as a first step to identify polymorphic DNA, from which primers or probes can be designed for a more robust and reproducible technique to be applied to clinical samples.

Frequently, fish pathogens are only distant relatives of organisms that have been studied in detail. This raises the possibility that PCR primers might cross-react with other closely related organisms, giving false positive results. As the genome of every single organism has not been sequenced, specificity cannot be guaranteed, but the chances of false positives can be reduced through judicious primer selection. The basic principles of primer design are widely known and should be adhered to wherever possible. For maximum specificity, primers should be targeted to regions of the genome that are likely to be specific only to the organism to be detected, such as a gene for a virulence factor.

When this is not possible, less specific amplification reactions can still be extremely valuable, particularly when dealing with a novel or poorly studied pathogen. In these cases, post-amplification analyses improve the specificity of the test.

Post-amplification Analyses

Obtaining greater amounts of DNA by methods such as PCR facilitates further analysis. Restriction fragment length polymorphism (RFLP) reveals differences in sequence that result in the gain or loss of recognition sites for restriction endonuclease enzymes. Single-stranded conformation polymorphism (SSCP),[11] denaturing gradient gel electrophoresis (DGGE),[12,13] and RNase protection assay (RPA)[14] can also demonstrate single nucleotide variations between fragments of nucleic acid of the same length. These methods all perform best on nucleic acid fragments of restricted size, such as those generated by PCR. All these methods, and others such as the application of probes, can assist in confirmation that an amplified product is specific or in validation of a new method.

Sequencing

The ability to determine the order of the four bases in a fragment of DNA[15,16] presently provides the greatest level of detail in analysis of genetic material from a pathogen. Although sequencing is currently a cumbersome method for disease detection, its value for developing molecular methods for diagnosis, and in epidemiology, is undisputed. Future innovation in hardware may bring sequencing into the range of rapid diagnostic techniques.[17]

Detecting Disease Agents in Clinical Samples

The ultimate aim of new tests is usually to improve the sensitivity and/or specificity of diagnosis. Amplification and detection of the nucleic

acid of a pathogen should theoretically be orders of magnitude more sensitive than some traditional methods. The analysis or exploitation of nucleotide sequences can provide much more detailed information on a pathogen than its phenotype or morphology. With these potential advantages, it is not surprising that molecular tests have been developed for all economically important fish pathogens. Development often proceeds via analysis of purified pathogen and comparison of test results from the target organism against results from other fish pathogens or other organisms likely to be present in the sample. A massive leap is then required to transfer a test from one that performs satisfactorily on materials prepared in the laboratory, to a method suitable for samples that are real or representative of clinical material received from the farm or sampling site. This is perhaps the greatest challenge to the application of any new diagnostic test, and molecular methodology has been subjected to intense scrutiny at this stage, possibly to the extent that application of molecular diagnostics has been hindered here. Frequently, the application of new methods is resisted until the technique has been properly validated.

Validation of Molecular Tests

The importance and processes of validation have been discussed in detail by Hiney and Smith[18–21] and are recognized by Office International des Epizooties (OIE) and other authorities.[22,23] Validation work lacks the excitement and kudos of initial development and laboratory-based testing of a diagnostic test. It is costly, time- and labor-intensive and routes for publishing the results of such work are not always obvious, particularly if a test proves less than satisfactory. These factors may go some way to explain why reports of rigorous validation trials are difficult to find. The application of molecular techniques to diagnostic testing seems to have incurred more intense scrutiny and calls for validation before application than any other diagnostic methodology. Perhaps this is due to the difficulty of validating a test that is more sensitive than any other, or the difficulties sometimes experienced when transferring a new test from the laboratory to field-based samples. Whatever the

rationale behind the calls for documented validation of molecular diagnostics, they are certainly more demanding than any applied to previous developments such as the enzyme-linked immunosorbent assay (ELISA) or culture of bacteria or viruses. We should not lose sight of the fact that practical application of a new method alongside existing techniques is the best validation of all, and that nothing can replace the experience of carrying out a diagnostic test for instilling confidence in the test or interpretation of its results.

Statistical comparisons of molecular and other methods of detecting aquatic pathogens have been reported for mollusc diseases.[24,25] These and other reports[26] are extremely valuable, indicating the relative sensitivity and specificity of individual and combinations of tests. Other methods have gained acceptance through necessity for their application, as described below.

Success Stories

An important example of field-based validation is the application of a PCR to detect infectious salmon anemia virus (ISAV) on a wide scale in Scotland. Mjaaland *et al.*[27] designed PCR primers from the first small segment of ISAV that was isolated. This was not designed as a diagnostic test, and there was no knowledge of the variability of this region of the genome or the possibility of cross-reaction as there was little data available on this type of virus, and none at all on ISAV itself. The method, being the only peer-reviewed technique available at the time, was applied on a large scale during outbreaks of infectious salmon anemia in Scotland in 1998 and 1999.[28] Further information, gathered after the 1997 publication, has shown that this segment is the most abundant target from ISAV,[29] therefore is likely to yield the greatest sensitivity in detection, and that the risk of cross-reaction with other common salmonid pathogens is low (see also Gregory[30]). However, when environmental samples are analyzed, the probability of cross-reaction increases significantly (Gregory, personal communication).

All PCRs Are Not Equal

The application of molecular diagnostics is slowly gaining acceptance and popularity. As this occurs and successful tests are lauded, requirements for PCR and molecular detection methods for other organisms grows. This has been seen in our laboratory, where the successful application of rapid and sensitive tests for ISAV have led to requests for PCR detection of other viruses and bacteria to be routinely available. In some cases, it has been a straightforward process of confirming that our reagents and equipment produce the desired results from material known to contain the pathogen. PCR detection of sleeping disease virus (SDV) has been implemented following published methods.[31,32] For other diseases or other tests, more extensive optimization may be required. Equipment, reagents and practices vary between laboratories and may require modification before a test performs satisfactorily. There can be no substitute for practical experience with a method for instilling confidence in the technique and in the interpretation of its results. There have been many instances of a single protocol performing differently in different laboratories and poor performance of a method "in our hands" can be disheartening but need not render the method inapplicable. Experienced personnel with a good grounding in the theory of the methods they are employing will be able to troubleshoot many problems, optimizing methods for the application and conditions in which they are to be employed. It should be remembered that not all PCR primer sets or methods will perform equally well. Sometimes this can be explained by different abundance of target material, such as the different segments of the Orthomyxoviridae, including ISAV, where segment 8 targets are present in the greatest abundance, rendering PCR detection of this segment more sensitive than PCR amplification of other segments. At other times, the reasons for differences in performance of different PCR methods may be unexplained, perhaps due to secondary structure, presence of inhibitors or equipment performance. The use of nested PCR protocols can improve sensitivity of detection. These methods are favored in some laboratories, but others consider that the increased risk of contamination outweighs the slight improvement in detection limits.

The fact that a single protocol may not suit all laboratories should not prohibit the application of different methods in different situations or locations. Indeed, application of more than one test may even instil greater confidence if the results concur. Also, comparison of methods is becoming more common and serves to provide some validation of the different tests.

As Quality Assurance and accreditation increases in molecular testing, the demand for inter-laboratory or ring testing will also grow. This is an excellent way of examining inter-laboratory or inter-test performance. During this process, great care must be taken to ensure that test materials sent to different laboratories are suitable and comparable; this is often the greatest challenge in setting up inter-laboratory comparisons.

Molecular Detection in Epidemiology and Pathophysiology

Aside from detection of pathogens in clinical samples, molecular tests and analysis have made a significant contribution to the study of the epidemiology and pathophysiology of aquatic animal diseases. Epidemiological analysis is greatly advanced by the ability to discriminate isolates of the same type or species of pathogen but from different geographical or host origins.[33–36]

The ability to discriminate different strains of virus or bacteria provides new opportunities for controlling only harmful types. Viral hemorrhagic septicemia (VHS) is a serious disease of rainbow trout in freshwater in Europe and therefore is listed in the European Communities (EC) Fish Health Legislation and controlled via a system of zones. However, other very similar rhabdoviruses have been found in marine waters[37] and the pathogenicity of different strains varies according to the fish species tested.[38] The correlation of genogroups, determined by molecular analysis,[39–43] with pathogenicity, opens the possibility of regulating only relevant strains of VHSV. Similar claims can be made for infectious hematopoietic necrosis virus (IHNV).[44] This regulation would require molecular testing, as virus culture and serotyping cannot discriminate these strains in sufficient detail.

A bacterial example, *Streptococcus iniae,* is difficult to diagnose by current commercially available systems. Typing isolates by analysis of the small subunit, or 16S, ribosomal RNA genes, appears a useful tool. 16S typing identified some *Streptococcus* isolates from human patients as *Streptococcus iniae*.[45]

The addition of data from molecular diagnostics can assist epidemiological analysis of disease outbreaks and disease management. Modeling results of PCR testing for ISAV in Scotland has provided strong evidence for a point source of infection and anthropogenic spread of the virus.[28,46,47]

In the study of pathophysiology, knowledge of the infection status and perhaps the infection load can be critical in interpreting results of physiological tests. Here, molecular tests can provide better sensitivity or quantification, and techniques such as *in situ* hybridization allow assessment of the location of the pathogen within the tissues of the host. Although largely restricted to experimental research studies at present, the results of this work may in future provide physiological testing methods to assess the health of fish. Other advantages in molecular approaches to studying pathogenesis are detailed in other chapters, thus will not be discussed here.

Use of Molecular Tests in Disease Management

On the Farm

On a fish farm, a rapid diagnosis can be vital for appropriate response and treatment. If culture of an organism is required, the time lapsed between the sample being taken and the results can allow spread of the pathogen and disease to all other cages. A swift identification might allow withdrawal of only infected cages, preventing infection and total loss of production from the others. Molecular testing provides significant advantages here, where culture of the organism is not normally required and PCR amplification can be carried out in 24 hours or less. Future developments in technology and methods (see below) are likely to provide probes, dipsticks, or hand-held thermocyclers that can be employed for an on-site diagnosis.

Identification of the strain of pathogen can be important for treatment. Antibiotic resistance can take some time to assess by conventional means, but could be detected through plasmid analysis in a shorter time frame. This would enable appropriate and fast treatment to prevent losses of fish and spread of the bacteria.

At a Regional Level

Worldwide movement of fish occurs and obviously presents a risk for disease transmission. Improved sensitivity and specificity of testing employed prior to certification for movement should assist pathogen control when operated within a scheme such as the European fish health regulations zone system.

One of the most significant improvements molecular diagnostics might make in regional, national and international control of pathogens, is the detection of carrier fish or infected ova.[48,49] Broodstock are valuable fish, sometimes representing the outcome of years of investment or a rare resource, especially in the case of species new to aquaculture. Where the regulatory regime renders reproduction from carrier fish inadvisable or uneconomic, the ability to detect fish carrying subclinical levels of pathogens is a great advantage. Carrier fish need not be reared to maturity and carriers could be removed from a population and reduce the risk of pathogen spread. Some diagnoses currently require lethal testing. For valuable repeat spawning fish, this can be an unacceptable burden, particularly in the early days of development when broodstock are rare and extremely valuable. The ability to test fish through non-lethal sampling of mucus, blood or biopsy is a significant benefit that may be offered via molecular methodology.[50]

Bottlenecks in the Application of Molecular Diagnostics

Major obstacles to the implementation of molecular diagnostics are discussed below. Once molecular testing is required, there are some limiting factors that presently restrict large-scale applications or

throughput. Scaling up PCR, hybridization, sequencing and other analyses has been possible through the use of 96-well formats, etc. However, procedures required before these can restrict the volume or number of samples handled.

Nucleic acid extraction is a good example of this. Phenol/chloroform-based methods perform well but are laborious and involve harmful chemicals. Various columns are produced commercially for extraction of DNA and/or RNA from a variety of starting material. If suitable, these kits can greatly improve the ease and efficiency of extraction. Before they are adopted, care must be taken to ensure they provide equivalent yields of nucleic acid to other methods[51] and to prevent cross-contamination, which is always a prime concern in clinical diagnostic testing.

Gel electrophoresis and other methods of analyzing nucleic acids can also restrict throughput. These problems have largely been overcome in systems such as real-time PCR which avoid the use of gels altogether.

Obstacles to Adoption of Molecular Diagnostics

With all the advantages of molecular methods for diagnostics, and a plethora of molecular tests for every notifiable or economically important fish pathogen, why have they not yet become the norm and replaced more traditional techniques?

Costs

Initially, molecular tests were largely developed in laboratories that were devoted mainly to research. Research laboratories are frequently separated from those that carry out routine diagnostic testing or screening. This separation may be due to the fact that different organizations or groups carry out different functions. Many molecular tests have been developed by university projects, whereas statutory surveillance and testing will be undertaken by government laboratories. When there is closer cooperation of research and diagnostic groups, the transfer of technology is often more straightforward and rapid.

The establishment of any diagnostic facility requires considerable investment in laboratories, equipment and trained personnel. The costs of maintaining such facilities is escalating as there is increased demand and pressure for these laboratories to be accredited and maintain Quality Assurance systems. Where organizations have already invested in facilities for histopathology, cell and bacteriological culture, and perhaps ELISA to establish diagnostic testing for bacterial and viral diseases, developing an additional department for molecular testing can seem to be an investment that may not necessarily yield sufficient benefits to justify the costs. Several trends are likely to demonstrate that the benefits of molecular diagnostic do indeed outweigh the expenditure.

Firstly, molecular tests are frequently more rapid than other methods. This is particularly true of virus isolation via cell culture, which may take over two weeks, but can also be said of the culture of some bacteria, such as *Renibacterium salmoninarum*.[22] A more rapid diagnosis will enable swift and appropriate treatment or action, so reducing production losses and spread of the pathogen.

The level of detail available following molecular testing and subsequent analysis, e.g. by nucleotide sequencing, is probably the most significant advantage at present. The identification of particular strains of pathogens, and especially the additional degree of epidemiological information yielded via molecular testing, frequently warrant the costs involved in setting up the facilities and carrying out the tests.

Finally, as molecular diagnostics have gradually developed and increased in popularity, peer pressure will also influence decisions to adopt these techniques. Given the significant investment required, and the sometimes variable performance of some tests in different laboratories, it may be more economically viable to have centers of expertise for certain pathogens or techniques, and to refer samples to these centers instead of having expensive equipment duplicated. This same argument might also be valid for other testing facilities such as tissue culture, but we have not seen concentration of these in specialist centers. At present it seems the prevailing culture will likely lead to development of molecular diagnostic capability in many laboratories with a sharing of methods and materials. This could be facilitated by

co-ordinating bodies such as the EC Community Reference Laboratories and augmented by central databases or laboratories that concentrate on certain aspects such as sequencing.

Validation

Understandably, there can be reluctance to invest heavily in new methodologies without proof of their value. There are several potential disadvantages in molecular tests. Concern over specificity of results is often a major obstacle for the adoption of PCR or probes for fish pathogens. It has been claimed that not enough is known about other organisms that may be present in the sample but are not pathogenic, yet may cross-react with primers or probes to yield a false positive result in a molecular test. This is indeed a possibility, but the probability of it occurring can be reduced in the initial development of an assay. Thorough knowledge of the type of organism the test aims to detect, together with information on the variability of the genome regions being targeted, will guide an appropriate choice of primer or probe.

Although every effort should be made to validate any new test, examples of practical application of a test providing validation in retrospect, such as PCR detection of ISAV discussed above, have probably made the greatest contribution towards the acceptance of molecular diagnostics to date. This has promoted a move away from the over-cautious approach that has prevailed to date. A more balanced view of the necessity of validation of molecular tests is welcomed and will further promote the application of this methodology, which to date has been lower than expected.

Future Developments in Molecular Diagnostics

Technologies to study aspects of pathogenesis or of certain pathogens are discussed in other chapters in this book. Many advances in methods and equipment are developed and applied first in research before being adopted for routine diagnostic use.

The amplification of nucleic acid has been the most significant development that enables molecular diagnostics, as discussed briefly above. Given the issues surrounding patents on PCR, other amplification methods may become more popular in diagnostics. Apart from NASBA, mentioned previously, rolling circle amplification and real-time PCR[52–56] have great potential in diagnostic tests, providing confirmatory tests at the same time as amplification. Nucleic acid amplification can be combined with antibody binding to amplify a signal and improve sensitivity of detection.[57]

Microarrays are transforming the generation and analysis of data on gene expression[58–61] and are also being applied in proteomics.[62,63] Despite the fact that there are some drawbacks with microarrays,[64] that workers must be aware of, as they should with any new methodology, chips have obvious applications in diagnostic testing. The main advantage at present is the ability to survey a huge number of probes in a single hybridization.[65] A criticism of current molecular techniques, in comparison to some traditional methods, is that they often detect only single pathogens. Therefore, assessment of a novel or unusual pathogen can be problematic. The use of microarrays can assess many genes or polymorphisms[66] at once, providing a more detailed picture of the organism. A dramatic illustration of this was the classification of the virus causing severe acute respiratory syndrome (SARS) following microarray analysis.[67,68] Arrays are now being applied using antibody–antigen binding[69] and these open up another avenue for analysis, alongside host–pathogen interactions.[70]

Microarrays will dramatically alter the speed and scale of molecular analysis of pathogens. The other major advance in the near future is likely to be the development of "laboratory-on-a-chip" devices that will enable on-site molecular detection and analysis. Although extraction of starting material can be a bottleneck in laboratory analysis, tiny electrodes may facilitate this and enable development of hand-held equipment.[71] Further electronic chips could carry out amplification and hybridization[72–74] and if these devices can be fabricated in a robust format, they could permit analysis on-site that currently requires several days in specialized laboratories. These lab-on-a-chip developments have been summarized elsewhere[75] and there is even a journal devoted to the subject (*http://www.rsc.org/is/journals/current/loc/locabout.htm*).

Conclusions

The application of molecular methods in diagnostic testing offers ever-increasing advantages as further techniques and equipment are developed. Yet the adoption of molecular testing for fish and shellfish has, overall, been much slower than expected. Several factors will promote the use of molecular diagnostics and these should be encouraged wherever possible. Data from validation trials should be made available, if not via traditional publications then at least through release on websites, etc. This will prevent duplication and allow ready assessment of suitable methods to adopt for each particular circumstance. The use of parallel testing as a means of validating molecular diagnostics should not be underestimated and should be readily accepted as a means of validation. Finally, practical experience of molecular tests, and their advantages in terms of sensitivity, speed or detail of results, may be their best publicity and encourage investment in facilities that will soon render PCR as routine as virus isolation in fish and shellfish health.

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Chapter 12

Current Trends in Immunotherapy and Vaccine Development for Bacterial Diseases of Fish

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Introduction

Aquaculture fish production has increased significantly over the past few decades and with it the incidence of bacterial disease outbreaks, often associated with an intensification of the culture conditions. Bacterial disease causes substantial economic losses to the industry. Although antibiotics and chemotherapeutants are extensively used to control disease outbreaks, there is increasing concern about the use of these substances in aquaculture because of drug residues in food, the development of antimicrobial drug resistance, and the detrimental effect on water microbial ecosystems and populations. Increased attention is now being given to disease prevention as a means of controlling disease outbreaks based on improved husbandry and biological control methods such as vaccination and immunostimulation.

Vaccination, or immunoprophylaxis, is based on the principle that when a foreign organism, such as a bacterium or virus, invades it host, the animal's immune response reacts against it in an attempt to remove it. If the fish is re-exposed to the same organism, the immune response is primed to response against it. This is referred to as a memory response or adaptive immunity. Vaccination mimics the invasion of pathogens and primes the animals' immune system for re-encounter with the pathogen without causing disease.

The first published evidence that fish are able to elicit an immune response to bacterial pathogens dates back as far as 1935.[1] Around this time, a number of authors showed that fish were able to produce antibodies against various bacterial fish pathogens. It was Duff,[2] however, working with *Aeromonas salmonicida*, who first showed that the antibody response elicited by fish was capable of protecting the animal from disease outbreaks. It took another 30 years before vaccination was actually used as a means of controlling fish disease. Serious outbreaks of Enteric Redmouth (ERM) in rainbow trout (*Oncorhynchus mykiss*) farms in the Hagerman Valley in Idaho, USA, and Vibriosis in salmon farms nearby led to a renewed interest in the potential of fish vaccines.[3]

The first commercial vaccines for aquaculture were licensed in the US in the 1970s and offered protection against ERM[4] and Vibriosis,[5] and later Furunculosis.[5] The introduction of vaccines to aquaculture in Scotland and Scandinavia followed in the mid-1980s. Early vaccines were based on formalin-killed preparations of bacteria, administered by immersion. However, *Aeromonas salmonicida*, the causative agent of Furunculosis, proved less immunogenic than *Yersinia ruckeri* (causing ERM) and *Vibrio anguillarum* and *V. ordalii* (causing Vibriosis), and was therefore mixed with adjuvant and administered by injection to improve its immunogenicity.[6]

Vaccination is now a part of routine husbandry management in many aquaculture systems, used as a means of controlling bacterial disease outbreaks, and the use of vaccines is steadily increasing as the diversity of species being farmed expands and new vaccines are developed for additional microbial agents. Both salmon and rainbow trout are vaccinated against three to five diseases during their production cycle, often with a multivalent vaccine, and productivity has increased as a result of vaccination.[7] Commercial vaccines are now available against Vibriosis, Furunculosis, Yersiniosis, Pasteurellosis, Cold Water Vibriosis, Winter Ulcer Disease, Edwardsiellosis, Streptococcosis and Lactococcosis, and many more experimental vaccines are in the process of being field-tested. The protection elicited by these commercial products is generally good,[8] and as a result of their use the levels of antibiotics applied to control bacterial disease in fish farms has been reduced. In Norway, for example, antibiotic application has decreased from 47 tons to around one ton.[9]

Not all diseases are successfully controlled by simple bacterin vaccine preparations, and alternative approaches are necessary to develop effective vaccines against the more "stubborn" pathogens. A greater understanding of the humoral and cellular responses of fish and identification of putative protective antigens on the pathogens will help in the development of efficacious vaccines.

Development of Vaccines for Bacterial Diseases of Fish

The primary considerations for any successful vaccine for aquaculture are cost-effectiveness and safety. To accomplish this, the vaccine must provide long-term protection against the disease under the intensive rearing conditions found on commercial fish farms. Consideration must be given to all the serotypic variants of the disease agent, the time/age when the animal is most susceptible to disease, the route of administration and the method of vaccine preparation (i.e. killed, attenuated, subunit or recombinant).

Most of the commercial vaccines presently available comprise inactivated (killed) disease agents. When such an approach has failed in the development of an efficacious vaccine, then live-attenuated vaccines have been tried. Whenever a live vaccine is used, there is always concern that the attenuated strain (usually as a result of gene deletion) may back mutate and revert to the virulent wild type.[10] Many of the successful vaccines against viral diseases in humans (e.g. rubella, measles and poliomyelitis) and in domestic animals (e.g. rabies and distemper) are live-attenuated organisms. Licensing of such vaccines may however prove to be very difficult in aquaculture. An alternative approach is to prepare subunit vaccines, where specific components of the disease-causing agent are isolated and then used in the vaccines.[11] In order to increase the amount of available antigens, the recent trend has been to clone up the genes encoding for specific antigens and then to incorporate them into bacterial DNA where they are expressed, i.e. recombinant vaccines.[12]

Pathogenesis

Effective control of fish disease by biological control methods can only be accomplished if we have a sound basic knowledge of the defence system of these animals. The pathogenesis of infection involves a complex series of interactions between the pathogen and host. The outcome of an infection depends on a combination of factors including virulence of the pathogen, the health of the host, and the innate resistance of the host to the pathogen. Important stages in pathogenicity and virulence include the ability of the invading pathogen to attach and enter the host, rapid multiplication of the pathogen by overcoming both specific and non-specific host defence mechanisms, and production of disease, causing damage to the host. The aim of vaccination is to induce long-term immunity by stimulating the memory component of the specific immune response. Vaccination protects against disease, and may not protect against infection, i.e. carriers may still exist.[13]

Development of Vaccines

Two essential components are required for the development of an effective vaccine. These are the identification of the protective antigens and determination of the protective response. The latter may be antibody-mediated, cell-mediated or both depending on the vaccine components. Identification of these two components is not an easy task. Once these have been identified then the immunogenicity of the antigen needs to be confirmed in the host species, and a practical method of administration and an inexpensive method of vaccine production need to be established. It is important in vaccine development to work with the antigens that are expressed during infection rather than antigens expressed in the laboratory. Many salmon vaccines from the past are based on inactivated (whole cell) cultures of the pathogenic organism (usually inactivated in formalin), grown *in vitro*. In these cases, the vaccines have resulted in good protection (e.g. *Vibrio* vaccine), however many pathogens appear to switch off important protective antigens when cultured *in vitro*. In such cases alternative methods of culture (e.g. the

inclusion of an iron-chelating agent) are required so that expression of the important "protective" antigens is induced.[14] This can be achieved by modifying the culture medium of the pathogen *in vitro*. Another approach that has been used is to place bacterial pathogens inside filter capsules in the peritoneal cavity of fish so that antigen expression *in vivo* can be determined.[15–17] Application of sera from fish recovering from infection in Western blot analysis can be used to pinpoint potential vaccine candidates. Of course, these antigens may be expressed and the fish may respond by producing antibodies to them, but they may or may not be protective. In fact in some cases they may be suppressive, e.g. P57 antigen from *Renibacterium salmoninarum*.[18,19] Thus, challenge of vaccinated and non-vaccinated fish is then performed to establish if the vaccine is protective.

Vaccine Evaluation

Once a vaccine has been developed for use in aquaculture it needs to be fully evaluated before it can be used commercially. This is accomplished initially by testing in an aquarium, followed by field trials. In the aquarium, safety and efficacy testing is performed on each new batch of vaccines produced. At present, efficacy testing requires the use of live fish in a challenge test and the following formula is used to calculate a Relative Percentage Survival (RPS) (or Protection) Value.[20]

$$\text{RPS} = 1 - \frac{\%\ \text{vaccinate moratalities}}{\%\ \text{control moratalities}} \times 100 \qquad (1)$$

For efficacy testing, the control mortality levels should be above 60%, inter-tank variability $< 20\%$ and non-specific mortalities $< 10\%$. Efficacy testing for mammals normally involves analysis of serum samples (serology) for the detection of antibodies to the vaccine antigens. This may be done in conjunction with small scale challenge testing or in isolation if challenge testing is not possible.[21] Future efficacy testing in our aquarium will include measurement of the immune response in fish. If such serological tests can be correlated with protection this may bring fish vaccine efficacy testing more in line with the testing of other veterinary

vaccines so that serology replaces (in part) live challenge testing. This will reduce the number of fish required for efficacy testing and will also be more cost-effective for vaccine companies.

Three criteria are used to assess the effectiveness of any vaccine in the field. This includes the rate at which protection is achieved, the final degree of protection (RPS), and the duration of immunity. It is now possible to measure the humoral response of a variety of fish species following vaccination, using enzyme-linked immunosorbent assay (ELISA) with anti-fish species Immunoglobulin M (IgM) monoclonal antibodies that are commercially available. This provides crucial information on the rate and duration of response in individual fish species to specific vaccines and many vaccine companies are now adopting this approach.

In the absence of natural exposure, booster vaccination is needed to maintain immunity. Oral vaccine boosters have been used successfully in field trials and some are now available commercially.

Methods of Vaccine Delivery

Three different methods of administration are used to vaccinate fish, namely by injection, normally given intraperitoneally (i.p.); by immersion, placing the fish directly into the vaccine solution; or orally, by feeding the vaccine to the fish in their diet. There are pros and cons for each method with regard to the level of protection obtained, side-effects due to adjuvants, ease of use and cost-effectiveness.[7] Palm *et al.*[22] showed that the specific antibody response elicited against *V. anguillarum* following immunization by injection, immersion and oral administration, correlated with protective immunity. However, a booster vaccination was needed to be able to detect the antibodies in fish vaccinated by the immersion or oral routes.

Injection Administration

Injection administration yields a good immune response in vaccinated fish, and the response it elicits is better than that obtained with immersion or oral vaccination.[3] It also has the advantage that all types of antigens can be used at doses adequate to elicit a strong immune response. Adjuvants and immunostimulants can be added to enhance the level and

duration of the response,[3] however adhesions and granulomas are associated with the adjuvant as discussed in the later section. The procedure is also more labor-intensive compared to the other two methods of administration. Fish must first be anesthetized and then passed to an operator equipped with a repeater syringe. Approximately 1000 fish per hour can be vaccinated per operator using specially designed vaccination tables and fish chutes. The injection procedure induces unavoidable stress in fish with increased corticosteroid production associated with the injection procedure.[23] The production of corticosteroids by fish during stress has been associated with immunosuppression, although the fish are still able to elicit an immune response against the vaccine.[24] Also, injection vaccination is not suitable for fish much below 15 grams.

Immersion Administration

Vaccination by immersion (and also spraying) provides intermediate levels of protection compared to injection and oral administration.[3] However the method has many advantages compared to injection vaccination.[25] It is less stressful for the fish and is less labor-intensive since it is possible to vaccinate large numbers of fish simultaneously. It can only really be used to vaccinate small fish although larger fish can be vaccinated by spraying.

A variety of immersion methods are available, including direct immersion, hyperosmotic flush, flush exposure and spray.[25] Of the four methods, direct immersion is the method most often recommended for administration of commercial vaccines. A variety of factors can influence antigen uptake during the immersion process, including antigen concentration in the vaccine, the length of immersion, the size of fish, stress, pH and salt concentration of the vaccine, water temperature, whether an anesthetic or adjuvant is used and the physical nature of the antigen.[25] The first two points are the most important with regard to antigen uptake and protection, with less effective vaccination achieved with shorter immersion times.[26]

Commercially, immersion vaccines are widely used to immunize small fish, especially since they are easy to use and highly cost-effective. Immersion vaccines, used to immunize salmonids against ERM and Furunculosis, are generally administered when the fish are around

3.5 to 5.0 grams. The duration of protection for 2.0- to 20-gram salmonids has been shown to be between nine and 12 months, while 0.5- to 2.0-gram salmonids appear to be protected for less than four months. If disease is present in the early stages of post-hatching or is present in the hatchery, then it may be possible to vaccinate at 0.5 grams or earlier, however boosting with the vaccine may be required.[27]

Controversy still exists as to the primary route of antigen uptake. Some authors claim that the lateral line is the primary site of uptake,[28] while others believe it to be the gills,[29–32] the gut[33–35] or the skin.[25] Dos Santos *et al.*[32] found very high numbers of antibody secreting cells (ASC) in the gills of sea bass fry immunized by direct immersion in a *Photobacterium damselae* spp. *piscicida* bacterin, while relatively low ASC numbers were detected in the head kidney and spleen, suggesting that the systemic immune compartment was only slightly stimulated upon immersion vaccination.

Immersion immunization clearly stimulates specific long-term protection, but how it does this is still unclear. The role of humoral antibodies in protection after immersion vaccination is also unclear. Some authors are unable to detect specific humoral antibodies after immersion vaccination, while others can detect elevated levels of specific humoral antibodies in the serum of immersion vaccinated fish. However, the antibodies measured do not always correlate with protection (see Nakanishi and Ototake[25] for relevant references). Antibodies have been detected in skin mucus[36–38] and the gill[39,40] after immersion vaccination, suggesting stimulation of mucosal immunity. Further studies are required to investigate the localized immune responses following immersion vaccination and the role they play in eliciting protection from the vaccine.

Oral Administration

Oral administration is "the ideal method" for administering vaccines to fish whereby the vaccine is incorporated into fish feed. It is less labor-intensive than the previous two methods and is suitable for vaccinating large numbers of fish of all sizes. It is also avoids the handling stressors experienced by the fish with the other two methods. The major

disadvantage with this route of administration is that lower levels of protection are achieved and the duration of protection elicited is shorter.

The fact that anal intubation resulted in a protective response in salmonids against *Y. ruckeri* and *V. anguillarum*[41] suggests that immune cells in the posterior gut are capable of antigen uptake and processing, and it has in fact been shown that fish possess gut associated lymphoid tissue (GALT) in the second gut segment.[42,43] One of the major problems associated with oral vaccination is the degradation of antigen by the gastric fluid in the stomach and anterior gut of the fish, and therefore the antigen may be inactivated by the time it reaches the posterior part of the intestine. Microencapsulation of the antigen is one approach being used to protect the antigen, but the next problem is to ensure sufficient quantities of antigen are transported across the gut wall to produce an effective humoral response.[43–45] Many studies have been carried out to examine the efficacy of oral vaccines in fish (reviewed by Quentel and Vigneulle[42]). These studies have looked at the types of immune responses stimulated by oral vaccination and the levels of protection obtained, but varying degrees of success have been reported in the literature. These variations are believed to be due to differences in experimental design between studies, including antigen preparation, the age and species of fish, the water temperature at the time of vaccination, the duration of feeding the vaccine and antigen integrity when it reaches the hind gut.

Few oral vaccines are available commercially, and where they are applied they tend to be used as a booster vaccination rather than as the primary form of immunization.

Types of Vaccines

The vaccine must be able to induce a successful immune response with the development of a reliable immunological memory. It must also be able to stimulate components of humoral and/or cell-mediated immunity depending on the type of pathogen the vaccine is against. Some vaccines appear to induce a good primary response without stimulating a memory response and in some cases an immune response is stimulated without necessarily eliciting a protective response. The

choice of vaccine preparation depends on its ability to induce both a protective and a memory response in vaccinated fish.

Whole-organism Inactivated Vaccines

The most common type of vaccine preparation used to immunize fish is inactivated preparations of bacteria, using heat or formalin for inactivation.[11] These types of vaccines are very affective at inducing a humoral antibody response, but are less affective at stimulating cell-mediated immunity or inducing a mucosal response. Formalin-inactivated preparations of *V. anguillarum, V. ordalii, V. salmonicida* and *Y. ruckerii* have been very successful in protecting fish against subsequent infections.

Attenuated Bacterial Vaccines

The bacteria present in live-attenuated vaccines have been modified so that they are no longer able to cause disease, but are still able to survive and grow within their hosts. Thus attenuated bacteria represent infection by the pathogen without disease. They provide the immune system with prolonged exposure to antigens present on the bacterium and are particularly effective at stimulating cellular immunity[46] and inducing a memory response.[11] One of the major disadvantages with attenuated vaccines, however, is the potential for the attenuated bacterium to revert to a virulent form. This has prevented live-attenuated vaccines from becoming commercially viable products, although live-attenuated vaccines have been permitted for field trial purposes in the catfish industry in the US.[7]

Advances in genetic engineering will undoubtedly improve the method for irreversible attenuating bacteria by removing genes essential for virulence.

Vaccines from Bacterial Components

In vaccines for human and veterinary use, macromolecules, such as inactivated exotoxins, capsular polysaccharides and recombinant antigens

are prepared from the pathogen and used as vaccine components.[11] These products eliminate the problems associated with attenuated bacteria reverting to a virulent form, and are used as an alternative where simple bacterin preparations have been unsuccessful. The advantage of immunizing with exotoxin preparations is that they stimulate the production of antitoxin antibodies, which neutralize exotoxins as the pathogen grows and multiplies within its host. Exotoxins, secreted by the bacteria, contain many of the products responsible for causing host damage during infection. They, therefore, need to be deactivated prior to injection, normally using formalin.[11]

Recombinant antigen vaccines are based on pathogen genes, which have been cloned into the genetic material of bacterial, yeast or mammalian expression systems, and are then expressed using the "genetic machinery" of the host organism.[12] Thus, large quantities of individual proteins from the pathogen can be synthesized, purified and used as vaccine candidates. The pathogen genes chosen need to be immunogenic and be able to induce protection and a memory response. Recombinant antigen vaccines are particularly useful for pathogens which are difficult to bulk culture, such as viruses, *Piscrickettsia* or *R. salmoninarum*. However, approval of their use has not yet been granted in Europe, although a licensed recombinant vaccine for fish is available in Norway for infectious pancreatic necrosis (IPN) using the VP2 gene of the virus.[47] There are no commercial bacterial recombinant antigen vaccines available, although some are under development.[48,49] Recombinant vector vaccines, whereby the pathogen is introduced into an attenuated bacterium and used as the vaccine, have been successfully used to eradicate smallpox.[11] The potential of using this type of vaccine for aquaculture has only recently been examined.[12]

DNA-based Vaccines

DNA-based vaccines include the recombinant antigen and recombinant vector vaccines described above together with DNA vaccination. DNA vaccination is a recently developed genetic immunization procedure in which naked DNA is injected directly into the skeletal muscle of the

fish. Appropriate pathogen genes are cloned into plasmid constructs, and upon injection of the construct into the fish, the genes are expressed extrachromosomally within the animal's muscle tissue. In mammals, experimental DNA vaccines have been successfully used to combat diseases including influenza and rabies,[7] while in fish, injection of plasmid DNA containing genes encoding glycoproteins or nucleocapsid protein have been shown to protect against infectious hematopoietic necrosis (IHN)[50] and viral hemorrhagic septicemia (VHS).[51]

DNA vaccines have many advantages over conventional vaccines in that they are easy to use and store, are cheap to produce (although their development can be costly), and there is no possibility of reversion to virulence by the pathogen. From mammalian-related literature, DNA vaccines are able to induce a specific immune response through antibodies, T-helper cells, as well as cytotoxic cells.[7] However, licensing DNA vaccines is a primary concern in commercial development, partly because of the problem with the public's perception of DNA vaccination, who confuse it with genetically modified organisms. No DNA vaccine has yet been licensed for human or veterinary use.

Adjuvants

Initial fish vaccines developed in the 1970s were fortunate enough to contain powerful immunogens, *Y. ruckeri* or *V. anguillarum* and *V. ordalii*, which resulted in a strong immune response in vaccinated rainbow trout.[4,5] However *A. salmonicida*, the constituent of the Furunculosis vaccine, was found to be a much weaker antigen in contrast and did not produce the same levels of protection in immunized fish.[6] Adjuvants have been used extensively in mammalian and poultry vaccines to strengthen the immune response to weak antigens and increase the duration of protection. Thus, when a mineral oil-based adjuvant was added to the Furunculosis vaccine, it increased both the level of protection and the duration of immunity.

Adjuvants appear to work in a number of ways. The first point to note is that the antigen is emulsified in oil droplets within the vaccines, and as the emulsion breaks down within the peritoneal cavity of the

fish, the antigen is gradually released. Therefore, the adjuvant has a "depot" effect storing the antigen within the oil droplet. The constant release of antigen over a long period of time helps to elicit a stronger immune response in the fish.[52] The adjuvant also produces an inflammatory response within the peritoneal cavity of the animal, attracting leukocytes to the site of injection. These cells take up the antigen and transport it to the lymphoid tissues where antigen-presenting cells present the antigen to lymphocytes. Lymphocytes are ultimately responsible for producing a specific immune response and immunological memory against the pathogen.

Initially, adjuvanted vaccines were composed of water-in-oil emulsions (i.e. the oil phase contains droplets of aqueous antigen solution, using mineral oil for the emulsion). These adjuvants are viscous and can be difficult to inject. There is also the potential for the emulsion to breakdown with it becoming unstable if not correctly emulsified. This can lead to an incorrect dose of antigen being administered and thus reduce potency, or higher levels of mineral oil being injected increasing the risk of side effects.[52]

There have been great concerns about the side effects of water-in-oil emulsion adjuvants, which include local reactions, with the development of granulomas at the injection site, adhesions within the abdominal cavity and melanin deposition on the abdominal wall.[53] In some instances the growth rate, the welfare of the fish and the quality of the final product are affected, thus resulting in economic losses by the fish farmers.[7]

Alternative adjuvant systems are being developed to reduce the side effects seen with mineral oil adjuvants. Some vaccine companies are now using non-mineral oils, which although can still produce side effects, are much less reduced than seen with mineral oil adjuvants. Other companies are using oil-in-water emulsions, in which the antigen is contained in small oil droplets dispersed throughout an aqueous phase. This system potentially reduces the risk of side effects because the vaccine contains less oil.[52]

Research is now focusing on the use of microencapsulation adjuvant systems from which the antigen is slowly released.[52] The potential use of immunostimulants in vaccine preparation to increase the non-specific immune response to the antigens is also attracting interest.[54]

The Current Status of Bacterial Diseases for Fish

The present section examines the state of development for vaccines against the more predominant bacterial fish pathogens affecting aquaculture.

Vibriosis

Vibriosis, caused by bacteria belonging to the genus *Vibrio*, is considered as one of the most economically devastating diseases in aquaculture. The most significant *Vibrio* species to cause disease include *Vibrio anguillarum (Listonella anguillarum), V. ordalii, V. salmonicida* and *V. vulnificus* biotype 2, although several other *Vibrio* species are also associated with disease in fish and shellfish.[55]

Vibrio anguillarum and Vibrio ordalii

The impact of *V. anguillarum* (=*Listonella anguillarum*) on marine aquaculture is particularly notable, causing severe economic losses in Pacific salmon, Atlantic salmon (*Salmo salar*), rainbow trout, turbot (*Scophthalmus maximus*), sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*), striped bass (*Morone americanus*), cod (*Gadus morhua*), Japanese (*Anguilla japonica*) and European (*Anguilla anguilla*) eel, and ayu (*Seriola quinqueradiata*).[56] Ten different serotypes have been described for the bacterium, however, only serotypes 01, 02 and 03 (to a lesser extent) have been associated with mortality.[57] Serotype 01 is a very homogeneous group of bacteria based on their biochemical, serological and genetical characteristics, whereas two different antigenic groups can be found within serotypes 02 and 03. Serotype 02 can be further divided into subgroups 02α and 02β,[58] and serotype 02α bacteria appear to affect both salmonid and non-salmonid fish, while bacteria from the 02β group seem to affect only non-salmonid species. Serotype 03 is composed of serotypes 3A and 3B.[59,60] Only the former serotype has been isolated from diseased fish while the latter is an environmental species. Cell surface components on pathogenic strains, for example

outer-membrane proteins and lipopolysaccharides (LPS), have been associated with the serotype of isolates.[61,62]

V. ordalii (previously known as *V. anguillarum* biotype II) is another source of Vibriosis in salmonids in North America, Japan and Australia. It is phenotypically and genetically distinct from *V. anguillarum*.[63]

Traditionally, vaccines for *V. anguillarum* and *V. ordalii* consist of formalin-killed bacteria, with better protection achieved through i.p. injection, rather than immersion or oral vaccination.[64,65] However, the efficacy of these vaccines may be improved by enriching them with inactivated exotoxin,[66] or by using bacteria expressing iron-regulated outer membrane proteins (IROMPs).[57]

Attempts have also been made to develop a live-attenuated *V. anguillarum* vaccine.[67] These are based on mutants deficient in siderophore synthesis[68] or lacking outer membrane protein OM2,[69] or with impaired 2,3-dihydroxybenzoic acid, a precursor of plasmid-mediated siderophore anguibactin.[70] Singer *et al.*[71] showed that the mutants they had prepared were still present in fish nine days after inoculation.

Another important consideration for vaccine companies is the formulation of the *Vibrio* vaccine with respect to *V. anguillarum* serotypes and *Vibrio* species. Many commercial vaccines use both *V. anguillarum* serotypes 01 and 02 and *V. ordalii*, or combinations of these to reflect the geographic relevance of the different isolates and the fish species being vaccinated. However, it is unclear whether *V. anguillarum* serotypes 02α or 02β is added. Recently, *Vibrio* species taxonomically related to *V. anguillarum* (VAR) have been identified in vaccinated fish, and are biochemically and serologically distinguishable from *V. anguillarum*.[72–74] These have been placed into six serogroups (A, B, C, D, F and G). It is possible that these VAR stains of *Vibrio* should be considered in vaccine formulations for particular regions.[57]

Vibrio salmonicida

V. salmonicida is responsible for a cold water Vibriosis known as Hitra disease and has been reported to affect salmonids and cod in Norway,

Scotland and Canada.[57] The disease usually occurs in late autumn, winter and early spring,[75] with fish displaying signs of anemia, hemorrhages and general septicemia. The isolates of *V. salmonicida* appear very similar in their biochemical and serological characteristics,[76] but distinct to *V. anguillarum*.[77] The LPS and outer membrane profiles of *V. salmonicida* and *V. anguillarum* are different, and antiserum from Atlantic salmon detects antigenic differences between the two bacteria.[78] Also, vaccines against *V. salmonicida* do not protect against *V. anguillarum*.[79] Therefore, commercial multivalent vaccines have been prepared, which are composed of both *V. salmonicida* and predominant species of *V. anguillarum*.[57]

Vibrio vulnificus

V. vulnificus is associated with Vibriosis in eels,[80,81] with outbreaks recorded in Japan, Taiwan, Spain and the UK.[55,57] Two biotypes of the bacterium exist. Biotype 1 is a potential human pathogen,[82] while biotype 2 is pathogenic to eels, although this biotype can also be zoonotic to humans.[83]

Although the two biotypes share many similar virulence factors (e.g. production of a capsule and siderophores),[84] the biotype 1 group of *V. vulnificus* is antigenically very heterogeneous with the ten different serotypes identified, and the biotype 2 group has only one serotype.[57] The LPS of the two biotypes is antigenically different, while the outer membrane proteins show antigenic relatedness.[85]

An experimental vaccine consisting of an exotoxin-enriched preparation with capsulated strains of the pathogen has shown promising levels of protection, both in the laboratory and in the field.[57] To date, no commercial vaccine is available against *V. vulnificus*.

Vibrio viscosus (=Moritella viscosa)

V. viscosus and *V. wodanis* have recently been associated with "Winter Ulcer," affecting salmonid fish reared in saline water in Norway, Iceland, and recently, Scotland. *V. viscosus* isolated from diseased fish has been grouped into homogeneous subgroups according to their geographical

origin.[86] The authors recommended the re-classification of *V. viscosus* as *Moritella viscosa* based on 16S rRNA gene sequence similarity of 99.1%, and indeed the bacterium has since be re-classified. An inactivated bacterin vaccine of *M. viscosa* was shown to give protection,[87] and the bacterium is now included in some of the multivalent commercial vaccines for Norway and the UK.

Yersiniosis

Yersinia ruckeri is the causative agent of Enteric Redmouth (ERM) disease or Yersiniosis, and causes acute or chronic infections in salmonids.[88,89] The bacterium is a Gram-negative bacterium in the family Enterobacteriaceae. Disease outbreaks appear related to conditions of stress or poor water quality, possibly because a carrier state exists with the bacterium lying dormant in fish until times of stress.[90,91] The bacterium has been identified in the US, Canada, Australia, South Africa, Chile and much of Europe,[92] and at least eight different serological groups are thought to exist based on whole cell reactions, O-antigens and LPS profiles.[93] Two of the most predominant groups of *Y. ruckeri* belong to serovar type 1 (Hagerman) which is more commonly isolated from rainbow trout, and serovar type II (O'Leary) first isolated from chinook salmon (*Oncorhynchus kisutch*).[94,95] Serovar 1 was originally thought to be the most virulent serovar, but it has since been shown that serovar 2 can be as virulent as serovar 1.[96] Serological reactions to the LPS and the whole cells between the two groups is distinct.[97–99]

Yersiniosis is successfully controlled with commercial vaccines and in fact represents one of the first diseases to be controlled by vaccination.[90,100] Most vaccines are bacterin preparations using whole cell preparations of serovar 1 (the Hagerman strain and the major cause of disease outbreaks). Bacteria are generally inactivated with formalin and some times pH lysed at pH 9.8 to expose internal cell components. It is still unknown which antigens on *Y. ruckeri* are responsible for protection.[93,101] The LPS of serovar 1 elicits a weaker (or negligible) antibody response[93] and lower cell proliferation memory response compared to serovar 2.[102] Reports of the degree of cross-protection

by serovar 1 against other serovars appear mixed.[103,104] Serovar I appears to cross protect against serovar II in North America, but vaccines containing the Hagerman stain do not protect as well against Norwegian serogroups I and II, but do protect against Norwegian serogroup III.[103] The addition of Norwegian serogroup II to the Hagerman vaccine improved protection against Norwegian serogroup II bacteria.

Various routes of administration (intraperitoneal injection, direct immersion, shower or spray, feeding and anal intubation) have been evaluated and provide good levels of protection,[101] although commercial vaccines for Yersiniosis tend to be administered by i.p. injection or by immersion. The success of the vaccine has been reported to be variable under field conditions, and often does not completely prevent disease outbreaks when the level of infection is high, as seen when fish are stressed.[92] Clearly, a greater understanding of the fish immune response against *Y. ruckeri* and the virulence factors and antigens elicited by the bacterium would help improve this situation.[93]

Furunculosis

Aeromonas salmonicida is an important fish pathogen causing significant economic losses to aquaculture worldwide except for South America.[105] Typical *A. salmonicida* (*A. salmonicida* subsp. *salmonicida*), causing Furunculosis, has been associated with clinical or covert disease in salmonid species, although non-salmonid fish can also be infected. In the past, it was associated more with salmonid aquaculture since salmonids were the predominant species being cultured.[106] In the 1980s prior to effective vaccination, mortality levels could range between 15–20% per annum in sea water salmon farm sites.[105] On the other hand, atypical *A. salmonicida* (*A. salmonicida* subsp. *mascoucida* and *A. salmonicida* subsp. *achromogenes*), has been associated with atypical Furunculosis in Atlantic salmon in Iceland, ulcer disease in goldfish and carp erythrodermatitis.

The disease can either be acute or chronic,[107] and covert infection can exist within a population with no clinical signs of disease. The bacteria are believed to reside in a "carrier state" within the animal's gut and can lead to disease outbreaks once the fish experiences some

sort of stressor.[108] Both clinical and convert infections are often seen during smoltification and spawning, in the spring during increasing water temperatures, but Furunculosis has been seen in alevin and fry at water temperatures as low as 2–4°C.[106]

A. salmonicida is a Gram-negative rod. Typical and atypical stains have many biochemical, serological and antigenic traits in common, although it is possible to differentiate between them biochemically.[109] For example, typical stains usually produce a brown, melanin-like, pigment on nutrient agar. The outer membrane of the bacterium consists of a cell surface A-layer protein supported by the O-antigen polysaccharide of the LPS molecule. The A-layer protein is present in recently recovered isolates and is associated with the bacterium's ability to autoagglutinate and with virulence (adherence and invasion), although it disappears after repeated subculturing *in vitro*.[105] Beneath this lies the phospholipid bilayer and other membrane proteins including IROMPs.[105] A bacterial capsule has also been observed *in vivo*[16] and under glucose-enriched culture conditions,[110] which helps protect the pathogen from host defences.

Commercial vaccines are available and have been very successful in protecting against Furunculosis. Isolates of the bacterium appear serologically very homogenous, with immunogenicity linked to LPS and the cell surface A-layer protein. Oil-adjuvanted vaccines appear to give the longest lasting protection against the disease, but as discussed above, side effects remain problematic in vaccinated fish.[111,112] Unadjuvanted Furunculosis vaccines have been of mixed success, however,[113] but the introduction of an unadjuvanted iron-restricted bacterin, with increased IROMPs on the bacterium,[14] resulted in higher levels of protection, with RPS values of >80% recorded in laboratory trials.[105] Antibody levels in fish vaccinated with the unadjuvanted vaccine were of short duration, so alhydrogel (an alum-adjuvant) was added to increase the length of the antibody response to over a year.[105] As a point to note, it is unclear if vaccination can induce potential immune carriers or if covertly infected fish remain infected.[106]

Injectable, adjuvanted vaccines containing *A. salmonicida* subsp. *achromogenes* have been successful in inducing antibodies and a protective response against atypical Furunculosis in salmon in Iceland.[114]

Pasteurellosis

The Gram-negative bacterium *Photobacterium damselae* subsp. *piscicida* (*Ph.d.p.*) (previously known as *Pasteurella piscicida*) is the causative agent of fish Pasteurellosis. The disease was first reported in the US.[115] It became a major problem for the mariculture of yellowtail (*Seriola quinqueradiata*) in Japan,[116] and sea bass and sea bream (*Sparus aurata*) in the Mediterranean.[117] Currently, Pasteurellosis still causes major problems in Japan and Europe. Vaccines available on the market in the late 1990s did not appear to give satisfactory protection,[118] and although improved formulations are now commercially available, research into improvement of vaccine efficacy continues.[119,120]

Previous studies have demonstrated that although vaccination can lead to measurable antibody titers, these are not correlated with protection.[121] Reports by Bakopoulos *et al.*[120] and Fukuda and Kusuda[122] on serum from recovered fish giving only short-term protection suggests that humoral factors alone may not be sufficient to halt the development of the disease. Hamaguchi[123] also suggested that stimulation of cellular immunity was required for effective protection against Pasteurellosis in yellowtail, with live-attenuated bacteria conferring higher protection than heat-killed or formalin-killed cells.[124] It is well known that live vaccines are more efficient stimulators of cellular immune responses,[125] and although they are unlikely candidates for a commercial Pasteurellosis vaccine, they can assist in the identification of the protective antigens. A vaccine should contain antigens that are expressed *in vivo* and involved in disease pathogenesis.[126]

Despite the fact that soluble antigens are not good immunogens when compared to particulate ones,[127] it has been suggested that extracellular products (ECPs) should be included in the Pasteurellosis vaccine.[119,128] In fact, several authors have reported that the inclusion of ECPs in experimental vaccine mixtures comprising inactivated whole cell bacterial cells administered via immersion or i.p. injection improved protection of challenged fish.[128,129] Recently vaccination trials using a variety of vaccine candidates (including ECPs) have indicated that there appears to be a correlation between the effectiveness of the route of

vaccine administration and the development of the fish immune system.[130]

Streptococcosis

Streptococcal infections of fish are caused by a range of Gram-positive cocci consisting of a variety of genera and species. These include *Streptococcus iniae*, *S. difficile*, *Lactococcus garvieae* (=*Enterococcus seriolicida*), *L. piscium* and *Vagococcus salmoninarum*. Originally, Streptococcosis was associated with yellowtail culture in the Far East, but it has since been responsible for significant economic losses in a variety of fish species in the US, South Africa, Australia, Israel and parts of Europe.[131] Infections by *S. iniae* have resulted in significant economic losses in warm water species such as tilapia (*Oreochromis niloticus*).[132] Some of the bacteria mentioned above are also zoonotic to humans.

Initial vaccine development for Streptococcosis used formalin-killed and/or heat-killed bacteria, and inactivated ECP. The preparations were administered by i.p. injection, orally or by spray in ayu or rainbow trout, and they gave mixed levels of protection.[133–136]

A number of laboratory and field studies were then carried out by researchers in Europe,[137–139] using whole-cell formalin-inactivated preparations of *S. iniae, S. difficile* and *L. garvieae*. The authors obtained good levels of protection with autogenous *S. iniae* and *L. garvieae* vaccines in rainbow tout and *S. difficile* vaccine in tilapia under laboratory conditions. They also showed the vaccines to be effective in field trials carried out in Israel and Italy, but the duration of the response in rainbow trout to *L. garvieae* was not as long lasting as seen in the laboratory studies.[131]

More recently, researchers in the US have carried out a number of trials looking at the immune response of tilapia to killed *S. iniae*.[140–142] They were unable to correlate antibody responses with protection in these experiments, but they did show a correlation between specific antibodies and protection using passive immunization.[132]

Schering-Plough Aquaculture is now marketing an immersion vaccine for Streptococcosis.

Bacterial Kidney Disease

The causative agent of bacterial kidney disease (BKD), *Renibacterium salmoninarum* has been isolated from a variety of salmonid species cultured in the US, Canada, Europe, Japan, Scandinavia and South America,[143] where the disease has been responsible for serious economic losses in intensively cultured fish populations. The disease generally appears as a chronic bacteremia with focal lesions in the viscera, although acute outbreaks have been observed.[144] It can affect salmonids at all stages of their life cycle in both fresh and salt water, although most epizootic episodes seem to occur during the first winter or early spring of the fish's life cycle, either during the final hatchery holding stage or on transfer to sea water.[144,145]

R. salmoninarum is able to survive and multiply within mononuclear phagocytes[146–149] (Fig. 1). Treatment and control of BKD is difficult, due partly to the fact that it is an intracellular pathogen, and is transmitted vertically via maternal infection.[151]

Chemotherapy as a means of controlling the disease has been largely unsatisfactory, possible because of the bacterium's intracellular nature.[152] Vaccination would be the ideal method for controlling the disease in

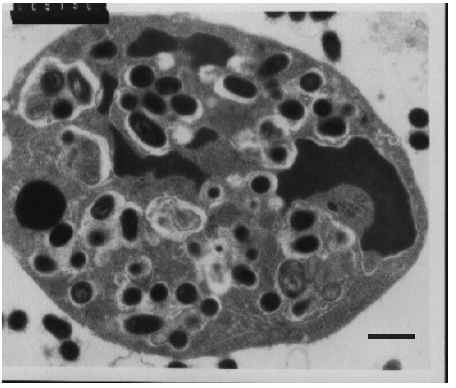

Fig.1 Transmission electron micrograph of *Renibacterium salmoninarum* 1113 growing within a macrophage (scale bar = 1 μm).[150]

aquaculture, but unfortunately attempts to immunize fish against BKD have been largely unsuccessful.[143]

Fish appear able to elicit both non-specific and specific immune responses against *R. salmoninarum*. Complement-mediated opsonization of *R. salmoninarum* has been shown to enhance bacterial adherence to rainbow trout macrophages and subsequent phagocytosis.[153] Its intracellular survival and multiplication is also enhanced by prior treatment with normal and immune serum, and also heat-inactivated serum.[154] Opsonization of the bacterium leads to increased levels of respiratory burst and nitric oxide production by macrophages immediately following ingestion of the bacterium.[155,156] Sakai *et al.*[157] found greatest protection with vaccine preparations in groups of fish that had the highest phagocytic activity and chemiluminescent response.

Fish are also able to elicit a specific immune response against *R. salmoninarum* during both natural infection and in response to experimental vaccination.[158–161] In an early study by Evelyn,[162] sockeye salmon were found to produce antibodies for 16 months following a single injection of killed *R. salmoninarum* mixed with Freund's Complete Adjuvant (FCA). In another study by Bartholomew *et al.*,[161] the specific antibody response against *R. salmoninarum* in immunized chinook salmon and naturally infected coho (*Oncorhynchus kisutch*) and chinook salmon appeared to be primarily against the 57 kDa protein of the bacterium. However, there does not appear to be any correlation between the specific antibodies and protection against the disease.

The success of vaccination against BKD has been mixed, with conflicting results existing between studies. In a study by Paterson *et al.*,[159] Atlantic salmon parr injected i.p. with inactivated *R. salmoninarum* cells mixed 1:1 with FCA had a strong, immune response and appeared able to reduce the level of infection in fish, but was not able to completely eliminate it. The results of McCarthy *et al.*[163] appeared more successful using pH-lysed bacteria without adjuvant, given by a single i.p. injection. However, hyperosmotic and immersion vaccinations with this preparation were unsuccessful. Sakai *et al.*[157,164,165] found formalin-killed *R. salmoninarum* administered by injection

stimulated both the humoral and cellular immune responses in immunized rainbow trout, but no protection was elicited by the vaccination upon challenging vaccinated fish.

The fact that whole cell bacterin preparations are problematic in producing consistent level of protection and antibodies, has led to alternative approaches being used in an attempt to develop a vaccine for BKD. Initial work focused on the p57 antigen.[143] The p57 antigen is the predominant protein and immunodominant antigen present on the cell surface of the bacterium, and is a major component of its extracellular proteins.[166–169] The protein processes a number of virulence characteristics, including immunosuppression. Turaga *et al.*[170] reported that it can suppress the antibody production of lymphocytes *in vitro*. Later, Fredriksen *et al.*[19] also demonstrated that *R. salmoninarum* surface proteins p22 and p57 caused immunosuppression of antibody production *in vitro* by B-cells stimulated *in vivo*. Wood and Kaattari[171] demonstrated an elevated antibody response to *R. salmoninarum* after removal of the bacterial cell surface associated p57 protein by heat treatment. Potentially vaccines may prove more efficacious if this immunosuppressive molecule could be removed from the bacterium. Recently, Piganelli *et al.*[172] demonstrated significant protection in coho salmon with an orally delivered vaccine consisting of formalin-killed *R. salmoninarum* with the p57 antigen removed by heat treatment ($p57^-$). However, fish injected i.p. with the $p57^-$ bacterium or fed oral vaccine with p57 cells did not demonstrate the same levels of protection.[172] Thompson *et al.*[49] found recombinant antigens prepared against the p57 antigen to cause immunosuppression in immunized rainbow trout challenged i.p. with *R. salmoninarum*. They did, however, obtain protection (between 30.8% and 59.3%) with recombinant antigens against the metalloprotease.

Other groups have looked at the possibility of using live-attenuated isolates of *R. salmoninarum*. Griffiths *et al.*[173] and Daly *et al.*[174] used a live-attenuated *R. salmoninarum* TSA1 strain as a live vaccine in Atlantic salmon, which resulted in reduced mortalities among fish exposed to an i.p. challenge of *R. salmoninarum*.

Kaattari and Piganelli[143] suggest that the efficacious vaccination preparations will probably be required to induce a vigorous cell-mediated response to destroy intracellular *R. salmoninarum* and to provide protective immunity.

Norvatis has developed a vaccine against the disease using live *Arthrobacter* spp., while Bayotec markets an autogenous vaccine for use in Canada.

Edwardsiellosis

Two major species of bacteria are responsible for Edwardsiellosis in fish, *Edwardsiella tarda*[175] and *E. ictaluri*,[176] although the diseases that they cause are quite distinct from each other. The former causes *Edwardsiella* septicemia (ES) and the latter, enteric septicemia (ESC) of catfish. Both bacteria are Gram-negative rods belonging to the family Enterobacteriaceae.

ES is a systemic disease of warm water species, although it has been isolated from migrating chinook salmon[177] and Atlantic salmon.[178] It is most prominent in cultured eels in Japan and Taiwan,[179] channel catfish (*Ictaluri punctatus*) in America, and Japanese flounder and other cultured fish in Asia.[180] It can also be zoonotic.[180] *E. tarda* may be divided into different serological groups based on O-agglutination tests, with four possible serotypes (A, B, C and D).[181] The most predominant serotype isolated from the kidneys of infected eels[181] and flounder[182] are of serotype A, suggesting that this serotype may frequently be associated with disease in fish.

The disease is currently controlled in the US using oxytetracycline and a potentiated sulphamide, although attempts have been made to develop a vaccine against the bacterium. In early immunization experiments, Song *et al.*[183] immunized elvers, 6 grams in weight, with a single immersion of a whole-cell bacterin preparation against which they some found protection, but two or three further immersion vaccinations were needed to increase the duration of the protection. In the following year, Salati *et al.*[184] immunized eels with formalin-inactivated whole-cell bacterin, bacterial LPS and culture filtrates, and found LPS

to be the most immunogenic. They later showed that the carbohydrate of the LPS was more immunogenic.[185] However, other researchers found formalin-inactivated whole cells and LPS administered by injection to only give slight to moderate levels of protection.[186] Likewise in Japanese flounder, formalin-inactivated whole cells administered by immersion or injection did not elicit protection although death was delayed in vaccinated fish,[187] and therefore further vaccine development is required for *E. tarda*.

ESC of catfish, caused by *E. ictaluri*, has become the most important bacterial disease of catfish farming in the US.[188] The host specificity of *E. ictaluri* is quite narrow and affects mainly catfish species. The disease usually takes the form of an acute septicemia, which rapidly develops, especially at temperature of 22–28°C, while a more chronic disease results outside this range.[188]

The bacterium is homogenous with regard to its biochemical, biophysical[189,190] and serological characteristics.[191–194] The organism has two major outer membrane proteins of 60 and 30 kDa, and the LPS of the bacterium contains no "O" side-chains.[180] The bacterium is very immunogenic and the fact that it is antigenically very homogenous has led researchers to look at the potential of using whole-cell bacterin preparation to protect fish,[195] and although antibodies are produced against these preparations, the antibody titers do not correlate with protection. However, Thune *et al.*[188] did find some correlation if the antibody response of fish was greater than 1/2048. Vinitnantharat and Plumb[194] also found increased protection in fish with antibody titers greater than 1/256 (agglutination titer). Thune *et al.*[188] has reviewed the various vaccination trials performed for *E. ictaluri* and found very mixed levels of protection. However, different vaccine preparations were used, many of which were based on whole-cell bacterin preparations, while some were component vaccines or live vaccines, administered by immersion, injection or orally. Live-attenuated vaccines are being developed for *E. ictaluri* based on deletion mutations in the aroA gene, the purA gene, or both, and ought to be incapable of reversion to virulence because of these mutations. It is intended to use these vaccines not only to vaccinate fish against *E. ictaluri*, but also to serve as vectors to present antigens from other fish pathogens.[195]

Two oral vaccines against *E. ictaluri* have been licensed for use in the US.

Piscrickettsiosis

Rickettsia-like organisms (RLO) have been observed or isolated from a variety of both fresh and sea water species worldwide.[196] The importance of rickettsiae as an emerging fish pathogen first became apparent in 1989 when an estimated 1.5 million coho salmon died in Chile of an unknown etiology.[197] The main hosts of the disease appeared to be salmonids,[198] and for this reason the disease has been called "salmonid rickettsial septicemia,"[199] caused by an obligately intracellular pathogen *Piscirickettsia salmonis*, although it is also been referred to as Piscirickettsiosis[200] (Fig. 2).

Vaccination is potentially very useful for controlling Piscirickettsiosis, but few vaccine studies have been reported in the literature for the

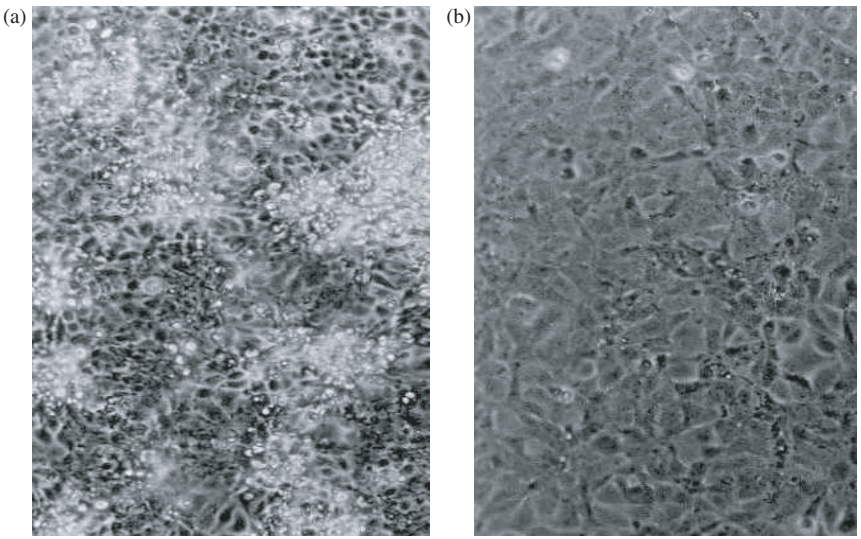

Fig. 2 Development of CPE by *Piscirickettsia salmonis* in CHSE cells. **(a)** CPE produced by *P. salmonis*, 7 days post-inoculation (magnification x100); and **(b)** 100% confluent CHSE-214 cell line (5 days old) (magnification x100).[201]

disease.[198] Little is known about the immune response of salmonid fish to *P. salmonis*, but a weak humoral response is elicited during both natural and experimental challenges.[198] However, antibodies against the organism have been detected in convalescent fish.[202] As well as reacting with a range of proteins and a carbohydrate antigen (at 11 kDa), the antibodies in these sera recognize the OspA lipoprotein on the bacterium.[203] In initial vaccination trials by Smith *et al.*[204,205] fish were vaccinated i.p. with formalin-killed preparations of *P. salmonis*. A lower cumulative mortality was observed in fish vaccinated with a non-concentrated bacterin compared to that of the control group, while a slightly higher mortality rate was obtained with fish vaccinated with a concentrated antigen emulsified in FCA. Although the results with non-concentrated bacterin appear encouraging, the extent of natural challenge was unknown and *R. salmoninarium* as well as *P. salmonis* were detected in experimental fish.

Relative percentage survival values of 35% were obtained in a trial where fish were injected with whole-cell preparations of *P. salmonis*, emulsified in an oil-in-water adjuvant. However, RPS values of 83% were obtained in the same trial with fish immunized with recombinant OspA lipoprotein fused with T-cell epitopes from tetanus toxin and measles fusion protein.[48] The results of this trial are potentially very encouraging and may offer potential control of the disease if licensing of this vaccine is approved.

Flavobacterium and Flexibacter Infections

Chromogenic, Gram-negative, gliding bacteria, belonging to the family Flavobacteriaceae, have been found to be pathogenic for many fresh water and marine fish species cultured worldwide.[206] These organisms include bacteria in the genera *Cytophaga*, *Flexibacter* and *Flavobacterium*, and currently seven different species among these genera are considered to be pathogenic to fish.[207] Four of these have caused substantial economic losses to aquaculture stock worldwide, namely *Flavobacterium branchiophilum, Flavobacterium psychrophilum, Flavobacterium columnare* and *Flexibacter maritmus* (=*Tenacibaculum maritimum*), with the latter

being a problem in marine species. Although no commercial vaccines are available for these pathogens, except *Flexibacter maritmus*, active research is underway in the development of a vaccine for all four pathogens.

Flavobacterium branchiophilum

Flavobacterium branchiophilum is the causative agent of bacterial gill disease (BDG) and has been isolated from a variety of different fresh water species of fish in the US, Japan, Hungary and Canada.[207]

During infection, severe epithelial lesions can be seen on the gills of infected fish[208,209] with large numbers of the bacterium colonizing the surface of the gill lamella.[208,210] However, the bacterium does not appear to be able to invade the gill tissue of its host, since the bacterium has not been isolated from all tissue except the gill,[211] and no systemic infections have been reported in fish with colonized gills.

The disease has been successfully transmitted by bath challenge, although transmission only appears successful when a recently isolated strain of *F. branchiophilum* has been used.[212] Both virulent and avirulent strains of the bacterium have been found.[213] Antigenic variations have also been found between strains,[210,214] with differences existing between isolates from different geographic locations and also between isolates from the same geographic location.[210,215] Common antigens have also been identified between islolates.[209,210,214,215]

Fish do not appear to produce serum antibodies against the bacterium during infection (natural or artificial).[39,216] Presumably since the bacterium only affects the gills with no apparent systemic infection, a systemic antibody response would be less effective against the organism than a localized gill immune response, and indeed specific gill-surface antibodies have been observed in brook trout during artificial infection.[39] However, fish that had recovered from infection do not appear to be protected from subsequent infections.[216]

Attempts have been made to develop a vaccine against BDG, with promising results. In trials carried out by Lumsden *et al.*,[211] rainbow trout were vaccinated several times at six-week intervals by bath using acetone-killed *F. branchiophilum*. When fish were challenged four weeks

after the last vaccination, cumulative mortalities were significantly reduced compared to the non-vaccinated controls, and almost complete protection was obtained when fish had been bath-vaccinated three times. The authors noted a significant decrease in the amount of gill-associated *F. branchiophilum* antigen throughout the first three days of the challenge presumably due to a localized gill immune response.

Flavobacterium psychrophilum

Bacterial cold water disease (BCWD) is a serious septicemic infection of hatchery-reared salmonids (*Oncorhynchu*s spp. *Salvelinus* spp. and *Salmo* spp.). The disease is known as BCWD because outbreaks normally occurred at temperatures below 10°C.[217] Borg[218] experimentally reproduced the disease using an isolate obtained from diseased fish, which he classified as *Cytophaga psychrophila*, and this has since been re-classified as *Flavobacterium psychrophilum*. BCWD is a condition responsible for large mortalities in salmonids in the US and Canada, particularly in coho salmon.[219]

More recently, the organism has been associated with a systemic disease affecting hatchery-reared rainbow trout fry and fingerlings in most European countries, and has come to be known as rainbow trout fry syndrome (RTFS).[220–225] It also affects fish in Japan, Tasmania and Chile.[217] It generally affects rainbow trout weighing between 0.2 to 10.0 grams at water temperatures between 6°C and 16°C,[227] and is believed to be transmitted both horizontally and vertically.[219,227–231]

Despite its importance as a fish pathogen, the serology, antigenicity and pathogenicity of *F. psychrophilum* is still poorly understood. Several authors have found common antigens existing among *F. psychrophilum* isolates.[226,231–234] It is clear from the literature that serotypic differences exist between the different isolates of *F. pychrophilum*, although the exact number has not yet been fully established. Depending on the immunological analysis used and the geographical origin of the strains, two,[234,235] three[225,236,237] or more serotypes[226] (Fig. 4) have been reported to exist.

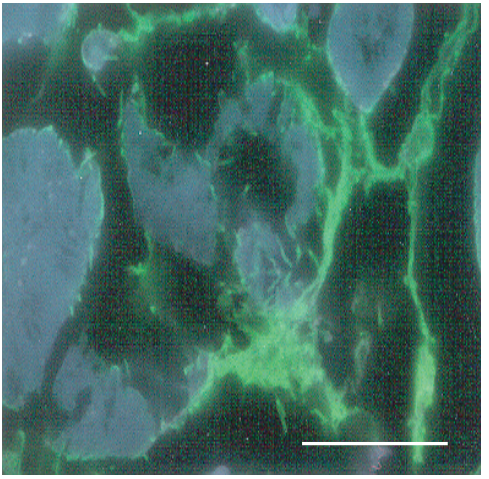

Fig. 3 Analysis of tissues from rainbow trout fry artificially challenged with an intramuscular injection of *Flavobacterium psychrophilum* isolate B97026P1 by indirect fluorescent antibody technique using rabbit anti-F. *psychrophilum* B97026P1 serum: muscle 5 days post-injection (scale bar = 10 μm).[226]

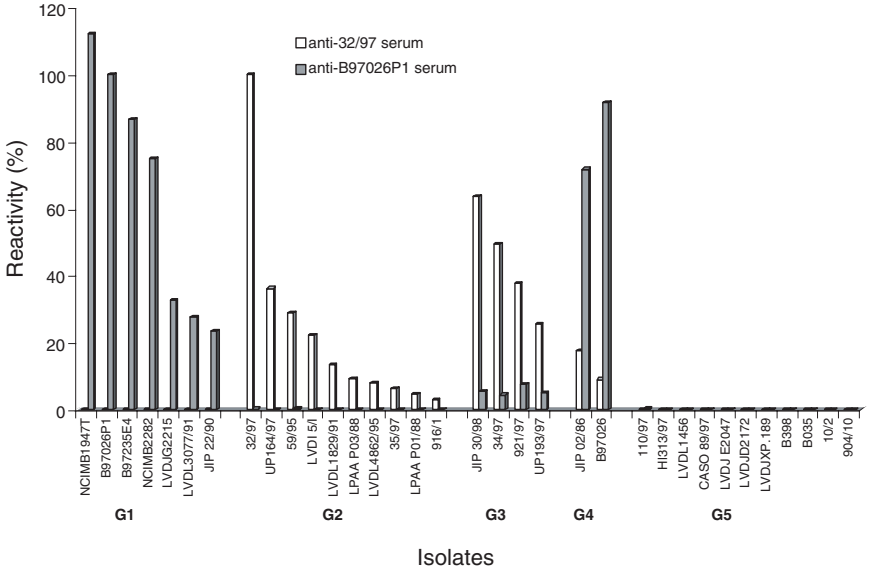

Fig. 4 Percentage reactivity of the rabbit anti-*Flavobacterium psychrophilum* sera against a variety of *F. psychrophilum* strains using an ELISA method (G: group).[226]

The use of antibiotics is not ideal for the treatment of RTFS, not only because of the development of antibiotic-resistant strains, but also because it does not eliminate the source of infection, which is likely to be infected brood stock fish.[238]

No commercial vaccine is currently available to protect against *F. psychrophilum* infection. There are a number of problems in developing a vaccine against this pathogen. It is a fastidious organism, which has proven difficult to culture under laboratory conditions. Also the number of existing serotypes has not yet been fully defined and the mechanisms involved in the defence of fish against *F. psychrophilum* infection are poorly understood. Intraperitoneal injection is not suitable for vaccinating small fry so an effective immersion or oral vaccination is required to vaccinate fry. Protection against the bacterium has been shown with i.p. injections of formalin-inactivated *F. psychrophilum* mixed with FCA and also with the bacterin by immersion.[235,239,240] The outer membrane fraction of the bacterium containing LPS and proteins has also been shown to be highly protective against BCWD in rainbow trout and ayu.[241] Vaccination as a measure to prevent outbreaks of RTFS in very young fry may not be completely successful, however, vaccination of brood stock may help to prevent vertical transfer of the pathogen.

Other Bacterial Pathogens

Some other significant bacterial diseases of fish include Mycobacteriosis (caused by predominantly *Mycobacterium marinum, M. fortuitum* and *M. chelonae*), Nacardiosis (caused mainly by *Nocardia asteroides and N. seriolae*), Motile Aeronomas Septicemia (agents include *Aeromonas hydrophila*) and infection by *Pseudomonas anguilliseptica*.[242] No commercial vaccines are as yet available for these diseases, although development is underway for all these pathogens, particularly *A. hydrophila*, which represents a very antigenically heterogeneous group of bacteria. This is one of the main factors hindering universal vaccine protection for *A. hydrophila*, although autogenous vaccines are available.

Commercial Vaccines Against Bacterial Pathogens of Fish

There are currently five internationally recognized fish vaccine-producing companies (Table 1), although a number of other vaccine companies can be found supplying a national market. The major market for the

Table 1 International fish vaccine manufacturers.

Fish vaccine manufacturer	Base	Parent company
Alpharma Aquatic Animal Health Division, PO Box 158 Skoyen N0212 OSLO, Norway. Tel: (+47) 2252-9079, Fax: (+47) 2252-9680.	Norway	Alpharma Inc.
Norvatis Animal Vaccines Enterprise House, Springkerse House Park, Stirling, FK7 7UF, Scotland. Tel: (+44) 1786-448200, Fax: (+44) 1786-448206.	Canada	Novartis
Schering Plough Aquaculture Aquaculture Centre, 24-26 Gold Street, Saffron Walden, Essex, CB10 1EJ, UK. Tel: (+44) 1799-528167, Fax: (+44) 1799-525546.	UK	Schering Plough
Intervet Norbio AS Thormøhlensgate 55, N-5008 Bergen, Norway. Tel: (+47) 5554-3750, Fax: (+47) 5596-0135.	Norway	Invervet International (Akzo Nobel)
Bayotek International Inc. 6761 Kirkpatrick Crescent, Saanichton, B.C. Canada, V8M 1Z8. Tel: (+01) 250-652-4482, Fax: (+01) 250-652-4802	Canada	Bayer AG

vaccine producers is salmon, however the trout market is also significant, and there is increasing opportunities for development in marine aquaculture for species such as sea bass, sea bream, turbot, halibut, yellowtail and cod.

Commercial vaccines are now available for a range of bacterial diseases, many of which are caused by Gram-negative bacteria, although the number of available commercial vaccines varies between countries because of licensing legislation. Fully licensed vaccines are commercially available for *Aeromonas salmonicida, Photobacterium damselae* subsp. *piscicida, Vibrio anguillarum* (serotypes 1 and 2), *V. ordalii, V. salmonicida, V. viscosus* (=*Moritella viscosa*), *Yersinia ruckeri, Flavobacterium columnare, Edwardsiella ictaluri, Streptococcus iniae and Lactococcus garvieae.*

Many of the vaccines produced commercially are multivalent, offering protection against a variety of pathogens.[243] The multivalent vaccines appear as effective as monovalent vaccines, and have the advantage over monovalent vaccines in that they lower the overall cost of vaccination and reduce the number of the times the fish has to be handled. Vaccines containing a mixture of *V. anguillarum, V. salmonicida* and *A. salmonicida* antigens appear to provide better protection against Furunculosis than monovalent Furunculosis vaccines, and multivalent vaccines containing *Vibrio* and *A. salmonicida* in alum adjuvants seem more effective than monovalent *A. salmonicida* vaccines.[112]

Immunostimulants

Vaccination has been effective in controlling many of the diseases affecting the aquaculture industry, but only in conjunction with improvements in farm management, nutrition and other disease control methods. However, there are occasions when specific treatments may not be available or may be ineffective due to stress-induced immunosuppression or the immaturity of the animal. Routine fish husbandry is potentially very stressful, particularly during transportation when fish are subjected to stressors such as handling, crowding and changes in water quantity and water temperature. Carriers of disease may act as a reservoir for infection and

when subjected to stressful events, their immunosuppressive state allows the pathogen the chance to multiply and cause disease. In addition to disease-associated mortalities, immunosuppression can lead to decreased growth and condition.

The application of immunostimulants by the aquaculture industry appears useful in combating this stress-associated immunosuppression.[54] These substances have been reported to enhance the immune system of the fish in the short term, when applied either on their own or in vaccines as adjuvants, and are apparently very effective in stimulating the non-specific defence mechanisms of the animal. Examples of commercially available immunostimulant/immunomodulating products for aquaculture are shown in Table 2.

Table 2 Commercially available immunostimulant/immunomodulating products for aquaculture.

Company	Product/Comments
American-based companies	
American Standard Products Inc.	Energee IMS Gold (antiviral)
Aqua-In-Tech Inc.	Highly fortified diets specifically manufactured for acclimation: extra vitamins, living bacteria, yeast, bacterial and algal extracts
Bonimex, Bonimex Santiago	Worldwide suppliers of Bonuline animal feed immunostimulant
Centrovet Ltd., Av.	Supplementary vitamin mixes
Cenzone Tech Inc.	"Aquature" fish feed containing 1,3-beta-, 1,6-beta- and 1,6-D-glucans
ImmuDyne Inc.	ProTropica™ — and ImmuStim™
Levapan SA.	Producer of beta-glucan immunostimulant
M and M Inc.	Bountiful Harvest (fish formulations available for addition to feeds)
MST Enterprises	"Aquaplus" krill products for feed manufacture and use of chitin as an immunostimulant
Nutri-Agri Technologies Inc.	Producers of Dinamune, immunostimulant of shrimp and fish, contains 1,3-beta-glucans

Table 2 (*Continued*)

Company	Product/Comments
Zeigler Bros Inc.	Feed and immunostimulant producer, vitamin C supplier
European-based companies	
Schering Plough Aquaculture	AquaVac™ Ergosan, the algine-based immunostimulant is shown to have multiple benefits in all fish including salmon
Bassleer Biofish	Bio food /immunostimulant for ornamental fish
EWOS	Producers of Vextra boosterfeed
Inve Technologies NV	Immunopak, immunostimulant pre-mix
Park Tonks	Producers of LifeBoost Aq, salmon and trout fingerling immunostimulant and Aquamine XL for recovering fish
Rich Series	Rich Series, algal-rich immunostimulant
Alpharma Animal Health Ltd.	Vetregard (1,3-beta- and 1,6-beta-glucans, mannan oligosaccharide and peptidoglycan)
Asia-Pacific companies	
Aquatic Diagnostic Services International (ADSI)	Distributors of Aquaguard, immunostimulant (1,3-beta-glucans) feed additive for aquaculture and ornamental fish industries
Inve (Thailand) Ltd.	Regional office of producers of Immunopak, immunostimulant

Source: Stirling Aquaculture (2001).

Conclusion and Future Prospects

Investment in vaccine development for aquaculture is increasing as the industry grows and new vaccine technologies become available. The list of diseases that can currently be controlled through vaccination is steadily increasing and these are very successful in reducing mortalites, and in turn, the use of antibiotics used in aquaculture. Fish vaccines

have become much more sophisticated in recent years with a trend for the development of subunit recombinant vaccines, in preference to the original killed whole-cell preparations. This has been necessary for some diseases because the simpler bacterin approach has not been successful and attempts at attenuated vaccines in general have not been encouraging from a safety point of view. New technologies such as recombinant and DNA vaccines are powerful tools for future vaccine development as these enable the isolation of potential protective antigens from suppressive ones. Even with recombinant technology there is still a need to identify and characterize vaccine candidates. The application of Proteomics in vaccine development is also an exciting new development as vaccine antigens can be characterized with great precision.

The commercial availability of a variety of anti-fish species IgM antibodies to assist in the identification of potential protective antigens, efficacy testing and determining the rate and duration of the immune response in a large variety of cultured fish species will without doubt assist in the development of fish vaccines to protect against significant and emerging diseases.

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Chapter 13

Current Trends in Immunotherapy and Vaccine Development for Viral Diseases of Fish

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Introduction

Viral immunity and vaccine strategies designed to prevent or minimize the impact of diseases in intensive salmon and trout culture is intricate work requiring integration of biological and economic variance. These elements can be analyzed scientifically from a basic or applied approach with the latter approach being a conduit for the third element, the production, commercialization and use of the product to improve fish health. It is difficult to discern and integrate these elements during preliminary investigations, but for clarity it is useful to understand which stage of scientific research is being addressed by a particular experiment or experimental outline. Basic viral vaccine research in finfish aquaculture should not necessarily require a motive such as the development of a commercial product. Without this limitation, research continuity could be attained resulting in a deeper understanding of the host-pathogen interaction and the development of effective control strategies. In this chapter, some of each element will be addressed but the primary focus will be basic biology and a description of the importance and utility of using a model system for systematic analysis and comparison of therapeutics and vaccine types.

The process of vaccine research and development requires that the effect of an immunomodulator on viral pathogenesis can be measured experimentally. The performance of a vaccine or therapeutant is determined by measuring its potency, efficacy and safety performance using a well-defined experimental model system. A model system requires an understanding of the dynamics of interaction between the host, pathogen and environment. Points to consider when developing a model system include host range and susceptibility, virus strain characteristics, route and entry and progression of disease, virus load, water temperature and other environmental variables, as well as fish size, age and life stage. A well-characterized model system has been developed for infectious hematopoietic necrosis (IHN) virus, a pathogen of salmon and trout and a member of the family *Rhabdoviridae* and the genus *Novirhabdovirus*. The disease is most acute in young fry and mortalities can be as high as 100% in a given population. IHN virus infection progresses from two major sites of entry: from the gills to the circulatory system and from the oral cavity to the gastrointestinal tract and into the circulatory system. Roughly 3 to 5 days after exposure, IHN virus is found in virtually every tissue resulting in an acute systemic infection with the epizootic period occurring between 6 to 14 days.[1] The sequelae suggests that targeting innate or acquired immune effectors that disrupt the virus at primary sites of replication would be an extremely effective control strategy. In addition, pre-existing antibodies could be effective in preventing viremic spread to sites distal from primary infection.

It is not necessary to understand the mechanism or the identity of the effectors that mediate immunity upon treatment with a therapeutant or vaccine. As a result, there is much more information available about activators rather than effectors of the fish immune response. For IHN virus, the G protein is the single viral protein that is necessary and sufficient for eliciting an IHN virus protective immune response in fish.[2–5] Rhabdovirus G proteins form membrane-anchored, extracellular spikes that coat the surface of the 60–85 nm × 180 nm virus particle. The IHN virus G protein is composed of 488 amino acids; a 20 amino acid signal sequence is removed from the N-terminal sequence during protein maturation.[6,7]

The IHN virus G protein, like other rhabdovirus G proteins, is an integral membrane protein. It is believed to be a homotrimer that protrudes from the virion particle, binds cell surface receptors and facilitates entry into the host cell.[6] The G protein of *Novirhabdoviruses* contains conserved cysteines that form six extracellular disulfide bonds, the position of the bonds being distinct from those found in the G protein of other rhabdoviruses.[8] IHN virus G protein amino acids involved in virus neutralization have been mapped using a panel of 12 neutralizing monoclonal antibodies.[9] Comparison of amino acid changes in the G protein of monoclonal antibody escape mutants with wild-type virus, led to the discovery of two primary antigenic sites composed of linear and discontinuous epitopes. Carbohydrate antigens are not involved in the binding of the monoclonal antibodies. Antigenic site 1, amino acids 230–231, is a conformation-dependent epitope that does not react with monoclonal antibodies when the glycoprotein is analyzed under reducing conditions. Antigenic site 2, amino acids 272–276, is composed of a linear and a discontinuous epitope and is the major neutralizing epitope of IHN virus. The discontinuous epitope in antigenic site 2 may possibly interact with amino acids 78–81 via a folded loop structure on individual glycoprotein monomers.

Vaccines and therapeutants for the control of IHN virus can elicit a potent immunoprotective response in trout and salmon resulting in sterilizing immunity. Attenuated,[10,11] killed,[12,13] recombinant,[14–18] peptide[5,19] and DNA vaccines[2,20–23] have been investigated for the control of IHN virus.[24] Compounds that act as immune-modulators, such as double-stranded poly I:C RNA,[25] Immune Stimulating Complexes (ISCOMS) and recombinant human interleukin (IL)-2[26] have been tested for their ability to elicit an antiviral state in fish. In addition, passive immunization, probiotics, antiviral drugs and adjuvants as well as co-infection with avirulent heterologous virus has been evaluated as a control method.[27,28] The studies have established that IHN virus vaccine(s) can protect fish against IHN disease with varying degrees of success,[4,5] that protection involves activation of select effectors of the immune system[29,30] and that the antiviral state may be established soon after vaccination and last for a considerable length of time.[13,22]

Therapeutants

Passive Immunization

Passive serum transfer has been examined as a prophylactic and/or therapeutic strategy for the control of viral diseases of finfish.[31] It was previously reported that protection against IHN virus could be attained after passive transfer of anti-IHN virus rainbow trout immune serum.[29,32] When rainbow trout (mean weight, 1 gram) were injected with 50 to 200 μl of pooled serum from juveniles (IHN virus neutralizing titer 1280) or adults (titer 640) that had experienced a natural infection with IHN virus and had survived, relative protection was 63% to 100% against IHN virus compared to rainbow trout injected with normal serum (titer < 20) or saline. LaPatra *et al.*[33] also showed that when rainbow trout (mean weight, 4.3 grams) were injected with 100 μl of anti-IHN virus immune serum (titer, 640) immediately following waterborne exposure to 10^5 plaque-forming units ml^{-1} of IHN virus, relative protection was 91% compared to fish injected with normal serum (titer < 20). Fish injected with immune serum at 24 and 48 hours post-exposure to virus exthibited relative protection of 88% and 75%, respectively. Less protection was observed in fish injected 3 or 4 days post-exposure. Endogenously produced virus neutralizing titers in surviving fish injected at 0 and 24 hours post-exposure were not detected but fish injected at 48 hours produced a significant humoral response. Juvenile rainbow trout (mean weight 1 gram) receiving injections of 50 μl of serum having a low plaque neutralization titer of 20 to 40 resulted in relative protection of 67% to 82% compared to fish injected with saline. When the same volume of a higher-titered serum was injected, greater protection was generally observed. These results suggested that low neutralization titers could be sufficient for significant protection against IHN disease.

Passive immunization could be used as an emergency management strategy if an important group of fish were diagnosed with IHN in the early stages of an epizootic. The results of one field trial indicated that approximately 44% fewer fish were lost in the immune serum-injected group compared to the group that was not injected. However, in both cases the mortality

was low. The fish were large (64 grams) when mortality due to IHN was detected and were divided into two separate raceways. Reducing fish densities possibly helped moderate the severity of the epizootic in both groups. Handling stresses associated with injection may also have exacerbated the disease. However, the passively immunized group that was injected and moved to a different raceway still had less mortality than the unhandled controls. Similar trends of antibody titers and prevalence of antibody-positive fish detected in the passively immunized group were also observed in experimental groups injected at different times after waterborne exposure to IHN virus. Passively transferred antibodies were used in defense of an IHN virus infection and reduced a specific immune response because antigen concentration was kept low. The results from the untreated fish are also in agreement with previous studies that examined the antibody kinetics in unimmunized fish exposed to IHN virus. Low titers and prevalences were detected at 2 weeks but all fish responded with high titers by 6 weeks.[29]

Additionally, LaPatra *et al.*[34] showed that passive immunization of rainbow trout with convalescent (immune) serum from fish exposed to one antigenic variant provided significant protection after challenge with representative isolates from other variant groups. Relative protection of fish injected with immune serum compared to normal serum ranged from 91% to 100%. The results indicated that neutralizing activity produced against one antigenic variant of IHN virus would cross-protect against other antigenic variants. A vaccine against a single type of IHN virus may be efficacious against all IHN virus strains.

Although injection is required and duration of protection may be limited, this strategy could be useful on valuable or small fish populations. Additionally, passive immunization studies can provide important information about immunogenic proteins, requirement for multiple vaccine strains, pathogenesis of microorganisms, and titer of antibody required for protection.

Probiotics

Co-infection with two viruses has been reported among salmonid fish but the potential interactions between these agents and the host's

immune response are poorly understood. The first information on the potential interaction of these agents in rainbow trout was reported by de Kinkelin *et al.*[35] They were able to show in co-infections with infectious pancreatic necrosis (IPN) virus and viral hemorrhagic septicemia (VHS) virus that young rainbow trout infected with IPN virus and later exposed to VHS virus showed significantly greater survival than parallel groups of trout not previously infected with IPN virus. The basis for the virus-mediated interference was suspected to be due to interferon (IFN) but none was detected at the levels of sensitivity for the IFN assay in groups of trout prior to challenge with VHS virus. The ability of trout alevins to produce IFN in response to IPN virus infections, however, has been reported.[36] Hedrick *et al.*[27] showed that juvenile rainbow trout exposed to waterborne live cutthroat trout (CT) virus showed increased resistance to experimental challenge with IHN virus. Pre-exposure to CT virus caused a relative percent survival (RPS) of 70 when compared to CT virus-mock-treated groups challenged with IHN virus. Additionally, the mean day to death was 10 days for CT virus-exposed versus 8 days for the CT virus-mock-treated group. Protection was obtained following exposures as brief as 5 minutes but was greatest among trout exposed for 1 hour to CT virus and then challenged 1 week later with IHN virus. Protection was observed for up to 4 weeks following CT virus exposures but absent at 6 weeks. Concentrations of serum anti-IHN virus neutralizing antibodies were significantly higher ($p = 0.007$) among trout previously exposed to CT virus when compared to the mock-treated group 5 weeks following challenge with IHN virus. Both groups (CT virus and CT virus-mock) surviving the first IHN virus exposure were solidly protected to a second IHN virus challenge. The mechanisms for the viral-mediated resistance induced by CT virus is unknown, but the virus was shown to be a potent inducer of interferon-like activity in anterior kidney cells isolated from rainbow trout.

LaPatra *et al.*[28] also showed that host defenses could be stimulated against IHNV by prior exposure to an avirulent reovirus (chum salmon reovirus; CSV). Pre-exposure of rainbow trout to CSV for 1 hour resulted in an RPS that ranged from 68% to 100% when fish were challenged with IHN virus over an 8-week period. The levels of serum

neutralizing antibodies detected 28 days after the IHN virus challenge were significantly lower among fish receiving prior exposures to CSV than among non-CSV treated fish. The differences in the humoral response to IHN virus in the CSV-treated fish suggested that other immune defense mechanisms may be involved.

Cumulative mortality detected in the CSV-exposed and IHN virus-challenged group was 9% and 26% in the IHN virus-challenged only group. The RPS was 65%, which is consistent with results obtained in a previous experiment that investigated interference-mediated IHN virus resistance with a picorna-like virus, CT virus.[27] The protection afforded by 1 hour bath exposures to CT virus provided up to 69% RPS following IHN virus challenge. This effect was present at 1, 2 and 4 weeks post-exposure to CT virus but was absent when the fish were tested at 6 weeks. Major differences were observed in the cumulative percent mortality between our two treatment groups at each of the time points tested. Strong protection was still observed in fish that were pre-exposed to CSV 8 weeks post-exposure.

No IHN virus neutralizing activity was detected in any of the control fish. However, the CSV-exposed IHN survivors had significantly ($p < 0.001$) lower neutralization titers than the surviving fish exposed only to IHN virus. This is contrary to what was observed during the course of similar experiments with the CT virus. The levels of IHN virus antibodies were significantly higher among the fish receiving prior exposure to CT virus than among the non-CT virus-treated fish challenged with IHN virus. These results indicated that not only was CSV capable of providing excellent protection to IHN virus challenge but specific immunity (serum neutralizing activity) also developed. However, the response appeared to be significantly depressed in treated fish.

Several possibilities for the CT virus and CSV stimulation of non-specific immune functions have been postulated. These include interferon induction and/or stimulation of the macrophage or natural killer cell functions. Cytokine activity is central to theses responses but unfortunately, in salmonids, many of these functions are poorly understood. As has been demonstrated for CT virus,[27] CSV has been shown to be a potent inducer of antiviral-like activity in the anterior

kidney cells isolated from rainbow trout (Congelton JL, National Biological Survey, University of Idaho, Moscow, ID, USA, personal communication). It is possible that cell-mediated immunity was also induced by CSV and provided protection against IHNV. A preliminary model, which was postulated based on our initial results, suggested that CSV induced the release of a T-cell cytokine that enhanced the cell-mediated immunity through the stimulation of T-cytotoxic cells. This was supported by our IHN virus neutralization titer data.

A low level of mortality has also been observed in fish stocks exposed to CSV (data not shown). Gross signs of a potential viral infection were observed and CSV was isolated from dead fish where high concentrations (10^6 to 10^7 pfu/ml) of the virus were detected. Randomly collected histological specimens were analyzed. In previous studies that examined the pathogenesis of CSV, no death occurred in 1 to 2 grams chum, chinook, kokanee salmon fry or rainbow trout injected with 10^4 $TCID_{50}$/ml. However, a slight focal necrotizing hepatitis was observed in the liver sections taken from infected fish.[37] Multi-focal to complete liver necrosis was observed in our specimens (Hedrick RP, University of California, Davis, USA, personal communication). Microscopic tissue changes were also observed in the endothelial cells and sinusoids of the kidney. We have also been able to detect CSV in fish 42 days post-exposure as previously reported.[37] The chum salmon reovirus appears to be very successful at establishing a persistent infection that is possibly confined to the liver. A non-lymphoid chronic acute-phase response may account for the RPS results for fish previously exposed to CSV.[38] By examining the levels of non-lymphoid ("natural") immune defense factors in the sera from CSV and mock-exposed fish, it might be possible to obtain information supporting this hypothesis. Characterization and production of the natural immune defense factors could potentially be developed into a broad-based viral disease control strategy.

Immunostimulants

An enhanced ability to resist infection can be obtained also by activation of the innate immune system with immune potentiators. The

components activated include both cellular (i.e. phagocytes, including both macrophages and granulocytes, and natural cytotoxic or natural killer cells) and humoral components. The latter include opsonins, lytic systems, antiproteases, antibacterial peptides, metal-binding proteins and interferons. Humoral alterations also include an hepatic acute phase response in which concentrations of specific plasma proteins rise to levels higher than those that characterize resting individuals.

The number of compounds known to have the ability to induce phagocyte activation and acute phase responses is large and growing. Many may eventually be found to achieve their effects by way of a smaller number of activation pathways such as IL-6 and tumor necrosis factor (TNF-α). A major group of these so-called immune potentiators is comprised of complex carbohydrates.

The objective of a study by LaPatra *et al.*[39] was to determine if a β,1-3-linked glucan had the capability to improve the ability of fish to derive benefits from passively transferred convalescent serum as measured by survival after a subsequent challenge with IHN virus. They found that glucan treatment did have a beneficial effect. However, it was also evident that glucan alone enhanced resistance to IHN virus and minimized the induction of neutralization activity in sera from fish that survived IHN virus challenge. It remains to be determined if glucan-induced reduction in mortality is due to antiviral cytokines, macrophage activation, or other cell-mediated immune defense mechanisms, to altered levels of humoral components, or to a combination of these. Obviously, understanding this will hasten development of more effective strategies for treatment of fish viral infections.

Recently, Sakai[40] reviewed the current status of research into the use of immunostimulants in fish. Mainly, substances such as glucan, chitin, lactoferrin and levamisole, as well as nutritional factors like vitamins B and C, growth hormone and prolactin are immunostimulatory because of their direct positive influence on non-specific immune elements such as phagocytic cell activity, natural killer cell activity, lysozyme levels, complement levels and total immunoglobulin (Ig) levels.

More recently, nucleotides, precursors of DNA replication, have long been recognized as important elements in mammalian nutrition. With

regard to resistance to infections, it has been shown that groups of mice fed diets supplemented with nucleotides had less mortality following challenge infection with *Staphylococcus aureus* and *Candida albicans* compared with groups of mice fed nucleotide-free diets.[41] This increase in resistance to infection is reported to be as a consequence of increased phagocytic activity of murine peritoneal macrophages, increased T-cell dependent antibody production, enhanced IL-2 production and elevated bone marrow cell and peripheral neutrophil numbers.

Burrells *et al.*[42] performed a number of studies to determine what effect the inclusion of exogenous nucleotides in aquaculture diets had on the resistance of fish to various challenge infections. When added to normal fish feed formulations at a combined inclusion level of 0.03%, these additional nucleotides were shown to increase resistance to challenge infections with bacterial, viral and rickettsial disease as well as ectoparasitic infestation. When the nucleotide-supplemented diet was fed for 3 weeks prior to challenge, mortality resulting from infections with infectious salmon anemia (ISA) virus was reduced (RPS = 25.7%).

The authors go on to say that nucleotides are provided endogenously by *de novo* synthesis in tissues such as the liver. This, however, is an energy-expensive process and the salvage pathway is preferentially utilized when there is an exogenous source of nucleotides such as in the diet. Although most tissues can synthesize nucleotides *de novo*, other cells such as immune cells and cells in the intestine are lacking in this capacity and must depend on pre-formed nucleotides. Mammalian requirements for exogenous nucleotides can vary considerably and can increase rapidly at times of rapid growth or physiological stress. This may also be the case in farmed fish where the exogenous supply of nucleotides in fish feeds is sufficient for normal requirements of health and growth. However, the stressful events associated with aquaculture management (vaccination, handling, disease, etc.) may lead to similar increases in reliance on the exogenous supply for optimal functions and responses. Supplemental dietary nucleotides applied in anticipation of these events could, therefore, ensure an adequate circulating nucleotide pool.

Antiviral Drugs

Antiviral drugs that have been useful in medical and veterinary practices have been tested in fish. The work has been reviewed by Winton.[5] Twenty-four compounds were tested *in vitro* in CHSE-214 cells and 11 were found to be selectively toxic for the virus.[43] Of these, five were tested in steelhead trout fry and there were more survivors in the treatment groups (14%–34%) than in the control, untreated group (8%). The five compounds (6-thioinosine, 5-hydoxyuridine, 9-[S]-(2,3-dihydroxypropyl, adenine, virazole and chloroquin) were added to the water at daily or alternate day intervals after infection during the treatment process. Hudson *et al.*[44] examined the antiviral compounds amantadine, mitisazone, bisbenzimidazole and ribavirin. Amantandine was very effective against IHN virus in rainbow trout cells in culture. The other compounds were also effective but had some associated cytotoxicity. Further studies have not been reported and it is more likely that the costs of these antiviral compounds make their use prohibitive for aquaculture. An antiviral compound produced by *Pseudomonas fluorescens* has also been found to be effective against IHN virus and *Oncorhynchus masou* (OM) virus, a fish herpes virus.[45,46] The compound, a cyclic peptide has been shown to be very effective *in vitro*, producing a 94% plaque reduction at concentrations of 12.5 mg/ml. The compound did not inhibit the fish viral pathogen IPN virus, a non-enveloped birnavirus, suggesting to the investigators that the inhibitor is specific for enveloped viruses.

Adjuvants

Initially, researchers experimented with a wide variety of organic and inorganic compounds including aluminum salts, mineral oil and killed mycobacteria to improve the immunogenicity of vaccines. More recently, hundreds of natural and synthetic compounds have been evaluated as vaccine adjuvants. After extensive safety and toxicity testing, many of these novel adjuvants have proven to be acceptable for further evaluation. During the same time, investigations into the mechanisms of action of

adjuvants have increased. Today, a major goal of adjuvant research is to apply the increased understanding of basic immunobiology to adjuvant development. Improved understanding of adjuvant mechanisms of action will provide a basis for the rational selection of adjuvants for use with new vaccines.

There is very little published work on the effect of adjuvants on viral diseases of fish. Recently, we conducted a study to evaluate the effect of Freund's complete (FCA) and incomplete (FCI) adjuvants on the protection of IHN virus at seven and 28 days after intraperitoneal injection. Four groups of 160 rainbow trout were either left unhandled, or injected with 100 μl of mineral oil, FCA or FIA. At seven and 28 days after injection, duplicate 25-fish groups were waterborne-challenged with IHN virus using a standardized method described previously.[33] A single 25-fish group from each treatment was mock-infected and served as a negative control. Mortality was monitored daily and virus was re-isolated from at least 20% of the fish that died each day.

The results indicated that when fish from each treatment group were challenged at 7 days after injection that the groups that received FCA and FIA exhibited significantly less ($p < 0.05$) mortality then fish that were either left unhandled or were injected with mineral oil (Table 1). When additional fish were challenged at 28 days post-injection, there were no differences amongst the treatments (Table 1). Virus was re-isolated from 98% (110/112) of the dead fish that were tested during the study.

Table 1 Cumulative percent mortality in duplicate 25-fish groups of rainbow trout that were either left unhandled or were injected with 100 μl of mineral oil, Freunds complete adjuvant (FCA) or Freund's incomplete adjuvant (FIA). At 7 and 28 days after injection, fish were challenged with infectious hematopoietic necrosis virus (IHNV).

Challenge day post-injection	FCA	FIA	Mineral oil	Unhandled
7	27% (14/52)	20% (9/46)	62% (33/53)	55% (27/49)
28	91% (42/46)	96% (47/49)	89% (41/46)	92% (44/48)

Adjuvants are necessary to activate and direct the innate and adaptive immune responses against relatively poor vaccine antigens. In general, the antigen can be thought of as a passive element while the adjuvant represents the activating and modulating intermediate, operating at the interface between the immune system of the host and the vaccine. The results of the study described above illustrate the effectiveness of adjuvants at eliciting innate antiviral defense mechanisms that pave the way for the induction of potent and long duration adaptive immune responses. These results also suggest that this is an area of fish immunotherapy that definitely should be targeted for further research.

Vaccines

Live-attenuated Vaccines

Live-attenuated vaccines illustrate some of the commercial limitations of otherwise potentially efficacious finfish viral vaccines. These vaccines replicate in the host, but are typically attenuated in their pathogenicity and do not cause disease. Approximately 60% of the viral vaccines listed in the semi-annual publication of veterinary biological products licensed by the United States Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS) are live-modified vaccines. Long-lasting immunity, stimulation of innate and well-balanced acquired immune response including humoral and cellular immune response, and the exceedingly small amount of vaccine required for optimum potency are often cited as advantages of the live-attenuated vaccines. However, there are no licensed live-attenuated viral vaccines for use in finfish aquaculture, and in the future it will likely become increasingly difficult to license such vaccines for aquaculture due to several drawbacks. These include risk of reversion to pathogenicity, risk of transmission to non-farmed fish in the surrounding waters or retention of virulence in certain fish species. In addition, assurance that the attenuated vaccine genotype remains stable when amplified *in vitro* requires extensive quality control measures.

Fryer *et al.*[10] created an attenuated IHN virus vaccine by passage of an IHN virus isolate originally obtained in 1971 from a rainbow

trout in Nan Scott Lake, Oregon, USA, in a cell line derived from steelhead trout embryos (STE-137). After 41 passages, the virus was 100-fold less virulent in sockeye salmon than the parental wild type isolate. Immersion of sockeye salmon fry in roughly 1000 pfu/ml attenuated virus for 48 hours at 18°C and challenge of the fry 25 days later with heterologous wild type virus administered either by immersion or intraperitoneal injection resulted in 95%–100% survival of the vaccinated fish compared to 10%–15% survival of mock-vaccinated fish. Similar results were obtained if the interval between vaccination and exposure was 110 days. Unfortunately, further basic research of the live-attenuated vaccine was discontinued when it was found to be virulent to rainbow trout.

A different problem is encountered when considering an attenuated vaccine targeted against a segmented virus such as the ISA virus or IPN virus. The difficulty arises because a genetic shift could occur upon re-assortment of the genomic segments if the attenuated vaccine and virulent virus co-exist in a single host. For example, genetic shifts have been responsible for major influenza virus pandemics in humans. These hurdles will make licensing of an attenuated viral vaccine for finfish unlikely.

Whole-killed Vaccines

Whole-killed vaccines, unlike the attenuated vaccines, cannot replicate or revert to virulence in the host. The commonly cited drawbacks of killed vaccines are that adjuvants are required and a booster vaccination is sometimes needed to prime the humoral immune response. To produce a killed vaccine, virus is first amplified in a cell line and then inactivated, typically using chemicals. A rational choice of inactivating agents and the conditions for inactivation of virus infectivity are critical components in developing killed vaccines. Chemical inactivation agents are of two types: reticulating and alkylating. Common reticulating agents include formaldehyde and glutaraldehyde and the alkylating agents include binary ethylenimine and β-propiolactone. The action of any inactivating agent causes simultaneous modification of viral components responsible for immunogenicity and viral polynucleotides that are

responsible for infectivity. The degree of modification of virion components and consequently the reduction of the infectivity as well as the immunogenicity of the viral suspension is proportional to the increase of time of treatment with the inactivating agent.[47] The most dramatic effect of inappropriate inactivation of the virus during preparation of a vaccine is the lack of recognition of an antigen by the host immune system due to chemical ablation of critical epitopes.[48,49] For example, formaldehyde inactivation of the rhabdovirus vesicular stomatitis (VSV) virus impairs select T-cell responses.[48] In addition, inactivation can result in preferential inactivation of antigenicity, a phenomenon observed with the surface glycoproteins hemagglutinin and neuraminidase of the influenza virus.[50] Epitope modifications can also lead to potentiation of disease due to an imbalance in the host immune response.[51]

Nishimura *et al.*[13] investigated in a systematic manner the inactivation of infectivity and immunogenicity of formaldehyde-killed IHN virus in rainbow trout fry. The infectivity of concentrated virus (10^{10} $TCID_{50}$/ml) was completely inactivated in 24 hours when incubated at 4°C in the presence of 0.8%–0.4% formaldehyde. Infectious virus remained viable for 5, 8 and at least 13 days when incubated under the same conditions but with 0.2%, 0.1% or 0.05% formaldehyde, respectively. When the killed vaccine preparations were injected into the intraperitoneal cavity of 2.5, 1.1 or 1.4 grams rainbow trout (0.05 ml/fish) and the fish were subsequently challenged by immersion 27–28 days later with $10^{6.4–1.2}$ significant protection against IHN disease was observed. Immunity appeared 1 week post-immunization when the fish were held and challenged at 5.2°C and the immunity remained for at least 56 days. A dose of 0.05 ml of $0.5 \times 10^{1–2}$ $TCID_{50}$/ml formaldehyde-killed virus elicited a protective immune response in vaccinated fish. Immersion of fish in a hyperosmotic solution containing formaldehyde-killed virus resulted in minimal protection against IHN virus challenge. In a different study, Amend[12] reported that IHN virus ($10^{7.3}$ $TCID_{50}$/ml) inactivated with 1 part β-propiolactone to 6000 parts virus suspension for 96 hours at room temperature and injected into rainbow trout (0.05 ml/fish) protected the fish from subcutaneous virus challenge ($10^{6.2}$ $TCID_{50}$/ml)

32 days post-vaccination. The numbers of animals used in the study were not sufficient for significant statistical analysis. However, the average mortality of the vaccinated fish was 4% whereas the positive control group was 70%. The results from these two studies suggest that the minimum effective dose is not commercially cost prohibitive. On the other hand, whether these doses of killed vaccine are effective in larger fish (>10 grams) or other species, e.g. Atlantic salmon is not known. Neither study directly addresses whether higher concentrations of the inactivating agent or storage temperature and time influences vaccine potency via epitope modification. In addition, the vaccines were not formulated with adjuvant and duration of immunity was not evaluated. Incorporation of adjuvant in the killed virus preparations could decrease the minimum effective dose of vaccine administered, an important issue given that expensive large-scale culturing of the virus *in vitro* is required.

Recombinant Vaccines

Recombinant vaccines, unlike the killed and attenuated vaccines, require prior knowledge of the identity as well as the nucleic acid and amino acid sequence of immunoprotective antigens. The IHN virus genome is a single strand of RNA composed of 11,131 nucleotide bases.[7] The RNA is a non-infectious, negative-sense molecule that encodes six proteins: 3′-nucleocapsid (N), polymerase-associated phosphoprotein (P or M1), matrix protein (M or M2), surface glycoprotein (G), non-virion protein (NV) and the virus polymerase (L)-5′. The virus is lipid-enveloped and covered evenly with "spikes" of the G protein.[6] As mentioned, the G protein is the single viral protein capable of eliciting an IHN virus-specific immunoprotective response in fish.[2,3,5,30]

Recombinant subunit vaccines

For the subunit recombinant vaccines, in principle, the gene(s) encoding a particular antigen are copied, cloned into a replicon and introduced into a permissive host, e.g. bacterium, yeast or insect cells,

which then synthesize the recombinant antigen. From this point, depending on the required purity of antigen, numerous strategies can be employed. These types of vaccines have the benefit of being well-defined, non-infectious and simple, as well as inexpensive to produce in large quantities. In addition, further engineering of the vaccine is relatively easy to do and can be used to enhance particular immune responses.

Gilmore *et al.*[15] were the first to exploit *Escherichia coli* for expression of the IHN virus G protein. They inserted a 329-base pair cDNA fragment encoding G protein amino acid residues 336–444 (Round Butte isolate) into the pATH 3 vector to form a *trpE*-G gene fusion. Western blot analysis of *E. coli* producing the recombinant G protein revealed an immunoreactive fusion protein between 48–49 kDa (37 kDa, TrpE; 11 kDa, G peptide) using rabbit anti-IHN virus polyclonal sera. Rainbow trout, 0.4 grams in weight, were immersed for one minute in a crude bacterial lysate normalized to 3 mg/ml total protein concentration followed by a two-minute immersion in a ten-fold dilution of the lysate. The fish were held for approximately 30 days at 10°C, separated into groups, and then challenged with four dilutions of either a Round Butte or Dworshak isolate of IHN virus. With homologous virus challenge, RPS ranged from 78% to 100% (the RPS values were inversely related to increasing doses of virus used in the challenge). Upon heterologous challenge, RPS values ranged from 5% to 87% with the same inverse relationship to challenge pressure. A similar trend was observed when chinook salmon fry were vaccinated with the recombinant protein and challenged with an Elk River IHN virus isolate. Subsequent work conducted in the laboratory examined whether a recombinant IHN virus N protein alone or in conjunction with the recombinant G protein provided protection from virus challenge.[17] The recombinant N protein did not seem to protect vaccinated rainbow trout from heterologous virus challenge (0%–2% RPS) whereas a modest protection was observed using homologous virus challenge (22.6% RPS). When the N and G recombinant vaccines were used in combination, there was a slight increase in the RPS values compared to either the N or G recombinant vaccine alone. Similar studies by Lorenzen *et al.*[52]

using VHS virus recombinant G protein vaccines extended this earlier work by analyzing the humoral immune response of rainbow trout to the recombinant G protein. The VHS virus G recombinant protein consisting of amino acid 17–445, produced in *E. coli*, was injected IP into rainbow trout twice, at 5-week intervals, using 2.5–5 μg protein in adjuvant followed by a boost with non-adjuvanted protein 4 weeks later. Ten days later, the booster serum collected from three of the four fish contained neutralizing antibodies ranging in titer from 1280–2560 (50% plaque neutralizing titer).

Several groups investigated the use of baculovirus to synthesize recombinant IHN or VHS virus G protein in insect cells.[14,53,54] The rationale for using this system is that insect cells have the ability to glycosylate proteins. It was believed that improper glycosylation using the prokaryotic system, explained at least in part, the observed inconsistent efficacy of the recombinant vaccines in the laboratory and in field trials, as well as poor stimulation of the humoral immune response. Lecocq-Xhonneux *et al.*[53] cloned the VHS virus G gene, derived from the 07.71 virus isolate, downstream of the AcNPV baculovirus polyhedrin promoter in pBacSHVG and introduced the construct into the insect cell line Sf9. The transfected cells displayed multinucleate syncytia that may have been due to the presence of the G protein on the cell surface. Cain *et al.*[54] using a similar system with the IHN virus G protein derived from the Round Butte isolate found that cell surface G protein expression was temperature-dependent. Recombinant G protein was surface-localized when the transfected cells were grown at 20°C, whereas the G protein was sequestered in the cell if the cells were grown at 27°C. Using the VHS virus G protein recombinant vaccine, RPS values between 33.9% and 80.2% were achieved when rainbow trout fry were vaccinated by IP injection and a negative RPS value was observed following bath exposure. Plaque neutralizing antibodies were present in 20%–40% of the IP-vaccinated fish. The wide variation in efficacy could have been attributed to challenge pressure. In contrast, the IHN virus G protein recombinant vaccine produced by Cain *et al.*[14] provided limited protection in rainbow trout challenged with IHN virus. No seroconversion was evident in

adult rainbow trout immunized with the recombinant IHN virus G protein.

While the results with the first generation recombinant subunit vaccines were somewhat disappointing, significant progress has been made in recent years towards the development of recombinant vaccines based upon the work of Kuzyk *et al.*[55,56] with the bacterial pathogen *Piscirickettsia salmonis.* Four factors were essential to the success of their project and will be important in the design of future antiviral vaccines for the finfish aquaculture industry. First, the nucleic acid sequence of the *P. salmonis* antigen, OspA, was optimized for high level expression in *E. coli*.[55] Analysis of the codon usage of OspA showed that 20% of the codons were rare *E. coli* codons. The polymerase chain reaction (PCR) was used to construct an *E. coli* codon optimized, synthetic version of the gene encoding OspA. Second, removal of the signal sequence and coupling of OspA with an N-terminal fusion partner resulted in improved expression of *ospA.* Third, recovery of the fusion product in inclusion bodies facilitated the concentration of the antigen. And finally, T-cell epitopes from either the measles virus fusion protein or the *Clostridium tetani* tetanus toxin were used to construct chimeric fusion proteins with OspA.[56] In the absence of the T-cell epitopes, the recombinant OspA vaccine stimulated a protective immune response in vaccinated coho salmon, resulting in an RPS value of 32% compared to 83% upon addition of the two T-cell epitopes.

An IHN virus recombinant vaccine is currently in development using the system described above, including synthetic optimization of the G gene. The IHN virus G protein, derived from the Western Regional Aquaculture Consortium type strain, was synthesized as a recombinant protein in *E. coli* with or without the measles virus epitope, formulated with adjuvant and used to vaccinate 1–3 grams rainbow trout fry or 10 grams Atlantic salmon by intraperitoneal injection. The vaccinated rainbow trout were held at 15°C for 28 days and then challenged by immersion. In a preliminary trial, RPS values between 48% and 68% were achieved relative to adjuvant and saline controls, respectively (unpublished results). In a separate trial, vaccinated Atlantic salmon were held in fresh water for 46 days at 13°C and an RPS value of 66%

relative to saline controls was obtained upon immersion challenge (unpublished results).

Recombinant DNA vaccines

Genetic immunization, or the use of antigen-encoding DNAs to vaccinate is one of the newest approaches for the development of vaccines. Since numerous reviews already cover this topic, this discussion will focus on a few aspects of the immune response elicited by DNA vaccines.[57–59] Rainbow trout and Atlantic salmon produce a protective antiviral response when inoculated with DNA vaccine encoding fish rhabdovirus glycoprotein. The G protein of the serologically unrelated IHN virus, viral hemorrhagic septicemia (VHS) virus, snakehead rhabdovirus (SHR) and *Vesiculovirus* spring viremia of carp (SVC) virus have been developed as fish DNA vaccines. The vaccines have been tested for their ability to elicit a protective antiviral response against challenge with IHN or VHS virus. The SHR and SVC virus have not been used in challenge studies because they are considered exotic pathogens in North America. The antiviral response elicited by the IHN and VHS virus G protein DNA vaccines is potent, conferring upwards of 100% RPS of vaccinated fish challenged with otherwise lethal doses of IHN or VHS virus. Rainbow trout are protected from IHN disease beginning 4 days after vaccination with the pIHNw-G vaccine (w indicates that the glycoprotein is derived from the Western Regional Aquaculture Consortium reference IHN virus isolate) or the VHS virus G DNA vaccine, and the protection lasts for at least 2 years for the pIHNw-G vaccinated fish. The state of immunity of IHN or VHS virus G DNA vaccine inoculated fish can be distinguished biologically into phases as a function of time following vaccination.

The early antiviral response (EVR) is cross-protective and is observed beginning 2 to 4 days after vaccination of rainbow trout held at 15°C. This is the earliest time for which protective immune responses have been observed following DNA vaccination in any model system thus far reported. Trout vaccinated with a single 0.1–10 μg dose of IHN or VHS virus G DNA vaccine and challenged 7 days later with

homologous or heterologous virus are protected from disease. The RPS seven days post-vaccination can approach 100% depending upon, among other things, the dose of vaccine administered. Vaccine doses of 0.1 μg are less efficacious than higher doses. The duration of the EVR may be influenced by the particular rhabdovirus G DNA vaccine that is used as well as water temperature. Inoculation of rainbow trout with 10 μg SVC and SHR virus G DNA vaccine elicits a cross-protective immunity against IHN virus 30 days after vaccination but not 70 days post-vaccination.

The specific antiviral response (SVR) is observed beginning approximately 28 days post-vaccination. The SVR provides protection against homologous virus but lacks the cross-protective immune function of the EVR. The transition from the EVR to the SVR happens between days 14–28 at water temperature of 15°C and is thought to be the effect of maturation of the EVR in response to the DNA-encoded antigen. The potency of the SVR decreases between 3 and 6 months post-vaccination. Fish vaccinated with the IHN virus DNA vaccine pIHNw-G and challenged with IHN virus are completely protected from disease (100% RPS) 3 months post-vaccination, whereas RPS values of 69% are observed 6 months post-vaccination.

The molecular components that mediate the EVR are not known, though some factors can be eliminated based upon current information. The EVR effector is not antibody. Generally, at water temperatures of 10–12°C, trout antibodies to a specific antigen appear in serum 3 to 4 weeks after antigen exposure. Serum collected from pIHNw-G vaccinated fish 7 and 21 days after vaccination does not contain detectable antibodies by the enzyme-linked immunosorbent assay (ELISA), Western blot analysis or serum neutralization test. Further, serum from pIHNw-G vaccinated fish collected 7 days post-vaccination and passively transferred to naïve recipients does not confer immunity to infection with virus. Thus, serum factors as effectors of virus clearance are not specifically involved in the EVR or alternatively they require co-factors. It could be argued that the passive transfer procedure dilutes the factor and thereby reduces the reliability of the method for assessing whether serum factors are involved in the EVR. However, the method involves inoculation of

a quantity of serum 1/20th the weight of the fish used in the study; this amount of serum is large in comparison to the amount of serum *in situ* in 1 gram fish. That serum factors are probably not involved in their own right is reinforced by the finding that the serum factors do not inhibit viral infection of cells grown *in vitro*.

The host effectors that contribute to the EVR can be inferred from deductive reasoning. First, the rapid onset and cross-protective nature of the EVR suggests that trout have pre-programmed, germ line-encoded proteins/cells that recognize and eliminate IHN and VHS virus. By analogy, this means that the EVR is part of the innate immune response of rainbow trout. This inference also stems from the observation that IHN and VHS viruses cause an aggressive, systemic infection in fish. Typically, waterborne exposure (in the laboratory) of susceptible fish to lethal doses of IHN virus results in systemic infection within 3–5 days post-exposure and death from disease 7–14 days post-exposure. Fish vaccinated with pIHNw-G and exposed to virus do not support virus replication. Further, infectious virus particles are cleared from vaccinated fish. It may be that the EVR acts at the primary site(s) of IHN virus infection abrogating a systemic infection. Interestingly, if the natural route of infection is breached, through direct intraperitoneal virus inoculation, the EVR is still effective, conferring 90% RPS. A number of studies using DNA vaccines as well as live virus have shown that interferon-related proteins and other virally induced cytokines are activated soon after treatment. It would not be surprising if interferon-like molecules and pathways mediate the EVR.

The question arises as to what component(s) of the vaccine is responsible for eliciting the EVR. The answer is that it is almost certainly an intrinsic component of the IHN virus glycoprotein and may be a pathogen-associated molecular pattern. Replacing the IHN virus G gene in pIHNw-G with the IHN virus nucleocapsid, phosphoprotein, matrix protein or non-virion protein-encoding gene, the rabies glycoprotein or reporter protein encoding genes abolishes the efficacy of the DNA vaccine. In addition, Atlantic salmon and rainbow trout inoculated with DNA encoding the surface hemagglutinin or the P3 protein of ISA virus followed by homologous or heterologous virus challenge did not

exhibit an EVR. Furthermore, the EVR is not a unique property of DNA vaccines. For example, IHN virus attenuated and killed vaccines, poly I:C, and pre-exposure of trout to viruses unrelated to the rhabdoviruses elicit a response reminiscent of the EVR but whether identical mediators of immunity are involved is not known. It is also not known if other antigens such as recombinant or purified IHN virus G protein can elicit an EVR, but they do elicit an SVR as evidenced by their efficacy in vaccine trials.

Antibodies are a component of the SVR elicited by the rhabdovirus DNA vaccines. However, there is not a discernible correlation between the percentage of DNA-vaccinated fish that seroconvert with the potency of the antiviral response when measured prior to virus exposure. For example, Traxler *et al.*[23] reported that only 33% of the Atlantic salmon vaccinated with pCMV-G had seroconverted at 8 weeks post-vaccination but an RPS of 90%–100% was observed when the vaccinated fish were challenged with virus. This suggests that the DNA vaccine does not restrict subsequent humoral immune response, and in fact the humoral immune system in DNA-vaccinated fish seems to be poised to respond upon virus exposure since antibody titers increase when vaccinated fish are exposed to virus. The increase in neutralizing antibody titer in vaccinated and virus-exposed fish is influenced by the amount of vaccine administered. Fish inoculated with 25 μg of the pIHNw-G vaccine have higher neutralizing titers than fish inoculated with 0.1 μg DNA vaccine. However, even the lowest dose of vaccine confers RPS values greater than 80%.

Conclusions

The crucial roles of cellular and humoral immune responses in controlling viral diseases of fish are being elucidated, providing targets for immunotherapeutic intervention and defining new goals for vaccine strategies. The large array of immune activators that have been shown to alter IHN virus pathogenesis in trout and salmon will form the basis for future work elucidating the relationship between the innate and acquired immune responses. DNA vaccines that encode the IHN

or VHS virus G protein will play a central role in these studies. The well-characterized model system and potency of the vaccine will result in a deeper understanding and appreciation of the complexity of the fish immune response to viruses. In the near future, it is certain that the unusual antigenic nature of the IHN and VHS virus glycoprotein will become evident and may result in the development of a new generation of vaccine types.

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Chapter 14

Role of Probiotics in Fish Disease Prevention

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Introduction

The demand for wild-caught fish kept pace with world demand between 1950 and 1990, rising steadily from approximately 20 to 90 million tons per annum[1] and this was mainly achieved through advances in fishing technology. However, it is now generally accepted that the maximum sustainable catch from natural sources has been reached and will, at best, remain stable at approximately 90 to 100 million tons per annum, or even decrease.[1] Increasingly, the world looks to aquaculture to satisfy the constantly increasing demand for fish. Although practiced in a basic form for over 2000 years, large-scale development of aquaculture has only taken place over the past 50 years with output increasing from approximately one million tons in 1950 to over 36 million tons in 2000.[1] The bulk of aquaculture is carried out in freshwater in small units in Southeast Asia and the above figures include production of molluscan shellfish and algae. In Europe, and in North and South America, aquaculture has focused on the high intensity commercial production of "high value" marine species such as Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*), turbot (*Scophthalmus maximus*), halibut (*Hippoglossus hippoglossus*) and cod (*Gadus morhua*), but much larger quantities of freshwater fish such as tilapia, carp (*Cyprinus carpio*) and trout (mainly *Oncorhynchus mykiss*) are produced. Thus, worldwide aquaculture

production in the year 2000 included 15.6, 1.3, 1.0 and 0.5 million tons of carp, tilapia, salmon and trout, respectively.[1]

Factors limiting production of fish in the aquaculture industry include infectious diseases which can cause losses of juvenile and adult fish at on-growing sites as well as limiting the provision of sufficient numbers of juvenile fish for on-growing.[2]

In comparison with wild fish, those held in aquaculture sites will almost inevitably be more susceptible to infectious diseases since the fish will normally be more stressed than those in the wild. The holding of very large numbers of fish, often up to one million in cages on a single site, provides ideal conditions for any new infectious agent to initiate infection, transmit disease due to the close proximity of high numbers of susceptible hosts, and increase virulence due to continued passage through many fish in the site. Unless rapidly removed, moribund fish remaining in the vicinity may shed very large quantities of infectious agent.[3]

Control of fish disease has involved a combination of improved husbandry and diets, the use of vaccination, where possible, and recourse to antibiotics. Apart from the problems that arise for the aquaculture industry through the evolution of antibiotic-resistant bacteria, there are more widespread concerns for human health and environmental pollution. Thus, there is pressure to reduce drastically the use of antibiotics in aquaculture and this has led to widespread interest in the application of probiotics to improve fish health. This is particularly so in the larval rearing stages where the immune system is insufficiently developed to allow the use of vaccines.

Although written from the perspective of finfish, this review will consider the use of probiotics in shellfish and crustaceans where these provide good examples for development of probiotics in finfish. A number of recent reviews contain useful details of particular aspects of probiotic use in fish.[4–8]

Infectious Diseases of Major Importance in Cultured Fish

A very large number of infectious agents have been implicated in losses of cultured fish and comprehensive reviews have recently been

provided.[9–12] The most significant losses have arisen from bacterial diseases such as vibriosis, furunculosis, enteric redmouth, edwardsiellosis, piscirickettsiosis and streptococcosis, and viral infections, particularly channel catfish virus (CCV) disease, infectious hematopoietic necrosis (IHN), infectious pancreatic necrosis (IPN), infectious salmon anemia (ISA) and viral hemorrhagic septicemia (VHS). The fungal pathogen *Saprolegnia* also causes problems in freshwater and these are increasing following the withdrawal from use of the very effective treatment agent, malachite green.[13]

Vibriosis, due mainly to *Vibrio anguillarum,* has been one of the most widespread and serious problems in marine aquaculture. As an example, prior or the development of effective vaccines against *V. anguillarum*, the cost to Norwegian aquaculture of vibriosis during 1984 to 1988 was estimated to be £67 million[14] and similar losses occurred in Japan during this time. At least 12 other *Vibrio* species are pathogenic for fish, causing significant losses in warm and temperate regions (e.g. *V. vulnificus, V. alginolyticus* and *V. harveyi*); in colder waters diseases such as "cold-water vibriosis" due to *V. salmonicida*[15] and "winter ulcers" due to *Moritella viscosa* (*V. viscosus*) are an increasing problem.[16,17] Although disease due to *V. anguillarum* can be largely controlled by vaccination, vibriosis due to other *Vibrio* species remains a major problem in many sectors of aquaculture. Invertebrates too are very susceptible to vibriosis, particularly at the larval stages where *V. tubiashii* can cause high mortalities in rearing of oysters and other bivalve larvae[18,19] and *V. harveyi* affects shrimp rearing adversely.[20]

Furunculosis, due to *Aeromonas salmonicida*, has caused very severe losses of Atlantic salmon in the wild and in aquaculture[9,21–23] and at its peak it was reported that up to 20% of post-smolt salmon were lost in Scottish aquaculture.[21] Such extreme losses highlighted the limitations of antibiotic therapy due to the rapid emergence of multiple-antibiotic resistant strains of *A. salmonicida*[24] but the development of effective furunculosis vaccines[22] has alleviated this problem and dramatically reduced losses due to this disease.

Other bacterial diseases currently causing significant losses in aquaculture include streptococcosis, caused by a range of Gram-positive

cocci,[9] and piscirickettsiosis, caused by *Piscirickettsia salmonis*,[9,25] for which effective vaccines have yet to be produced commercially.

As noted by Plumb,[10] "Comparatively few fish viruses cause severe disease in aquaculture, but when they do, results can be devastating." Not only are effective vaccines unavailable for fish viral diseases but antibiotics such as those used to control bacterial infections are not available either. IPN, once regarded as generally a subacute disease of salmonids in freshwater is now a major cause of losses in marine aquaculture of salmon, currently causing very high losses of post-smolt Atlantic salmon.[26,27] ISA also has the potential to cause serious damage to salmon aquaculture.[26,28]

Therefore, with the expansion of world aquaculture, particularly with attempts to increase the range of species in commercial culture, existing and newly discovered infectious agents pose a risk to the cultured species as well as to wild fish stocks.[29]

Current Disease Prevention Strategies

Vaccines

Effective vaccines against several of the most serious bacterial infections have proved relatively easy to develop. Thus, long-term protection against *V. anguillarum* can be induced using vaccines comprised of killed bacterial cells or cultures, and the vaccine can be administered simply by dip or bath immersion.[30,31] The protective factor is lipopolysaccharide,[32] the major component of the Gram-negative cell outer membrane and although 23 serotypes of *V. anguillarum* are currently recognized[33] only three, serotypes O1 and O2 and to a lesser extent O3, are associated with significant mortalities in fish.[33]

Vaccines against enteric redmouth, caused by *Yersinia ruckeri*,[34] and furunculosis[22] have also proved very effective. The protective antigen in *Y. ruckeri* is also lipopolysaccharide,[34] but in *A. salmonicida* vaccine protection seems to be obtained via iron-regulated outer membrane proteins and a capsular polysaccharide[35] and the vaccine

is only effective if administered by injection in an adjuvanted oil emulsion.[22] Significant bacterial diseases for which effective commercial vaccines are not yet available include those due to the intracellular pathogens *Renibacterium salmoninarum* (bacterial kidney disease)[36] and *Piscirickettsia salmonis.*[37]

Because of the difficulty in developing vaccines against viruses and certain bacteria, other strategies have been investigated for disease control. Also, larval fish rearing, where large losses can occur, is not amenable to intervention with vaccines as the larval immune system will normally be immunologically immature, and administration of vaccine may suppress later immune responsiveness to an infectious agent.[38]

Antibiotics

Antibiotics have proved highly effective in controlling bacterial infections in humans, animals and fish during the past 50 years. However, there are concerns about the possible effects on human health of the large-scale use of antibiotics in aquaculture as the increasing emergence of antibiotic-resistant strains of bacteria has led to the need for more stringent control over their use in aquaculture.[39] At the peak of the furunculosis epidemic in Norway, over 40 tons of antibiotics were used in Norwegian aquaculture[40] but stringent control on use, coupled with the introduction of effective vaccines, has reduced this to 591 kg in 1999.[41]

As some antibiotics, such as oxytetracycline, have a very long half-life[42] residues can accumulate in marine sediments close to fish farms and these can persist for a considerable time. This could lead to the development of antibiotic resistance in marine bacteria and the potential transfer of resistance to other bacterial species more significant to human health.

Diet and Immunostimulants

In addition to the introduction of vaccines, the most significant improvements in fish health have perhaps been due to revised, lower-stress management procedures to improve general health and, indirectly,

enhance the fitness of the immune system. The addition of immunostimulants to diets to enhance the non-specific immune system is a further way of enhancing disease resistance and this has been reviewed recently.[43] Numerous factors have been considered as immunostimulants, ranging from synthetic chemicals such as levamisole (an anti-helminthic drug) to vitamins, hormones, animal and plant extracts and polysaccharides.[43] However, much attention has focused on microbial cells or cell fractions (Table 1). The use of adjuvants, e.g. mineral oil emulsions (Freund's adjuvant, with or without killed *Mycobacterium tuberculosis* cells), alum and lipopolysaccharide, is well established in mammalian immunology to enhance the specific immune response to a co-injected antigen.[44] However, as Janeway[45] has proposed, the efficiency of the non-specific immune response is dependent on rapid recognition of invading microorganisms and this can best be achieved by identifying highly conserved structures only found in such microorganisms. These encompass the active components of the immunostimulants shown in Table 1, which enhance the non-specific immune response by upregulating phagocytic cell functions (chemotaxis, phagocytosis and intracellular killing) and in some instances elevating serum lysozyme and complement activities (see Sakai[43] for references). Whilst most of the studies demonstrating effectiveness of immunostimulants have involved injection of the agents, some success has been reported by oral administration in the diet. This approach is being explored increasingly in aquaculture diets but it is recognized that excessive doses can lead to suppression of the immune response.[43]

A more general approach was taken by Burrells *et al.*,[46,47] who incorporated enhanced levels of nucleotides into the diet of rainbow trout and Atlantic salmon. It was hypothesized that rather than directly stimulating cells of the non-specific immune response as β-glucans do, provision of greater levels of dietary nucleotides would allow the entire immune system to respond more rapidly and effectively to infection. In a series of well-controlled studies, the nucleotide diet enhanced protection of fish from challenge with *V. anguillarum*, *P. salmonis* and ISA virus[46] and enhanced the antibody response of salmon to furunculosis vaccine.[47] In summary, there is great potential for

Table 1 Selected examples of immunostimulants of microbial origin which have been used in fish.

Immunostimulant	Origin	Effect	Reference
Bacterial cells	*Achromobacter stenohalis*	Enhanced resistance to *A. salmonicida*	121
	Clostridium butyricum	Enhanced resistance to *V. anguillarum*	122
	Vibrio anguillarum	Enhanced resistance to *Enterococcus*	122
Freund's Complete Adjuvant	Oil adjuvant incorporating killed *Mycobacterium tuberculosis*	Enhanced resistance to *A. salmonicida* and *V. ordalii*	123
Muramyl dipeptide (MDP)	*Mycobacterium tuberculosis*	Enhanced resistance to *A. salmonicida*	123
		Enhanced resistance to *V. anguillarum*	124
Lipopolysaccharide	Gram-negative bacteria	Enhanced immune response	125
Peptidoglycan	*Brevibacterium lactofermentum*	Enhanced resistance to *Enterococcus seriola*	126
FK-565*	*Streptomyces olivaceogriseus*	Enhanced resistance to *A. salmonicida*	127
β-glucan	*Saccharomyces cerevissiae*	Enhanced resistance to *V. anguillarum*, *V. salmonicida* and *Y. ruckeri*	128

*Synthetic analogue of a naturally occurring component of the bacterial cell wall.

optimization of the diet of culture fish to enhance the effectiveness of the immune response, and this approach[46,47] appears more applicable than the broader range of immunostimulants.

Probiotics

The health benefits of administering probiotics to humans, animals and birds have been widely promoted but until recently well-controlled studies producing statistically robust data have been the exception.[48,49] In addition, there is disagreement about what constitutes a probiotic and many different definitions have been proposed. The term "probiotic," derived from the Greek, and meaning "for life", was introduced in 1965[50] to describe factors which had the opposite effect to antibiotics, *viz.* growth-promoting factors produced by microorganisms and of benefit to other microorganisms. The definition by Fuller[51] of a probiotic being "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" is commonly used. However, Salminen *et al.*[49] proposed that a better definition would cover "microbial cell preparations or components of microbial cells that had a beneficial effect on the health and well-being of the host." This was intended to include non-viable cells and cell components, but to exclude metabolites such as antibiotics. Irianto and Austin[7] reviewed various definitions of probiotics and, in relation to aquaculture, broadly agreed with the definition of Salimen *et al.*[49] Although evidence is accumulating that many recognized human and animal probiotics may act via enhancement of the immune response,[52] the definition of Salminen *et al.* [49] seems unrealistically wide-ranging. Not only would it include most of the immunostimulants and nucleotide supplements mentioned above, but also vaccines (as no method or route of administration is defined) and is not considered useful here for discussions on fish health.

So as to accommodate the fact that fish exist in an aqueous environment where the skin and gill microflora, just as much as the intestinal flora, could be involved in disease prevention Gram *et al.*[53]

amended Fuller's definition to "a live microbial supplement which beneficially affects the host animal by improving its microbial balance." Such a definition would cover both addition of microbes to food and direct addition to rearing tanks and ponds where this alters the microbial balance in a beneficial way for fish or shellfish reared in such tanks.

Probiotics in the Rearing of Bivalve Molluscs

Shellfish account for a significant proportion of world aquaculture production[1] and the industry is dependent on the hatchery cultivation of large quantities of spat of oysters, clams and scallops for on-growing in the sea. Very heavy losses of larvae often occur in routine rearing hatcheries due to bacterial infection[54] and antibiotics have frequently been used to control pathogenic bacteria. Such a strategy is not sustainable in the long term due to the eventual development of antibiotic resistance in the pathogens, the desire to minimize release of antibiotics into the environment, and the need to minimize antibiotic use in species for human consumption. Thus, several groups have attempted to identify probiotic bacteria that could prevent losses due to pathogenic bacteria and alleviate the need for intervention with antibiotics. Douillet and Langdon[55] used axenic cultures of oyster, *Crassostrea gigas* larvae to identify a bacterial isolate, CA2 that enhanced growth and survival during larval rearing. In several experiments, addition of strain CA2 enhanced growth of oyster larvae and the organism appeared to act as a growth supplement and an effective probiotic.[56] In the culture of scallops, *Pecten maximus* hatcheries routinely used chloramphenicol to control bacteria otherwise survival of larvae would be extremely low.[57,58] A novel organism, *Roseobacter gallaeciensis* isolated from a scallop rearing unit was antagonistic to a range of *Vibrio* spp., including the scallop pathogen *V. pectenicida*.[59,60] Extracts of the bacterial culture significantly enhanced larval survival but this effect was not found with whole bacteria.[60] In similar studies with the scallop *Argopecten purpuratus*, Riquleme *et al.*[61] screened over 500 bacterial strains for *in vitro* inhibition of a *V. anguillarum*-related larval pathogen, and isolated 11 inhibitory bacteria of which one, *Pseudomonas* sp. 11, gave significant protection of larvae

from experimental infection by the *V. anguillarum*-related pathogen. In large-scale trials at a commercial hatchery over a complete rearing season, addition of three probiotic bacteria, *Bacillus* sp. B2, *Vibrio* sp. C33 and *Pseudomonas* sp. 11 every two to three days over the rearing cycle gave survivals better than parallel batches routinely treated with antibiotics.[62] In contrast to small-scale laboratory experiments reported by most workers, the scale of these trials, with an initial stocking of 120 million larvae per treatment and involving eight replicate rearing cycles throughout the year is impressive and provides very firm evidence that probiotic bacteria can provide an effective substitute for antibiotic treatment.

Probiotics in the Rearing of Crabs and Shrimp

Production of crab larvae is carried out on a significant scale in Japan but high mortalities were reported in young larvae.[63] After repeated losses due to a *Vibrio* infection, a bacterial strain PM-4, probably a *Pseudomonas* species, was identified[64] that inhibited growth of *V. anguillarum* in sea water and enhanced survival of crab larvae in routine hatchery rearing. In seven full-scale rearing trials using approximately three million larvae per batch, bacteria were added daily to a final concentration of approximately 10^6 ml^{-1} resulting in a final mean survival of 27.2% of larvae compared with 6.8% in nine control trials. In the latter control trials, six of the nine had no survivors. The added PM-4 dominated the flora of the tank water and *Vibrio* concentrations were much reduced or undetectable. Similar results were obtained through a second rearing season and no resistant *Vibrio* sp. emerged during this time.

The problems of prolonged use of antibiotics in shrimp culture, resulting in antibiotic residues in the farmed shrimps and in rapid emergence of antibiotic-resistant bacteria, are well documented,[65] leading to many attempts to modify the microbial flora of the shrimp larval rearing systems.[66,67]

Use of Probiotics in Larval Finfish Rearing

Pathogenic bacteria have been identified readily in rearing of invertebrate larvae,[18,19] crabs[63] and shrimps[20] but this has proved more difficult where

large losses have occurred routinely in larval finfish rearing for several species, e.g. with turbot and halibut. Early studies of the development of the microbial flora of fish larvae, their rearing water and food failed to identify recognized bacterial pathogens in Japanese flounder,[68] rockfish, tiger puffer and red grouper,[69] turbot,[70–72] halibut[73,74] but recent work has identified *V. splendidus*[75,76] and *V. pelagius*[77] as causing losses in turbot larval rearing. *Tenacibacter* (*Flexibacter*) *ovolyticus* has also been identified as a pathogen of halibut eggs and larvae.[78]

As noted by Gatesoupe,[4] probiotic treatments are particularly appropriate at the larval rearing stages of fish and shellfish larvae as they begin feeding before the digestive tract is fully developed. Prior to the initiation of feeding, low numbers of bacteria may seed the intestinal tract from the egg[79] or be ingested via drinking[71,73,74] which is necessary to maintain osmotic balance.[80] Once live food is ingested, its attendant microflora leads to a dramatic increase in bacterial concentrations in the gut within a few days, and the gut microbial flora changes when the food source is altered, e.g. from rotifers to *Artemia*.[71,73,74] Large-scale losses in larval cultures, or "crashes" can occur in hatcheries several days after such changes in diet during this period of instability in the gut flora. The importance of bacteria in causing larval crashes is inferred from the extremely high levels of larval survival when larvae are reared in the absence of a range of defined bacteria[81] and the beneficial effects of administering antibiotics.[74,82] In laboratory-based larval rearing systems, very high survival of turbot or halibut larvae can be obtained in the presence of high concentrations of specific bacteria (10^7 ml^{-1}) derived from the larval gut microflora[81,83] showing that most bacterial isolates from the larval gut are probably innocuous and that only a small proportion of the bacteria in the digestive tract are harmful to larvae.[83] Bacteria pathogenic for turbot larvae and isolated from instances of high losses of larvae in hatcheries have been identified recently as *V. splendidus* biovar 1.[75–77]

Many groups have begun the search for effective probiotics to enhance larval survival in finfish culture in the absence of identified pathogens. However, no successful application to large-scale rearing has yet been published, although many groups have reported the

identification of "potential probiotics" (see later section on "Discovery of New Probiotics").

A typical approach is that of Huys *et al.*[84] who exposed turbot larvae to a selection of bacteria in 1 litre beakers containing 500 ml seawater. Larvae exposed to one organism, a *V. mediterranei* strain isolated from sea bream larvae, showed greater larval survival up to day 5 than unexposed control larvae and the organism was considered a prospective probiotic. Results from such a test system must be regarded as preliminary since larvae were not fed and all died of starvation by day 8; in hatcheries it is generally considered that mortalities occur particularly after first feeding or changes of diet in larvae. Hansen and Olafsen[79] reported that their attempts to colonize cod eggs with "antibiotic-producing bacterial isolates" had not altered the colonizing bacteria from those found in untreated controls (although no data was shown).

Several studies have been reported by Gatesoupe in which commercial food additives containing live lactic acid bacteria were used in larval rearing.[85–89] Thus, a preparation containing *Streptococcus thermophilus* and *Lactobacillus helveticus* was added to rotifer cultures fed on algae and bakers' yeast and this enhanced the rate of production of rotifers in the culture significantly, whereas a second preparation, derived from lactic acid fermented cereal grain (Acosil) had no effect on rotifer growth.[88] Acosil-treated rotifers did, however, produce a small, but significant, increase in growth of Japanese flounder larvae over 18 days but no increase in larval survival.[88] Similar experiments with turbot larvae showed no increase in survival of groups fed on rotifers cultured with lactic acid bacteria but the mean weight of larvae fed on supplemented diets was significantly greater than that of control groups indicating a benefit from addition of these probiotic bacteria. Similar experiments with pollack, *Pollachius pollachius* showed that *Saccharomyces cerevisiae* and *Pediococcus acidilactici* significantly enhanced growth of larvae but did not improve survival.[89] In addition to testing commercial preparations of lactic acid bacteria (LAB) intended for use as animal probiotics, LAB were also isolated from the bacterial flora of rotifer cultures.[86] Incubation of rotifers in suspensions of these bacteria enhanced the proportion of LAB associated with the rotifers, and turbot

larvae fed LAB-enhanced rotifers showed enhanced survival compared with control groups.

Use of Probiotics with Juvenile and Adult Fish

Although vaccines have been developed which are effective against a range of bacterial diseases (see above), there is increasing interest in disease prevention by other means. Thus, several authors have reported success in protecting fish from infectious diseases by prior treatment with bacteria described as probiotics, although by most definitions some of these preparations might be better described as immunostimulants.

In Atlantic salmon infected with *A. salmonicida*, a very high proportion of fish which survive infection become asymptomatic carriers of the organism for a prolonged period.[90] Overt infection can be induced in carrier fish by subjecting them to stress, and Smith and Davey[91] showed that bathing such fish for one hour in a suspension of *Pseudomonas fluorescens* gave highly significant protection ($p < 0.01$ in each of four replicate experiments) from stress-inducible furunculosis. The *P. fluorescens* isolate was obtained during screening of bacteria from brown trout and it inhibited growth of *A. salmonicida* both on agar plates and in broth co-cultures. From other experiments, it was concluded that *P. fluorescens* inhibited growth of *A. salmonicida* due to competition for iron.

In a large systematic screening survey of bacteria from the intestinal tract of fish, *Pseudomonas fluorescens* strain AH2 was identified as a potential probiont to protect fish from pathogenic bacteria.[92,93] Strain AH2 inhibited growth of both *V. anguillarum* and *A. salmonicida* on agar plates and in broth co-cultures.[92,93] When rainbow trout were exposed to *P. fluorescens* AH2 for five days, added to tank water to a concentration of 10^5 ml^{-1}, highly significant ($p < 0.01$) protection was conferred from challenge with *V. anguillarum*[92] but not from a cohabitation challenge with *A. salmonicida*.[94] Protection from *V. anguillarum* could be obtained either by longer-term (five days) exposure of fish to probiotic bacteria or by addition of higher concentrations during exposure to *V. anguillarum*, the effects being

additive. The results indicate that this organism may provide an alternative strategy for control of vibriosis; the lack of success in controlling furunculosis may indicate a different route of infection by this organism and/or a different mode of pathogenesis of infection. Evidence was adduced that *P. fluorescens* AH-2 inhibited growth of *V. anguillarum in vitro* by competition for iron[95], as noted in other studies described above.[91] Iron is a crucial element for growth of almost all microbes[96] and the above evidence suggests it as a promising target for probiotic intervention.

Many algae are known to inhibit growth of bacteria[97] and *Tetraselmis suecica* has been shown to inhibit growth of several bacterial pathogens *in vitro*, and *in vivo*.[98] Atlantic salmon fed on a diet containing 1% algae showed mortalities of only 0% to 15% when challenged with a range of bacterial pathogens (*A hydrophila*, *A. salmonicida*, *Serratia liquefaciens*, *V. anguillarum*, *V. salmonicida* and *Y. ruckeri* type 1), whereas control groups of fish showed 85% to 100% mortalities. The mode of action was not determined but the possibility of a β-glucan-like action with elevation of the non-specific immune responses, i.e. an immunostimulant, was discussed.[98]

Further work by Austin's group has shown that feeding rainbow trout a diet containing four bacterial isolates from the intestinal tract of rainbow trout gave better survival of fish from challenge with *A. salmonicida*.[99] Similar effects were noted with live or dead cells.[100] Some non-specific immune defence parameters, e.g. phagocytic and lysozyme activities, were enhanced, but not a specific antibody response, again suggesting that these bacteria were acting as general immunostimulants.[7,99,100] Similar findings have been described by Nikoskelainen *et al.*[101] who fed rainbow trout on a diet containing 10^9 or 10^{12} colony forming units of *L. rhamnosus* g^{-1} feed for 51 days. In comparison with untreated control groups, the fish receiving *L. rhamnosus* showed significant protection from challenge with *A. salmonicida*. The *L. rhamnosus* strain tested was able to adhere to fish mucus and it inhibited growth of *A. salmonicida in vitro* by competition for nutrients rather than by secretion of antagonistic factors.[102] Therefore, it seems probable that *L. rhamnosus* acts as a

true probiotic by inhibiting growth of, or colonization/invasion by, the pathogen, rather than via a direct immunostimulant action.

Lactic Acid Bacteria Isolated from Fish

The well-documented ability of LAB to inhibit growth of many species of bacteria[52] makes them an attractive focus for studies on probiotics in fish. LAB dominate the normal microflora of the early stages of life of endothermic animals but their incidence in the microflora of fish is low,[87] possibly due to the low ambient temperature of the host and the fastidious growth requirements of the bacteria.[87,103] However, a careful search has resulted in isolation of *Lactobacillus* and *Carnobacterium* species from the digestive tract of several species.[87]

Joborn *et al.*[104] isolated an LAB, later identified as *Carnobacterium inhibens*,[105] during screening of material from salmon intestinal tract for bacteria capable of inhibiting growth of the fish pathogens *A. salmonicida* and *V. anguillarum*. The organism was capable of colonizing the intestinal tract of fish, surviving gut passage and production of inhibitors during growth in extracts of mucus and feces. Such an organism shows promise as a probiotic in fish, and in later work the *C. inhibens* strain K-1 was incorporated at a level of $5 \times 10^7\ g^{-1}$ into feed for juvenile rainbow trout and Atlantic salmon.[106] High concentrations of the organism were achieved in the intestine ($7.4 \times 10^6\ g^{-1}$) but it did not persist in the digestive tract once withdrawn from the diet, falling in concentration by $> 99\%$ within two days and to an undetectable level by six days.[106] Fish fed on the *C. inhibens*-containing diet for 14 days were more resistant to disease when challenged with *A. salmonicida*, *Yersinia ruckeri* and *V. ordalii*, but not *V. anguillarum*. However, the statistical significance of the results was not stated. The protection obtained against these important bacterial pathogens is encouraging but two other points are of particular note. Firstly, there was no correlation between the ability of *C. inhibens* to inhibit growth of the above organisms *in vitro* and the protection found *in vivo*, since *A. salmonicida*, *V. ordalii* and *V. anguillarum* were inhibited *in vitro* but *Y. ruckeri* was not.[106] Also, given the close

relationship between *V. anguillarum* and *V. ordalii*, the difference in response to these two pathogens to the effects of the probiotic is interesting. The difficulty in maintaining a high level of LAB in the fish intestine is commensurate with the finding of low levels of such bacteria in the intestinal tract of normal fish,[87] but enhanced levels may not be essential to achieve short-term probiotic effects.

Alternative Applications of Bacteria

Bioremediation or Water Maturation

Enclosed aquaculture systems provide the opportunity to control the microbiology of the rearing environment whether this is for control of water quality or suppression of pathogens. The ability to deal with nitrogenous wastes by removing ammonia and nitrite will govern the capacity of both traditional, open aquaculture ponds of Southeast Asia and the high capacity recirculated water systems increasingly used for rearing marine flatfish or salmon smolts in the Northern hemisphere. In recirculated water systems, efficient biofilters are essential to maintain "water quality" and floating biofilters have been used in shrimp ponds to increase shrimp survival.[107] Moriarty has described the additional use of *Bacillus* species in aquaculture ponds to inhibit shrimp-pathogenic *V. harveyi* and has proposed the term "bioaugmentation" to describe this process.[4,108,109] Similar work by Kennedy resulted in isolation of *Bacillus subtilis* from the common snook[110] and use of this organism resulted in elimination of vibrios from fish larvae and enhanced larval survival.[110]

In addition to attempting to prevent the growth or colonization of pathogens directly, other approaches have been investigated to control the bacterial flora of larval rearing tanks, or shellfish and fish rearing ponds. In this context, Vadstein *et al.*[111] outlined a strategy for altering the microbial flora of flatfish larval rearing tanks. Based on ecological principles, bacteria harmful to larvae were considered *r* strategists capable of rapid growth on a range of organic substrates and forming unstable pioneer communities, as opposed to *K* strategists capable of growing more slowly, when nutrients are limited, and forming stable interacting communities.

In an attempt to enhance the proportion of *K* strategists in the larval rearing water, the input water was passed through a biofilter to "mature" the water, i.e. reduce available organic nutrients in the water. Enhanced growth rate and survival were achieved for turbot and halibut larvae in rearing trials conducted using such matured water and represent an alternative strategy for controlling the microbial flora of rearing systems.[111] However, more development is probably required before this method could be scaled up for use on commercial larval rearing operations.

A different approach was developed by Verner-Jeffreys *et al.*[112] who operated larval halibut rearing tanks with recirculation of the tank water through a biofilter to achieve a more varied larval gut microflora than with conventional flow-to-waste rearing tanks. In five replicate trials, the gut microbial load was higher in larvae from tanks with recirculated water (8.7×10^3 larva^{-1}) than from those with flow-to-waste water (3.1×10^2 larva^{-1}) but survival was significantly greater (mean survival 48% versus 29%). The bacterial flora of larvae from the recirculated water group was more diverse than from the flow-to-waste group and it was concluded that bacterial types, rather than numbers, were important in determining larval survival.

Altering the Microflora of Live Food Organisms

A further method of controlling the gut microbial flora of larva is through control of the bacterial flora of live food organisms as these are vectors for introducing large numbers of associated bacteria into the digestive tract. The commonly used live food organisms, such as rotifers and *Artemia*, contain sub-optimal levels of polyunsaturated fatty acids (PUFA) that are essential for fish larvae. Levels of PUFA in these organisms are boosted by incubation in emulsions rich in essential fish oils, after which they can contain high concentrations of associated bacteria. Disinfection is commonly used to inhibit bacterial colonization of live food organisms during enrichment,[113] but UV radiation[114] has also been used, resulting in $>99\%$ reduction in bacteria associated with rotifers and enhanced survival of turbot larvae in a low-intensity culture system.[114]

The inhibitory effect of certain microalgae on bacterial growth ("Use of Probiotics with Juvenile and Adult Fish" section) was exploited by Olsen *et al.*[115] by feeding *Artemia* on *Tetraselmis* for two hours prior to use as live food. This short exposure to microalgae reduced the bacterial load of *Artemia* by 75% leaving a more diverse bacterial flora and leading to a reduced number of bacteria associated with the gut of larval halibut fed on the *Artemia*. An alternative method to altering the bacterial flora of rotifers and *Artemia* is by incubation of the prey organisms in suspensions of bacteria and this has been pursued in several studies.[81,86,114,116]

Future Developments

Types of Diseases Amenable to Probiotic Intervention

In designing a strategy for discovery and use of probiotics in fish one must consider the types of infectious agents which might or might not be amenable to probiotic intervention. Whereas enteropathogenic bacteria should prove appropriate targets for intervention, viruses would be more difficult opponents, as once they have achieved the intracellular state they often spread directly from cell to cell. However, if nucleotide-enriched diets can induce enhanced resistance, then the possible benefits of probiotics should not be dismissed out of hand. Fungal infections are a particular problem in freshwater infections of fish and strains of *P. fluorescens* that inhibit growth of *Saprolegnia* have been isolated from catfish rearing pond water.[117] *P. fluorescens* has been considered by several workers[91–94] as a potential probiotic organism and may be of value in modifying the fish rearing environment as well as acting directly as a probiotic.

Discovery of New Probiotics

In the search for potential probiotic bacteria, Hjelm *et al.* [118] have recently developed a targeted approach that allows screening of very large numbers of environmental bacteria from which those capable of

inhibiting growth of pathogens, e.g. *V. anguillarum*, can be identified and isolated. This high throughput approach represents a major advance in identification of potential probiotic organisms from a wide range of sources. This now requires a similar advance in the screening of such organisms to establish that they are not harmful to larval fish, before commitment to large scale trials *in vivo*.

Identification of Suitable Probiotics — Lessons from Agriculture

The majority of studies aimed at identification of probiotics for mammals have concentrated on identification of individual organisms, usually LAB, which are capable of directly inhibiting growth of enteropathogens or their colonization of the alimentary tract.

However, to select probiotic bacteria that could protect chickens from carriage of salmonellae, Nisbet *et al.*[119] used a continuous culture approach to mimic conditions in the chicken digestive tract resulting in selection of a mixture of organisms that could colonize the digestive tract. The balanced mixture of organisms (PREEMPT^{TM}) was shown to be successful in large-scale commercial use and represents an excellent model for developing a true "normal" gut flora capable of withstanding the intrusion of pathogens. The challenge remains to develop such a mixture for fish, especially larvae, when the digestive tract continues to develop with concomitant changes in O_2 tension, pH and cell receptors.

Maintaining Probiotic Bacteria in the Fish Digestive Tract

One of the difficulties which must be overcome in the use of probiotics is how to achieve adequate levels of the prospective probiotic bacteria in the larval fish gut. If probiotic bacteria cannot compete for nutrients against other bacteria their numbers will decline rapidly unless replenished in substantial numbers via live food. This may not present a problem in finfish where the bacteria appear to induce non-specific stimulation of the immune system[100–102] but in larval rearing systems

it may be necessary to maintain significant concentrations of bacteria in the gut until a stable flora is established.

In small-scale trials, this may not be difficult to achieve if the live food has a low concentration of microorganisms competing with the added probiotic organism. Thus, high levels of defined bacteria can be loaded onto live food organisms, e.g. rotifers and introduced into the larval gut in high numbers without difficulty in the *absence* of competing bacteria.[81] Also, a single addition of *V. pelagius* to the water of larval rearing tanks to a concentration of 4×10^5 ml^{-1} on the day of hatching of the larvae led to this organism forming 60% of the bacterial gut flora (5×10^4 larva^{-1}) by day 14.[120]

However, with similar organisms in trials in a commercial hatchery, Munro *et al.*[114] found that three different types of potential probiotic bacteria administered to turbot larvae via the rotifer feed, could not subsequently be detected in the larval gut microflora, representing at least a 100-fold drop in bacterial concentration relative to other bacteria in the microbial flora.

This highlights the potential difficulties in moving from small- to farm-scale experiments, and careful choice of potential probiotics, or regular addition of an organism in large quantities may be required to maintain the organism in the gut microflora.

Commercial Production and Licensing of Probiotics

The discovery of probiotics capable of preventing disease in aquaculture, thereby reducing use of antibiotics and improving fish welfare represents an important scientific goal. However, once the efficacy of a probiotic has been proven in properly designed trials the feasibility of producing the organism in sufficient quantities and at an economic cost on a commercial scale must be considered. Also, whether classed as a medicine or a food supplement, the organism(s) chosen must meet regulatory requirements for safety before they could be licensed for use in most countries. These factors may provide hurdles equal to those of the scientific tasks before probiotics can be used routinely in aquaculture.

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