**Abstract**

**Introduction:** *Piscirickettsia salmonis* (*P. salmonis*) is the aetiological bacterium of the contagious disease piscirickettsiosis or salmonid rickettsial septicaemia (SRS) and causes significant economic losses to aquaculture production in Chile. Current strategies to control infection are i) indiscriminate antibiotic use and ii) vaccination with predominantly *P. salmonis* bacterin vaccines that do not provide acceptable levels of protection against piscirickettsiosis.

**Areas covered**: the basic biology of *P. salmonis*, clinical piscirickettsiosis and disease control, the development of current *P. salmonis* vaccines, innate and adaptive immunity and a 5 year plan to develop new piscirickettsiosis vaccines.

**Expert commentary:** fundamental knowledge is lacking on the complexities of *P. salmonis*-host interactions, relating to bacterial virulence and host innate and adaptive immune responses, which needs to be addressed. The development of new *P. salmonis* vaccines needs the application of comprehensive ‘omics’ technologies to identify candidate vaccine antigens capable of stimulating long-lasting protective immune responses.

**1. Introduction**

Aquaculture is a worldwide industry that provides ~50% of the farmed fish consumed by humans annually. As with other food industries, it suffers problems during the production phase. A major problem is transmissible disease caused by diverse viruses, prokaryotic (bacteria) and eukaryotic organisms (e.g. moulds, fungi, metazoa, unicellular and helminthic parasites). Transmissible diseases impact on fish immune systems and eventually lead to severe aquaculture production losses. In Chile, the farmed fishing (salmon and trout) industry has rapidly grown during the past two decades to become one of the most important factors in the country’s economic development and it is the largest in the world alongside Norway. The most important pathogens for these fish that are found in freshwater are *Flavobacter spp.* bacteria, fungal *Saprolegnia spp.* andinfectious pancreatic necrosis (IPN) virus*,* whereas those found in seawater are the bacterium *Piscirickettsia salmonis,* the sea louse ectoparasite *Caligus rogercresseyi* and the infectious salmon anaemia (ISA) virus [1]. Significant economic losses to Chilean aquaculture production occur annually due to the contagious bacterial disease piscirickettsiosis or salmonid rickettsial septicaemia (SRS), which is caused by *P. salmonis*. Piscirickettsiosis is a systemic infection that affects wild and cultured salmonids such as the coho salmon (*Oncorhynchus kisutch*), Atlantic salmon (*Salmo salar L*) and rainbow trout (*Oncorhynchus mykiss)* [2]. There have also been reports of *P. salmonis* infection in marine fish including the European seabass *Dicentrarchus labrax* [3], the white seabass *Atractoscion nobilis* [4], the grouper *Epinephelus melanostigma* [5], tilapia (*Oreochromis, Tilapia* and *Sarotherodon* *spp.*) and blue-eyed plecostomus *(Panaque suttoni*) [6].

Intesal (Instituto Tecnológico del Salmón, Salmon Tech Institute, Chile) estimates that piscirickettsiosis results in annual losses to Chilean farmed fisheries of USD $700 million. However, the disease is not restricted to Chile and in subsequent years, the disease has been reported in Ireland, Norway, Canada [7,8] and newly emergent in Turkey [9] . Interestingly, outbreaks in several parts of the world have not been as serious as the outbreaks in Chile and could be related to differences in virulence between *P.salmonis* geographical isolates [10]. Control and prophylactic strategies against *P. salmonis* have relied principally on antibiotic use and vaccination, but these strategies are inadequate. Our review begins with brief descriptions of the biology and known virulence factors of the causative organism *P. salmonis*, the clinical presentation of piscirickettsiosis and disease control and treatment through antibiotic use. We then focus on the nature and efficacy of the vaccines currently available commercially in Chile and recent developments in experimental vaccines. Our review closes with a discussion of the outstanding issues in *P. salmonis* research, including new strategies that could be used to produce more effective vaccine(s) to command a marketplace crowded with a large number of vaccine choices.

**1.1. Biology of *Piscirickettsia salmonis***

The first case of piscirickettsiosis was reported in 1988 in the Huito channel, Calbuco, Chile, where the pathogen was isolated from an outbreak of infection in coho salmon that caused >90% production losses [11,12]. The *Piscirickettsia salmonis* bacterium isolated in 1989 from Chilean coho salmon is referred to as the type strain LF-89 [13,14], located in the American Type Culture Collection as ATCC VR-1361 [15]. *P. salmonis* is a non-motile, non-encapsulated, pleomorphic but usually coccoid bacterium, found in pairs or ring-shaped structures with an approximate diameter of 0.5–1.5 μm. It is a facultative, intracellular Gram-negative bacterium and was classified initially within the *α-Proteobacteria* family *Rickettsiaceae* and referred to as *Rickettsia*-like, because of similarities with other family members. Subsequently, molecular phylogenetic methods based on 16S rRNA gene sequencing classified the bacterium into a new family *Piscirickettsiaceae* in the phylum *Proteobacteria*, class *γ-Proteobacteria*, order *Thiotrichales* and assigned a new genus *Piscirickettsia* and species *salmonis.*

For some time, it was considered that *P. salmonis* could not be cultured outside of eukaryotic host cells [13], but there are several examples of different agar and liquid culture media available for bacterial culture [16-18]. The ability to grow *P. salmonis* *in vitro* allows study of the growth characteristics, genetics and pathogenesis of this organism and is a significant step towards vaccine development. The first draft genome sequence of *P. salmonis* strain LF-89 VR-1361 was published in 2013 [19]; this genome sequence reported 3,388,517 bp and a G+C content of 39.2%. The same genome sequenced by Pulgar *et al.* reported a single circular chromosome of 3,184,851 bp and three plasmids (pPSLF89-1, 180,124 bp; pPSLF89-2, 33,516 bp; pPSLF89-3, 51,573 bp), 2850 protein-coding genes, 56 tRNAs and six copies of 5S-16S-23S rRNA [20]. For the latest genome assembly (NCBI ASM153472v1), the genome has 3,423,535bp, 3516 protein-coding genes and 74 RNAs. Within the major coding sequence subsystem categories (Figure 1), there are 362 subsystems and for the 3516 protein-coding sequences, 48% are recognised within the subsystems (1657 total protein-coding sequences, with 1604 non-hypothetical, 53 hypothetical) and 52% are not recognised within subsystems (1859 total protein-coding sequences, with 962 non-hypothetical, 897 hypothetical) . Draft genomes of the virulent *P. salmonis* strain AUSTRAL-005 [21], isolated from *O. mykiss* farms and strains A1-15972 (EM-90-like) and B1-32597 (LF-89-like), recovered in 2010 and 2012 from Atlantic and coho salmon, respectively [22] are also now available. However, at present, the known catalogue of potential vaccine antigens is limited, although the complete lists of protein-coding sequences provide a platform for comprehensive reverse vaccinology and proteomics approaches for developing *P. salmonis* vaccines.

**1.2. Clinical piscirickettsiosis and disease control**

The route of pathogen transmission and disease spread is not completely understood. The aquatic environment obviously protects *P. salmonis* from desiccation and transmission is generally considered to be via water [23]. Unlike *Rickettsiae* that are usually transmitted to hosts via an invertebrate vector or from a reservoir [24], no vector or reservoir has been established for *P. salmonis* [13]. However, *Ceratothoa gaudichaudii*, a parasitic isopod associated with farmed salmon in Chile, was identified as a potential host for *P. salmonis*. The potential for marine finfish species acting as a reservoir has been suspected but remains unproven [25] and molluscs such as *Mytilus chilensis*, *Choromytilus chorus* and *Aulocomya ater*, which are normally cultivated close to the fish farms and *Fisurella spp.* that are natural inhabitants of cages, do not seem to be involved as vectors/reservoirs of transmission [26]. It has been suggested that the main entry site of *P. salmonis* is through fish skin, whereby bacteria can penetrate the skin in the absence of injury, but that skin injuries could facilitate invasion [27], and possibly natural transmission of bacteria via the gills to the gill capillaries [28]. Oral exposure is not an important route of transmission as the low pH and digestive enzymes present in the fish stomach are capable of inactivating the bacterium [27].

Fish infected with *P. salmonis* are symptomatic and the clinical signs and pathological findings of piscirickettsiosis are summarized in Table 1. *P. salmonis* is capable of invading all major fish organs, but the bacterial virulence factors and the mechanisms of pathogenesis remain poorly understood. Recognizing how *P. salmonis* invades deeper tissues and adopts the intracellular lifestyle requires robust *in vivo* and *ex vivo* models for studying infective mechanisms. Table 2 contains descriptions of some key virulence factors that have been identified for *P. salmonis* and their putative pathophysiological functions. Recent studies are beginning to unravel the biological behaviour of this bacterium *in vivo* and *in vitro*. With regards to host invasion, *P. salmonis* may use macrophage infection as a strategy, common to other intracellular pathogens, to colonize and disseminate within host tissues. The molecular mechanisms used by *P. salmonis* to maintain an intracellular lifestyle are unknown, but roles have been attributed to various factors including the bacterial heat shock protein (HSP) protease ClpB, the virulence factor BipA, LPS and the Dot/Icm proteins (Table 2). *P. salmonis* is capable of surviving and replicating within complete membrane-bound cytoplasmic vacuoles in rainbow trout head kidney macrophages *in vivo* [29]. Recently, it has been shown that clathrin is required for macrophage internalization of *P. salmonis* and that the actin cytoskeleton plays a role throughout infection. *P. salmonis* appears to use actin monomers from the disorganized cytoskeleton and apparently the bacterium can induce *de novo* synthesis of actin in order to generate the vacuoles that harbour the bacteria [30]. However, intercellular motility of *P. salmonis* did not appear to be actin-based [29]. P. salmonis can also infect, survive and replicate within the macrophage/monocyte cell line RTS-11, derived from rainbow trout spleen, without inducing characteristic cytopathic effects [31]. Studies of *P. salmonis* interacting with CHSE-214 cells *in vitro* showed that attachment and invasion are rapid events [32]. However, it has been reported that *P. salmonis* can induce caspase-3 mediated apoptosis in trout macrophage and monocyte-like cells *in vitro* [33]. Taken together, the conflicting capabilities of *P. salmonis* to either survive intracellularly without inducing cytopathy, or to induce apoptosis – which may reflect different cell tropisms - both represent possible mechanisms of bacterial survival and evasion of host innate immunity.

According to the Chilean Fisheries and Aquaculture Service (Sernapesca), the main infectious cause of salmonid species mortality during the ‘on-growing phase’ in Chile is piscirickettsiosis [34]. Indiscriminate use of antibiotics has been used to control infection in these species since the beginning of farmed fisheries as an industry in Chile. In 2014, 563.2 kilo tonnes of antibiotics, particularly the anti-bacterial agent florfenicol (71%) and the bacteriostatic agent oxytetracycline (28%) were administered to control piscirickettsiosis [35] and this amount represents ~90% of the total antibiotics used in the country! Although *P. salmonis* is sensitive *in vitro* to many antibiotics commonly used to control other infectious diseases in fish [13,36], infected salmonids respond poorly to these treatments, likely due to the fact that the bacterium maintains an intracellular lifestyle and perhaps insufficient concentrations of antibiotic(s) reach the intracellular niche to effect pathogen removal. Antibiotics can be administrated to fish using immersion baths, in feed or by injection. Oral administration of oxolinic acid has been the drug of choice, but the fish response is slow and the drug has to be given repeatedly [23]. Treatment with florfenicol and oxytetracycline has shown sub-optimal efficacy in controlling piscirickettsiosis. Moreover, environmental pollution through indiscriminate antibiotic usage can promote the development and spread of antibiotic-resistant commensal and pathogenic bacteria in aquaculture ecosystems [37], as well as influence the genetic diversity of the intestinal microbiota of both salmonid and non-salmonid fish.

Studies to elucidate the total microbiota of fish species, including *O. mykiss* and other aquaculture species, are now increasingly being reported [38]. Microbiomes play important roles in teleost physiology and overall health, but microbiome homeostasis can be affected by changes to nutritional status, infectious diseases and stresses including antibiotic exposure. For example, using flavomycin and florfenicol reduced total intestinal bacterial counts in tilapia [39] and the use of oxytetracycline was reported to significantly reduce the diversity of the intestinal microbiota of *Salmo salar,* potentially favouring the proliferation of opportunistic pathogens [40]. Recently, *P. salmonis* isolates that are resistant to quinolones have been described, and polymorphism in the Quinolone Resistance-Determining Region of the DNA gyrase protein encoded by *gyrA* could be responsible for the phenotype [41]. The development of resistance has impacted negatively on Chilean salmon farming, but the industry has been pro-active since 2007 in adopting new biosecurity and aquaculture practices to minimize outbreaks of infections caused by ISA, *P. salmonis* and *C. rogercresseyi* [1]. International markets appreciate that antimicrobial exposure poses potential health risks on consumption and therefore set maximal residue limits (MRL) for antimicrobials in the flesh and skin of fish; for example, in the US and Japan, oxolinic acid is not permissible, whereas in the European Union and Chile, a MRL of 100µg oxolinic acid/Kg is allowable [1].

Proposed solutions to the profligate use of antibiotics in Chilean aquaculture include the use of probiotics, bacteriophages and effective vaccines. Elucidating total fish microbiota has the benefit of identifying probiotic bacteria, which could provide the basis for a strategy complementary to using antibiotics as growth promotors and sterilizing agents in farmed fish [42]. Probiotics could offer distinct advantages, e.g. improving the activity of the normal, commensal intestinal microbiota and optimizing food utilization, and enhancing factors such as humoral and mucosal immunity and resistance to infectious diseases in order to improve growth and survival [43]. As one example, recent studies have shown that commensal *Lactococcus lactis* isolates from *O.mykiss* intestineexerted a probiotic activity against the trout pathogen *Lactococcus garvieae* [44]. Though probiotics are not in general use for the farmed fishing industry, the major solution of vaccination has been successful in Norway, which moved from using antibiotics to vaccination [45], such that today, Norway uses only ~1000 Kg of antibiotics to treat disease in infected fish, coupled with hygiene measures to prevent cross-contamination. The variety of reasons why the vaccination strategy used in Norway is not being replicated successfully in Chile, with a multitude of commercially available vaccines at her disposal, is discussed below.

**2. The development of *P. salmonis* vaccines**

According to Servicio Agricola y Ganadero (SAG, May 2016), there are 33 commercially available vaccines against piscirickettsiosis (Table 3). The majority of these vaccines are composed of *P. salmonis* bacterin, which is strictly defined as a preparation of killed *P. salmonis* bacteria. Manufacture of bacterin involves growth of the pathogen by infection of tissue culture cells or growth in broth, with demonstration of a cytopathic effect, followed by bacterial collection by centrifugation. Bacterin can be prepared by heat-inactivation (e.g. 100°C for 30 min) or formalin treatment (1% (v/v) formaldehyde for 24h at 4°C) [46]. Formalin-inactivated bacterin is washed with phosphate-buffered saline prior to vaccination. *P. salmonis* bacterin vaccines are prepared with oil-based emulsion adjuvants, e.g. montanide ISA 763A, non-mineral oil adjuvants, Drakeol 6VR, Arlacel 83V, Tween 80, vegetal phospholipids, polysaccharides or glucans. Vaccination can be with monovalent bacterin or bi-, tri-, tetra- or pentavalent vaccines (Table 3). The multivalent vaccines can contain bacterins from *Vibrio ordalii* *(anguillarum)* and/or *Aeromonas salmonicida* and/or IPN virus, ISA virus and *C. rogercresseyi* (Table 3). There is one subunit vaccine, AQUAVAC® SARISTIN 2, which contains a recombinant IPN virus protein VP2 and a recombinant SRS lipoprotein ORF1-90kDa protein, and one live, attenuated vaccine, ALPHA JECT LiVac® SRS.

Commercial fish vaccines can be are administered orally in feed, by dip or bath immersion or by intraperitoneal (*ip*) or intramuscular (*im*) injection route [47]. The majority of the *P. salmonis* bacterin and other fish bacterin vaccines that are emulsified with oil adjuvants are injected *ip*, whereas only two *P. salmonis* vaccines are delivered orally. The *im* route is reserved generally for DNA vaccines, but no such vaccines are licensed for *P.salmonis*. In general, *ip* injection produces the highest levels of protection, but this route of delivery is associated with stress to the fish from intensive handling and the development of injection-site adverse effects such as inflammation and tissue necrosis caused by the oil adjuvant component. The vaccination route is also suggested by the age and size of the fish, such that small fish are usually vaccinated orally or by immersion, but followed by *ip* boosting with oil-based adjuvanted vaccine, which is the only regimen that induces long-lasting protection, despite the adverse effects.

The potency and safety of current and new fish vaccines are generally evaluated using *in vivo* methods with affected host species [48]. Ideally, the conditions for vaccine validation should most closely resemble the affected species’ rearing and natural environments [47]. Few studies have evaluated the protection afforded by *P. salmonis* vaccines against different pathogenic strains and via different potential routes of exposure to pathogen, such as *ip* injection, injection into the dorsal median sinus, infectivity by bath challenge, co-habitation or surface application of bacteria on paper discs applied to the skin or by transferring vaccinated fish to an environment with a history of *P. salmonis* outbreaks for natural infection [46,49-52]. In all these studies, only a few different *P. salmonis* strains were used for infection, namely *P. salmonis* PS2C [50], LF-89 [49,51,52] and SCO-95A [46]. For mimicking natural infection, Smith *et al.* [49] vaccinated pre-smolt coho salmon with bacterins and transferred the fish after smolting into a natural *P. salmonis* infection area. Protection observed with the bacterin, however, could be influenced by this infection model, since the amount of bacteria in the water was not controlled and probably insufficient to produce true infection, and potentially other pathogens could have caused the observed mortality and not specifically *P. salmonis*.

The model that mimics natural exposure most closely, whereby *P. salmonis* needs to overcome external and internal innate host defences, is probably cohabitation of infected and uninfected fish. This model is likely to be the most satisfactory challenge method for vaccine studies. However, in the study from Birbeck *et al.*, who reported the effects of vaccination with a *P. salmonis* SCO-95A bacterin on post-smolt Atlantic salmon and compared different routes of infection, co-habitation as well as bath challenge and surface application of bacteria all failed to induce mortality, whereas injection of pathogen into the dorsal median sinus resulted in significant mortality that could be used as a model to examine vaccine efficacy [46]. It should be stressed that the virulence of a single pathogen can also vary between different fish and that validation of any infection model should be done with several different strains. *P. salmonis* vaccine validation needs standardization and the use of surrogate strategies could be considered, e.g. transgenic fish cell lines that might inform immune activation by vaccine antigens and the use of zebrafish to study vaccine delivery and uptake [47]. However, these models may be useful for pre-screening, but are unlikely to replace the need for final testing with reliable and reproducible models that use the target fish species under field conditions.

There are considerable issues surrounding the efficacy of *P. salmonis* bacterin vaccines under field conditions. There is a lack of field information in Chile regarding the immune response and protection generated by these vaccines, although the general consensus appears to be that long-term efficacy is variable [25,53]. For Atlantic salmon and rainbow trout, a recent study has demonstrated that commercial vaccines do not completely prevent piscirickettsiosis; although the time-to-first outbreak was significantly delayed for vaccinated rainbow trout compared to unvaccinated fish, the total piscirickettsiosis mortality of vaccinated fish was not significantly different from unvaccinated fish [54]. However, the same study suggested that a booster vaccine strategy in Atlantic salmon provided significantly lower mortalities associated with piscirickettsiosis and a delay in the onset of disease.

The variable efficacy of *P. salmonis* bacterin vaccines has led to an increase in the number of injected vaccine doses for the control of piscirickettsiosis in Chile. Interestingly, the initial immunization event with these vaccines has a profound effect on the disease profile: the vaccines are reasonably effective at preventing the initial piscirickettsiosis outbreaks that occur in fish moved from fresh to sea water for the ‘on-growing’ stage [50]. However, the fish become susceptible, after surviving the initial outbreak, to new and more aggressive piscirickettsiosis outbreaks and this is believed to occur as a result of immuno-suppression following the first immunization event, though this mechanism remains unproven. Notably, these secondary piscirickettsiosis outbreaks affect large fish at 10–12 months after transfer, at which time vaccine protection may be redundant, and results in greater economic losses. Re-vaccination of larger fish with injectable bacterin vaccines to prevent or moderate these secondary outbreaks has been considered, but essentially dismissed as a viable strategy due to operational difficulties.

As an alternative strategy, oral immunization poses less risk to the fish and experimental approaches to developing oral vaccines against a wide variety of different fish pathogens have been reviewed extensively by Embregts and Forlenza [47]. It is worth noting that very few oral-mucosal vaccines are licensed for use in animals and humans. By contrast to the *ip* or *im* routes, vaccine antigens such as recombinant proteins, synthetic peptide-based vaccines and DNA vaccines are susceptible to breakdown in the fish gut when delivered orally. This has led to the development of vaccine antigen-protective microencapsulation strategies using liposomes, chitosan, alginates, Poly D,L-lactic-co-glycolic acid or the MicroMatrix™ Targeted Delivery System, all of which can be delivered in feed. In a recent study, salmonids were vaccinated with different commercial mono- or polyvalent vaccines against piscirickettsiosis and ISA, with a primary *ip.* injection of oil-adjuvanted vaccine(s) followed by booster vaccination with oral vaccines to simulate mucosal immunity [55]. Injected vaccines induced high levels of specific IgM antibodies and subsequent oral immunization maintained long-term high concentrations of anti-*P. salmonis* and anti-ISAv specific IgM antibodies. When the concentration of antibodies decreased below 2000 pg/mL, susceptibility to piscirickettsiosis infection was apparent. Thus, several oral immunizations in the field, which are considered facile to administer, would be necessary to maintain high levels of anti-*P. salmonis* antibodies during the whole productive cycle. However, it should be stressed that little is known about the balance between effective immune induction and the potential for inducing tolerance by oral immunization, and whether oral primary immunization could induce local and systemic responses. Moreover, how much immunity at the mucosae and gut surfaces is induced by *ip* vaccination needs to be addressed.

**3. Expert Commentary and Five Year Plan for developing *P. salmonis* vaccines**

Overall, ten years have passed since the first vaccine(s) against *P. salmonis* was introduced into the Chilean market. It is fair to say that most of these commercially available *P. salmonis* bacterin vaccines have had no significant effect on reducing farmed fish mortalities under true field conditions. Variable bacterin vaccine efficacy has been attributed to several inter-relating factors, namely i) the virulence of the organism, ii) the host factors of genetic resistance, quality of the smolt and the immune system, iii) the farmed environment and iv) the quality of fish husbandry [25]. When outbreaks do occur, the recourse is an indiscriminate use of antibiotics, which does not necessarily lead to pathogen removal. Tackling infection now requires a deeper understanding of the basic biology of the pathogen, the nature of the host innate and adaptive immune responses to infection/vaccination and the application of new ‘omics’ technologies to expand the sparse list of potential vaccine candidates.

**3.1. Innate immunity to *P. salmonis***

A renaissance in fish immunology research has provided an ever-increasing number of reviews describing many aspects of teleost innate immunity, including teleost immune-relevant genes, mucosal immunity and the role of phagocytes, neutrophils, T and NK cells and B-1 lineage cells [56-61], notwithstanding articles on innate responses of specific fish to specific viral, bacterial and helminthic infections. Nevertheless, there is still much to be learned of salmonid innate immune responses to infection with *P. salmonis*. The epithelial and mucosal barriers of salmonid skin, gills and intestinal tract provide the first physical barrier to *P. salmonis* and mucus secretions also contain a plethora of antimicrobial components [62]. There does appear to be significant genetic variation for resistance to piscirickettsiosis in *S. salar* and genetic improvement of disease resistance is still a viable strategy to control infection in farmed fish [63]. The identities of *P. salmonis* surface components and the specific host cell receptors that recognise them in the context of cellular adherence and invasion, as well as immune recognition, are still patently unclear. *P salmonis* does present some diverse pathogen associated molecular patterns (PAMPs) on its cell wall e.g. flagellin, LPS, proteins, lipoproteins and phospholipids [64] as well as intracellular CpG-DNA [51]. In fish, including *Salmo salar*, a few Toll-Like Receptors (TLRs) have been described at genome level (TLR1, TLR3, mTLR5, sTLR5, TLR7, TLR8a1, TLR8a2, TLR8b1, TLR8b2, TLR9, TLR13 [65]) and seven non-mammalian TLRs at the protein level (TLR18, TLR19, TLR20 (4 copies from a-d) and TLR21 [66]). There is some evidence that *P. salmonis* and other fish pathogens modulate *S. salar* TLR expression and innate immune responses [67]. Increased mRNA expression for m/sTLR5 that recognize flagellin was observed in Atlantic salmon infected with *Aeromonas salmonicida*, but no protein expression or functional responses was examined [68]. Cytokine responses of *ex vivo* fish cell cultures to different TLR agonists also suggest involvement of TLR signaling pathways in fish innate immune responses. Treatment of primary cell cultures from the head kidney of *S. salar* with the TLR1/2 and TLR2/6 agonists Pam3CSK3 and FSL-1, demonstrated up-regulated mRNA expression of IL-1β cytokine induced by Pam3CSK3, whereas FSL-1 was inactive. Significant up-regulation of IL-1β and IL-8 expression was also observed in fish cells incubated with the TLR4 agonist *Escherichia coli* K12 LPS and the sTLR5 agonist flagellin. Salmon primary cell cultures incubated with the TLR 9 agonist CpG ODN2006 also up-regulated IL-1β and IFN-α1 expression [65]. Up-regulation of TLR-1, TLR-9, TLR-22, Myd-88 and IL-1β was observed in SHK-1 cells and primary culture of trout head kidney leukocytes stimulated *in vitro* with *P. salmonis* [67,69], suggesting that *P. salmonis* can induce innate immune signaling pathways in the host. The nature of PAMP-TLR interactions during piscirickettsiosis and indeed other microbial infections of fish require further study, to elucidate the host signaling and defense pathways activated. However, progress in this area is hampered by the lack of diagnostic antibodies and reagents for fish immunology. It has been suggested that TLR ligands could be used as adjuvants in fish vaccine formulations [65] and their inclusion could contribute to improving the efficacy of the commercially available *P. salmonis* bacterin vaccines and experimental non-bacterin vaccines.

In addition to PAMP-TLR interactions, key molecules of the host innate immune response to *P. salmonis* and other fish pathogens include lysozyme, trypsin-like proteases, β2 macroglobulin, complement components C3, reactive protein-C and agglutinating lectins. An increase in serum lysozyme has been reported in *S. salar* infected with *A.* *salmonicida* for long periods and was present well before the development of an adaptive immune response [70]. A trypsin-like activity has also been identified in the mucus and skin of *S. salar* [71,72].The serine protease glandular kallikrein has been described in phagocytic and cytotoxic cells of fish that are analogous to mammalian natural killer (NK) cells [73]. Glandular kallikrein has been detected by immunohistochemistry in the gills, skin, head kidney and mucus of *S. salar* infected with *P. salmonis and V. ordalii* [74], though the antimicrobial function of this enzyme remains unclear but may involve activation of the complement cascade rather than a direct microbicidal effect. Further fundamental studies on innate immune response mechanisms of fish to microbial insults are essential.

**3.2. Understanding adaptive immunity to *P. salmonis* is critical for vaccine development**

Many components of the teleost adaptive immune system have been described and reviewed extensively elsewhere [56-58,75-77]. The teleost and mammalian adaptive immune systems shares several similarities [57], with the presence of lymphocyte cells, B and T cells and cytokine networks, and effective vaccination against *P. salmonis* must rely on stimulating adaptive immunity and long-term memory responses. Teleost B lymphocytes express immunoglobulin (Ig) on their surface and secrete antigen-specific antibodies in response to immune stimulation [78]. Three classes of immunoglobulin isotypes have been identified in teleost fish (IgM, IgD and IgT) according to their heavy chain isotype, namely μ, δ and τ (ζ for zebrafish), respectively. The most abundant is IgM produced by plasma cells and plasmablasts located in the head kidney. IgT appears to be the main Ig in the gut, skin and nasal mucosa, suggesting a specialized role for mucosal immunity [79]. B cell subsets can be distinguished according to their expression of distinct Ig class combinations. Both IgM+ and IgT+ B cells have been reported to possess potent phagocytic and anti-microbial properties. Teleost immunological memory is disputed: the adaptive response is slower in fish compared to mammals and specific antibodies are not normally detected in fish until three-to-four weeks after immunization. B cell affinity maturation and clonal expansion does exist in fish; hence, protection can persist for several years post-vaccination.

At least four different T-helper (Th) cell subsets - Th1, Th2, Th17 and T-regulatory cells - have been characterized in mammals in detail, with each subset expressing signature cytokines and transcription factors. A common feature of all Th cells is surface expression of CD4 glycoprotein, which provides specificity in the cellular interaction with MHC-II proteins. A structurally conserved ortholog of mammalian CD4 as well as additional genes (*cd4rel* or *cd4-2*) have been demonstrated in many fish [58]. Th cells also release cytokines that initiate and activate downstream effector mechanisms and cytokines such as IL-2, IL-4/13, IL-12, IL-17A/F and IFN-γ are important initiators of adaptive immunity in many fish. Although the identity and character of teleost T cells and T cell-related molecules is being studied, whether Th cells and Th cell subtypes exist and are capable of releasing cytokines to mediate adaptive immunity is not fully understood in teleosts. Cytotoxic T cells (Tc) express the T cell receptor (TCR) co-receptor CD8, recognize antigenic peptides via TCR/MHC class І interaction and produce effector molecules that kill infected cells. In different fish, two CD8 genes have been identified, as well as conserved structures in the intracellular region (including the CXC motif), a recruiting region for the Lck kinase and demonstrable specific cell-mediated cytotoxic activities, e.g. alloantigen- and virus-specific cytotoxicity.

It is clear that *P. salmonis* activation of fish adaptive immune responses is limited or abrogated, since the pathogen exhibits intracellular survival and evasion of the host immune response. Moreover, the exact mechanisms of vaccine-induced protection are unclear. It is unlikely that vaccine-generated antibody-mediated immune responses by themselves will protect against an intracellular pathogen, but must not be dismissed entirely, as they present one strategy to potentially block mucosal invasion by extracellular bacteria. The challenges of inducing cell-mediated immune responses towards *P. salmonis* are considerable and similar to the challenges presented by other intracellular pathogens, including *Francisella,* the *Chlamydiae* and the *Rickettsiae*. The importance of Type 1 immunity, consisting of T-bet+ IFNγ-producing group 1 innate lymphoid cells (ILC1 and NK cells), CD8+ cytotoxic T cells (TC1) and CD4+ TH1 cells [80], for protection against intracellular *P. salmonis* via activation of mononuclear phagocytes is not known. In addition, the role of T cell mediated immune functions against *P. salmonis* has not been fully explored.

Understanding immunity to *P. salmonis* shares many similarities with the biology of other intracellular pathogens. For the human pathogen *Francisella tularensis*, CD4+ and CD8+ T cells are essential for controlling primary infection and for optimal vaccine-induced protection and roles have been established also for B-1 lymphocytes, specific antibodies and new T cell subpopulations [81]. In the context of vaccination, antibody-mediated immune responses generated by immunization with live or killed *F.tularensis* vaccine strain LVS or antigenic *Francisella* preparations were only partially protective against virulent *Francisella* strains and T cell functions were identified as necessary for optimal protection. CD4+ and CD8+ T cells are also involved in protective immunity against *Rickettsiae* [82]. Whole killed bacterin, live attenuated bacteria and DNA vaccines as well as outer membrane protein OmpA and OmpB recombinant protein vaccines, have all been developed for human rickettsial infections [83-85]. Infection with *Rickettsia* induces long-lasting immunity against re-infection; by contrast, the vaccines showed low rates of protection and did not prevent infection, but acted to reduce the case fatality rate in vaccinees. Studies of an inbred mouse model of *R. conorii* infection demonstrated early on that immune CD8+ T cells were more important than CD4+ T cells and highlighted the critical role of CD8+ cytotoxic T cells in clearing infection [86]. For infections by *Chlamydiae spp*., protective immune responses needed to target the developmental cycle of the pathogens, including the intracellular stage, would include a primary T cell response, but antibodies may also be effective [87].

**3.3. Contribution of genomics and transcriptomics to *P. salmonis* vaccine development**

Vaccine development is hampered by poor understanding of the virulence factors and pathogenic mechanisms of *P. salmonis*. Transcriptomics could be one useful tool to examine *P. salmonis*-host cell interactions and recent studies have shed some light on host responses to infection. Tacchi and colleagues examined the transcriptional response of post-smolt *S. salar* to *P. salmonis* following infection in liver, head kidney and muscle tissue [88]. Infection induced large differential alterations of transcriptional activity in all three tissues. Genes involved in oxidative and inflammatory responses were up-regulated, suggesting activation of the innate immune response, whereas genes involved in the adaptive immune response, G protein signalling pathway and apoptosis were down-regulated, thus favouring *P. salmonis* survival and replication and the ability to evade host defences. Pulgar *et al.* analysed the head kidney transcriptional response of *S. salar* families with different levels of susceptibility to *P. salmonis* infection, in an attempt to reveal mechanisms that might confer resistance to infection [89]. Host gene expression changes associated with cellular iron suggested that iron-deprivation might be an innate immunity defence mechanism against *P. salmonis.*

However, more work is needed to examine the co-transcriptional changes occurring in *P. salmonis* and target host cells. Around 283 cell lines have been established from finfish worldwide and from a wide range of tissues, *e.g.* ovary, fin, swim bladder, heart, spleen, liver, eye muscle, vertebrae, brain and skin, although only 43 have been listed in international depositories such as ATCC and ECACC [90]. Thus, pathogen and host co-transcriptional responses can be studied using profiling techniques such as RNA-seq on cell lines related to sites of infection, e.g. the trout-derived gill epithelial cell line, RTgill-W1, or skin explant cell cultures, or persistence, e.g. primary macrophage cells. These are likely to reveal the complexities of inter-related transcriptional responses and potentially suggest pathogen-related virulence factors that could be vaccine targets. For example, Pulgar *et al.* identified several *P. salmonis* mechanisms for iron acquisition, suggesting that the pathogen can obtain iron from different sources. In common with many pathogens with iron-sequestration systems, putative iron-binding proteins represent one attractive target for vaccine development.

Increasing the number and availability of whole genome sequences for *P. salmonis* can be addressed readily. These genomes will be important for our understanding of the genetics and taxonomy of the pathogen and will provide insight into putative virulence properties. It would be useful to sequence the genomes of disease-causing isolates of wide geographical spread as a means to investigate why disease severity varies from country to country. Moreover, with high-throughput sequencing available today, a goal for the next 5 years would be the establishment of a publically-available database of *P. salmonis* genomes, *e.g.* on <http://pubmlst.org/>, which would be a valuable tool for comparative pan and core genomics and vaccinology studies. The development of the *Neisseria* PubMLST database ([http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=
pubmlst\_neisseria](http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_neisseria)\_isolates) provides an exemplar of what can be achieved and there is no reason why the same cannot be done for *P. salmonis.* Indeed, epidemiological studies of the *Neisseriae* have benefitted from robust surveillance and monitoring of disease outbreaks coupled with genome sequencing of disease isolates.

**3.4. Five year plan for *P. salmonis* vaccine development**

Worldwide, vaccines are available for more than 17 species of fish and protect against more than 22 different bacterial diseases and 6 viral diseases. The nature of these vaccines has been extensively reviewed elsewhere [91-93]. Licensed vaccines are dominated by bacterins and attenuated live bacterial vaccines, administered primarily in water-in-oil emulsions. Bacterin vaccines have been licensed for infections caused by *Aeromonas salmonicida*, *Vibrio spp. (anguillarum, ordalii, salmonicida*), *Flavobacterium spp. (columnare, psychrophilum, branchiophilum), Moritella viscosa, Renibacterium salmoninarum, Lactococcus garvieae* , *Streptococcus spp. (iniae, phocae)*, *Photobacterium damselae*  and *Piscirickettsia salmonis*. Examples of avirulent live culture vaccines include those for infections caused by *Arthobacter davidanieli, Edwardsiella* *ictaluri* and *F.columnare.* One future trend for fish vaccine development could be DNA vaccines, especially against viral diseases and bacterial pathogens with persistent intracellular lifestyles. However, DNA vaccination is not without its own issues, related to improving DNA transfection of host cells, antigen presentation and inherent adjuvanticity.

Vaccines studies for the next 5 years should include the development of non-bacterin based *P. salmonis* vaccines and/or significant improvement of currently-used bacterin vaccines, perhaps by the inclusion of recombinant outer surface proteins (OSPs). The availability of one licensed subunit SRS vaccine in Chile suggests that this is a promising strategy. Chronologically, the development of experimental non-bacterin *P. salmonis* vaccines can be traced back to the late 1990s and examples are presented in Table 4. Several OSP have been identified using genomic and proteomic approaches and other approaches investigated include using DNA fragment vaccines, a live attenuated non-pathogenic species vaccine and variations of bacterin preparations (Table 4). Again, in controlled protection trials, many recombinant OSP induce high relative percentage survival (RPS) rates in infected fish, but trials under field conditions are urgently needed. In terms of priority, of the components listed in Tables 2 and 4, we propose that field trials should be done within 5 years of i) LPS-depleted OMV, similar to the successful use of OMV for human vaccines, e.g. *Neisseria meningitidis* vaccines [94] and ii) any identified OSPs, including OspA and TbpB, since these are likely to be recognised by the fish immune system and are potentially immuno-dominant. There is an increasing literature with other fish bacterial pathogens that recognises the importance of surface-exposed membrane proteins as potential vaccine components, but comparative evaluation of these approaches and the nature of the identified antigens amongst the wide variety of fish pathogens currently being studied, is outside the scope of this review. We would also propose in secondary priority, field trials with *P. salmonis* TSS-associated proteins, e.g. the Dot/Icm proteins, which are upregulated early during infection, although this comes with the caveat that their expression during the pathogen’s life cycle is not clear. More promising could be the variety of *P. salmonis* HSP proteins, since these appear to be highly immunogenic *in vivo*. However, there are studies with HSPs from other fish pathogens, e.g. HSP60, HSP70 of *F. psychrophilum*, which did not demonstrate protection [95], although some evidence suggest that HSPs may have an adjuvant effect on vaccine-induced protection and would make useful adjuncts [96].

Realistically, for the next 5-10 years, we would also propose the following experimental studies related to the biology and vaccinology of *P. salmonis*:

1. A thorough *in silico* reverse vaccinology approach for *P. salmonis* in an attempt to identify candidate outer membrane/surface-exposed or secreted (lipo)proteins, extracellular toxins and putative adhesins/invasins, as vaccine antigens capable of stimulating humoral and cell-mediated immune responses [97]. Genome subsystems (Figure 1) and protein-coding sequences of *P. salmonis* isolates curated in http//pubmlst.org would be interrogated *in silico* for the presence of putative virulence factors and potential vaccine candidates within core and pan-genomes.

2. An immuno-proteomics approach in conjunction with pathogen transcriptomics to identify potential candidate *P. salmonis* antigens associated with virulence and immune responses. Ideal *P. salmonis* vaccines capable of providing the strongest protection and long-lasting immunity in salmonids would likely include antigens stimulating antibodies to conserved epitopes and the ability to stimulate memory CD4+/CD8+ T cells that recognize the shared antigens. For identifying the latter, the proteo-genomic approach could be useful, in which the *P. salmonis* transcriptome/proteome is determined during natural infection and predictive algorithms are then used to identify proteins containing potential T cell epitopes. However, determining and validating T cell epitope activity is necessary but the process of determination still remains empirical [98].

3. The development and validation of experimental fish models of *P. salmonis* infection**,** ideally using the target host species, and/or replacement surrogate models, in order to test new vaccines. Depending on the composition of the vaccine - whether a bacterin, OMV, recombinant protein, encapsulated DNA or live, attenuated organism – an examination of the various routes of immunization is warranted.

4. Testing priority vaccine antigens in field conditions. Prime-boost strategies using available *P. salmonis* bacterins (Table 3) with any new subunit vaccine antigen should also be considered.

5. Defining the complete cellular modulation roles in fish species of the full complement of effectors present in the *P. salmonis* pan-genome. Co-transcriptional studies would be extended to analyses at the co-translational level, which would provide fundamental knowledge about the complexities of *P. salmonis*-host interactions, relating to bacterial virulence and host innate and adaptive immune responses.

In conclusion, *P. salmonis* infection is a considerable burden to the farmed fisheries industry in Chile, impacting adversely on agricultural security, food security, the generation of individual and national wealth and on international export and trade. The indiscriminate use of antibiotics is catastrophic to the environment and potentially to human health. The general consensus is that the current prophylactic vaccines based on bacterin preparations provide some protection, although there is still limited documentation of their efficacy in the field. Control of piscirickettiosis in Chile will likely depend on a new generation of vaccines developed from a rigorous application of available ‘omics’ technologies. Furthermore, the development of improved vaccines will also benefit fisheries in other parts of the world that are affected by piscirickettsiosis.

**4. Key Issues**

* *Piscirickettsia salmonis* causes the contagious bacterial disease piscirickettsiosis or salmonid rickettsial septicemia (SRS), which results in significant economic losses to Chilean aquaculture production of USD $700 million annually.
* Indiscriminate use of antibiotics is environmentally damaging and needs to be curtailed.
* In Chile, there are 33 commercially available vaccines against piscirickettsiosis; the majority are composed of *P. salmonis* bacterin, but field information regarding the immune response and protection generated by these vaccines is lacking.
* Field studies are needed for the identified non-bacterin *P. salmonis* vaccine candidates.
* There are few reports on the virulence factors of *P. salmonis* and the mechanisms of pathogenesis remain poorly understood.
* More basic biology investigations on innate and adaptive immune responses to infection with *P. salmonis* are needed. The exact mechanisms of vaccine protection are unclear and validated, reproducible models for testing vaccine efficacy are needed.
* The complete cellular modulation roles in fish species of the full complement of effectors present in the *P. salmonis* pan-genome needs characterization.
* The paucity in available whole genome sequences for *P. salmonis* needs to be addressed in order to increase our understanding of the genetics and taxonomy of the pathogen and provide insight into putative virulence properties.
* *P. salmonis* vaccine development would benefit from thorough *in silico* reverse vaccinology and immuno-proteomics-pathogen transcriptomics approaches in order to identify candidate vaccine antigens capable of stimulating protective immune responses.
* Methods for identifying the appropriate *P. salmonis* CD4+/CD8+ T cell antigens are lacking.

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|  |  |  |
| --- | --- | --- |
| **Clinical signs** | **Pathological findings** | **References** |
| * Lethargy
* Loss of appetite
* Abnormal swimming behaviours
* Severity ranges from peracute to severe to chronic
 | * Pathogen can be observed in the liver, spleen, kidney, intestine, skin, gills, muscle and brain.
* Disseminated intravascular coagulation (DIC)
* Vasculitis, with fish appearing darker in colouration
* Pale gills due to anaemia accompanied by ascites and peritonitis to varying degrees
* Skin lesions and/or areas of necrosis resulting in nodules, haemorrhaging and ulceration
* Swollen, discoloured kidneys, enlarged spleens
* Petechial haemorrhage over the visceral fat, stomach, swim bladder or body musculature
* Chronic multifocal liver lesions – ‘doughnut’ lesions
* Thickening of liver capsule
* Fibrin thrombi, vacuolated hepatocytes and focal areas of ischemic necrosis; pathogen found within hepatocellular vacuoles, endothelial cells and leucocytes
* Occasional small white foci seen in the heart, skeletal musculature, fins and gills
 | [23,36,99-101][8,23] |

**Table 1. The clinical signs and pathological findings of piscirickettsiosis**

|  |  |  |
| --- | --- | --- |
| **Virulence Feature** | **Function** | **Chronology[Reference]** |
| Lipopolysaccharide (LPS) | Responsible for endotoxicity and DIC. Structurally, the major *P. salmonis* lipid A is represented by the hexaacyl form that resembles the classical lipid A found in the *Enterobacteriaceae* family and lipid A from *Rickettsia* *typhi* that confers potent endotoxicity. Endotoxicity of *P. salmonis* LPS is primarily determined by the number, nature and arrangement of acyl chains and phosphate groups on the lipid A moiety. Carbohydrate backbone structure of *P. salmonis* LPS has been described and two unique features were found - i) the presence of 4-aminoarabinose (Ara4N, 4-amino-4-deoxy-l-arabinopyranose) on O-4 of the alpha-GlcN1P of the lipid A moiety; ii) two consecutive residues of diacetylated pseudaminic acid (Pse 5,7 Ac, 5,7-diacetamido-3, 5, 7, 9-tetradeoxy-l-glycero-l-manno-non-2-ulosonic acid), which in particular appear to be distinctive amongst polysaccharides containing this acidic sugar.  | 2005-2013 [102-105] |
|  |  |  |
| Selection of infective variants resulting from variation within ITS region.  | Structurally, the standard size for *P. salmonis* LF-89 strain is 0.5-1.5 µM, but the pathogen has been recovered from naturally infected fish as well as from ageing post-infected CHSE-214 cell cultures as a novel genetically different small infective variant. Although the variant was infective, the *in vitro* progeny was identical to the prototype LF-89 strain. Selection of infective variants possibly represents a survival strategy of *P. salmonis* in response to limiting growth conditions.  | 2008 [106] |
|  |  |  |
| Heat shock protein protease ClpB and virulence factor BipA. | A study of *P.salmonis* infection of the SHK-1 cell line derived from the Atlantic salmon head kidney showed that during infection, the pathogen significantly increases the expression of ClpB and BipA. Infection and LPS treatment induced SHK-1 cell production of anti-bacterial reactive oxygen species via activation of NADPH oxidase. Both ClpB and BipA proteins have been widely characterized in other pathogens with an intracellular lifestyle, e.g. *Legionella pneumophila, Coxiella burnetii* and *Francisella tularensis*. Their up-regulation possibly may permit *P. salmonis* survival and replication within macrophages. | 2010-2014 [107,108] |
|  |  |  |
| Mobile genetic element ISPsa2 | Identified in *P. salmonis* isolates collected from different outbreaks of infection in Chile and considered to indicate high genomic plasticity of the bacterium. Description of a functional toxin-antitoxin operon (ps-Tox-Antox) in *P. salmonis* appears to be a natural consequence of this genome versatility. | [109,110] |
|  |  |  |
| Large cell aggregates that closely resemble typical biofilm structures.  | Produced under stress conditions: *P. salmonis* appear to be embedded within a matrix sensitive to cellulase. Strong association of the lectins ConA and WGA with this structure suggests an exopolysaccharide composition that is typical of biofilms. | 2012 [111] |
|  |  |  |
| Genes of the toxin/anti-toxin mazEF operon  | Induced at early stages of biofilm formation by *P. salmonis*. It is possible that this sessile lifestyle might be an adaptive strategy for *P. salmonis* survival and persistence under stress conditions in marine environments. | 2012 [111] |
|  |  |  |
| Secreted thermolabile extracellular products (circumstantially exotoxin in nature) | Cytotoxicity towards salmonid tissue-derived cell lines (Chinook salmon embryo-214 (CHSE-214) and ASK) but not against the mammalian cell line MDBK. | 2013-2015 [112,113] |
|  |  |  |
| The Dot/Icm (Deficient in organelle trafficking/Intracellular multiplication) proteins | Dot/Icm proteins are a specialized Type IV-B TSS encoded by the *dot/icm* genes. The Dot/Icm system is a major virulence mechanism of other intracellular pathogens such as *L. pneumophilia* and *C. burnetii*. *P. salmonis* encodes ORFs of *dot/icm* genes *dotA, dotB, icmE* and *icmK*. These genes were induced after 48 h *P. salmonis* infection of RTS11 cell line and the Sf21 insect cell line and induction was accompanied by lysosome acidification. *P. salmonis*-containing replicative vacuoles did not fuse with lysosomes, suggesting bacterial interference of the endosomal maturation process that ensures bacterial survival. Dot/Icm TSS may be responsible by delivering putative effector proteins/virulence factors within the host cell. | 2013-2016 [114,115] |
|  |  |  |
| Genes associated with Type Secretion Systems (TSS I-VI).  | TSS play roles in host cell intracellular survival and/or replication of *P. salmonis*. Recent comparative analysis of the LF-89-like strain B1-32597 and the EM-90-like strain A1-15972 showed differences in the number of Type IV TSS expressed. | 2014 [22] |
|  |  |  |
| Outer membrane vesicles (OMV).  | In common with many other Gram negative organisms, *P. salmonis* also sheds OMV, which were observed during infection of CHSE-214 cells and also during normal growth in liquid media and found to contain the chaperonin Hsp60. Purified OMV induced a cytopathic effect on CHSE-214 cells suggesting a potential role in pathogenesis. | 2016 [116] |

**Table 2. Putative virulence factors expressed by *P. salmonis* and their role during infection *in vivo* and *ex vivo***

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Name of Vaccine** | **Active Principle** | **Vaccine Composition** | **Date of introduction\*** | **Efficacy****(Months)\*\*** | **Route of injection** | **Dose** | **Strain** |
| AGROVAC SRS | Bacterin | Monovalent | December 2005 | 18  | *ip* | 2.5x105 TCID 50 | AG 5, AG 16 |
| BLUEGUARD SRS INYECTABLE | Bacterin | Monovalent | January 2007 | 18  | *ip*  | 108.0 - 108.5 TCID 50 | PS 2C |
| ALPHA JECT 4-1 | Bacterin | Tetravalent (*P.salmonis, A. salmonicida, V. ordalii*, IPN virus) | March 2007 | 12  | *ip*  | 0.11-0.25 mg  | AL 10005 |
| AGROVAC 4 | Bacterin | Tetravalent (*P.salmonis, A. salmonicida, V. ordalii*, IPN virus) | May 2007 | 18  | *ip*  | 2.5x105 TCID 50 | AG 5, AG 16 |
| BIRNAGEN FORTE 2 | Subunit | Bivalent (*P.salmonis,* IPN virus) | September 2007 | 18  | *ip*  | Hsp60 (2.5±0.125 g), Hsp70 (2.5±0.125 g), FlagG2 (2.5±0.125 g) | LF89 |
| AGROVAC 3 | Bacterin | Trivalent (*P.salmonis, V. ordalii*, IPN virus) | October 2007 | 18  | *ip*  | 2.5x105 TCID 50 | AG 5, AG 16 |
| ALPHA JECT MICRO 3 | Bacterin | Trivalent (*P.salmonis, V. ordalii*, IPN virus) | March 2008 | 18  | *ip*  | 0.075-0.20 mg  | AL 10015 |
| BLUEGUARD SRS+IPN+Vo Inyectable | Bacterin | Trivalent (*P.salmonis, V. ordalii*, IPN virus) | March 2008 | 18  | *ip*  | 108.47 - 108.77 TCID 50 | PS 2C |
| BLUEGUARD SRS+IPN INYECTABLE | Bacterin | Bivalent (*P.salmonis,* IPN virus) | June 2008 | 18  | *ip*  | 108.47 - 108.77 TCID 50 | PS 2C |
| ALPHA JECT MICRO 2 | Bacterin | Bivalent (*P.salmonis,* IPN virus) | December 2008 | 18  | *ip*  | 0.075-0.20 mg  | AL 10015 |
| BLUEGUARD SRS ORAL | Bacterin | Monovalent | March 2009 | 18  | Oral | 108.0 - 108.5 TCID 50 | PS 2C |
| BLUEGUARD SRS+IPN+Vo+As Inyectable | Bacterin | Tetravalent (*P.salmonis, A. salmonicida, V. ordalii*, IPN virus) | April 2009 | 18  | *ip*  | 108.5 - 108.7 TCID 50 | PS 2C |
| FAV | Bacterin | Trivalent (*P.salmonis, V. ordalii*, IPN virus) | Provisional registration | 18  | *ip*  | 1.5x105 - 1.5x108 TCID50 | *P.salmonis* 01-Chile -01 |
| ALPHA JECT 5-1 | Bacterin | Pentavalent (*P.salmonis, A. salmonicida, V. ordalii*, IPN virus, ISA virus) | May 2011 | 18  | *ip*  | 0.075-0.11 mg  | AL 10005 |
| FAV | Bacterin | Tetravalent (*P.salmonis, V. ordalii*, IPN virus, ISA virus) | Provisional registration | 18  | *ip*  | 1.5x105 - 1.5x108 TCID50 | *P.salmonis* 01-Chile -01 |
| AGROVAC 3 + ISA | Bacterin | Tetravalent (*P.salmonis, V. ordalii*, IPN virus, ISA virus) | April 2011 | 18  | *ip*  | 2.5x105 TCID50 | AG 55, AG 165 |
| AGROVAC 4 + ISA | Bacterin | Pentavalent (*P.salmonis, A. salmonicida, V. ordalii*, IPN virus, ISA virus) | April 2011 | 18  | *ip*  | 2.5x105 TCID50 | AG 55, AG 165 |
| BLUEGUARD IPN+SRS+As+Vo+ISA Inyectable | Bacterin | Pentavalent (*P.salmonis, A. salmonicida, V. ordalii*, IPN virus, ISA virus) | March 2010 | 18  | *ip*  | 108.5 - 108.8 TCID50 | PS 2C |
| FAV | Bacterin | Monovalent | Provisional registration | 18  | *ip*  | 1.5x105 - 1.5x108 TCID50 | *P.salmonis* 01-Chile -01 |
| AGROVAC IPN-SRS | Bacterin | Bivalent (*P.salmonis*, IPN virus) | August 2011 | 18  | *ip*  | 2.5x105 TCID50 | AG 5, AG 16 |
| FAV | Bacterin | Bivalent (*P.salmonis*, IPN virus)  | Provisional registration | 18  | *ip*  | 1.0x107 –1.0x108 bacteria | *P.salmonis* 01-Chile -01 |
| FAV | Bacterin | Pentavalent (*P.salmonis, A. salmonicida, V. ordalii*, IPN virus, ISA virus)  | Provisional registration | 18  | *ip*  | 1.0x107 –1.0x108 bacteria | *P.salmonis* 01-Chile -01 |
| RICKEMUNE-VAX | Bacterin | Monovalent | October 2011 | 18  | *ip*  | 5.7x105 –2.5x106 bacteria | Cepa 238 |
| BLUEGUARD SRS+ISA ORAL | Bacterin | Bivalent (*P.salmonis,* ISA virus) | December 2011 | 18  | oral | 108.0 - 108.4 TCID50 | PS 2C |
| PROVIDEAN AQUATEC 1 SRS | Bacterin | Monovalent | July 2012 | 24  | *ip*  | 1.0x107 –1.0x108 bacteria | AT-01-CHILE-01 |
| PROVIDEAN AQUATEC 2 SRS IPN | Bacterin | Bivalent (*P.salmonis*, IPN virus) | July 2012 | 24  | *ip*  | 1.0x107 –1.0x108 bacteria | AT-01-CHILE-01 |
| PROVIDEAN AQUATEC 3 SRS IPN Vibrio | Bacterin | Trivalent (*P.salmonis, V. ordalii*, IPN virus) | July 2012 | 24  | *ip*  | 1.0x107 –1.0x108 bacteria | AT-01-CHILE-01 |
| PROVIDEAN AQUATEC 5 SRS IPN *Vibrio* ISAv *Aeromonas* | Bacterin | Pentavalent (*P.salmonis, A. salmonicida, V. ordalii*, IPN virus, ISA virus) | May 2013 | 24  | *ip*  | 1.0x107 –1.0x108 bacteria | AT-01-CHILE-01 |
| PROVIDEAN AQUATEC 4 SRS IPN *Vibrio* ISAv | Bacterin | Tetravalent (*P.salmonis, V. ordalii*, IPN virus, ISA virus) | May 2013 | 24  | *ip*  | 1.0x107 –1.0x108 bacteria | AT-01-CHILE-01 |
| AQUAVAC SARISTIN 2 | Subunit | Bivalent (*P.salmonis,* IPN virus) | December 2013 | Not informed | *ip*  | 0.015 – 0.031 U | AL-ORF1-90kDa |
| RICKEMUNE-VAX INMERSIÓN | Bacterin | Monovalent | January 2014 | 18  | Immersion | 5.0x106 –1.0x107 CFU | Cepa 238 |
| RICKEMUNE PLUS | Bacterin | Bivalent (*P.salmonis,* IPN virus) | May 2014 | 18  | *ip*  | 5.7x105 –2.5x106 bacteria | Cepa 238 |
| Centrovet Inactived | Bacterin | Pentavalent (*P.salmonis, V. ordalii*, IPN virus, ISA virus, *C. rogercresseyi*) | August 2015 | 18  | *ip*  | 107.8 - 108.2 TCID50107.8 - 108.2 TCID50107.8 - 108.2 TCID50 | PS 2CPS G2PS GA |
| ALPHA JECT LiVac SRS | Live attenuated | Monovalent | February 2016 | 18  | *ip*  | 1.9 x105 – 4.9x106 TCID50 | AL 20542 |

**Table 3. A list of *P. salmonis* vaccines licensed for aquaculture use in Chile.** \*Source: Servicio Agrícola y Ganadero, Chile, May 2016. \*\*There are no published data for the duration of efficacy presented; these are provided by the pharmaceutical companies that manufacture the vaccines. *ip,* intraperitoneal; TCID­50, tissue culture infective dose: this endpoint dilution assay quantifies the amount of bacteria required to produce a cytopathic effect in 50% of inoculated tissue culture cells (CHSE-214). ISA, infectious salmon anaemia virus; IPN, infectious pancreatic necrosis virus. CFU, colony forming units; U, units.

|  |  |  |
| --- | --- | --- |
| **Putative vaccine/antigen** | **Properties** | **Chronology [Reference]** |
| Carbohydrate LOS antigens (11, 16 kDa) and *Mr* 20, 36 and 56 kDa protein antigens  | Identified from their reactivity with convalescent salmon sera or detection using polyclonal and monoclonal antibodies against salmon Ig. | 1996-1998 [117,118] |
|  |  |  |
| Non-concentrated bacterin | *O.kisutch* and *O. mykiss* vaccinated with formalin-killed bacterins showed lower cumulative mortalities that non-vaccinated fish groups. | 1997 [119] |
|  |  |  |
| Whole-cell bacterins | Defined as cell culture supernatants from *P. salmonis* infected CHSE-214 monolayers that display complete cytopathic effect, subsequently inactivated with formalin. Undiluted bacterin preparation provided ~35% RPS protection. | 2001 [52] |
|  |  |  |
| OspA | A 17 kDa antigenic outer surface protein, detected by convalescent serum antibodies from coho salmon. A recombinant OspA protein induced a RPS rate of 59% in vaccinated coho salmon and efficacy of this vaccine was increased three-fold by the incorporation of T cell epitopes (TCE's) from tetanus toxin and measles virus fusion protein. Improved RPS of ~83% was observed. | 2001 [52,120] |
|  |  |  |
| DNA fragments in expression vector pCMV-Bios | *P. salmonis* DNA fragments cloned into pCMV-Bios and injected intramuscularly into *O. kitusch.* Survival rates from *P. salmonis* challenge only 20%. | 2003[121] |
|  |  |  |
| HSP10, HSP16 | Reported to be highly immunogenic in salmon. | 2003 [122] |
|  |  |  |
| Transferrin binding protein (TbpB, membrane-bound transglycosylase (MltB) | Recombinant proteins inducing strong humoral immune responses in Atlantic salmon. | 2004 [123] |
|  |  |  |
| Heat-killed and formalin inactivated whole bacteria vaccines | *P. salmonis* SCO-95A heat-killed and formalin-inactivated vaccines conferred protection, with RPS values of 71% and 50% respectively. | 2004 [46] |
|  |  |  |
| HSP60, HSP70 | Recombinant proteins inducing immune responses in Atlantic salmon and conferring protection against *P. salmonis* infection. | 2005 [124] |
|  |  |  |
| *Arthrobacter davidanieli* live vaccine | Vaccine composed of live non-pathogenic Gram-variable *A. davidanieli* reduced mortality in coho salmon infected with *P. salmonis*, experimentally and in field trials. | 2005 [125] |
|  |  |  |
| HSP60, HSP70, flagellar protein FlgG | A comparative genomics strategy used on a *P. salmonis* genome library identified 15 ORFs that encoded HSPs, virulence factors, membrane bound and other surface exposed antigens. A vaccine formulated with recombinant chaperonin Hsp60 and Hsp70 proteins and FlgG induced a relative percent survival (RPS) rate of 95% in infected *S. salar*. | 2006 [51] |
|  |  |  |
| ChaPs HSP | A highly immunogenic protein of *P. salmonis* identified from naturally-infected salmonid fish using one and two-D PAGE gel electrophoresis and western blot with polyclonal anti-*P. salmonis* antibodies. The protein was identified as a 57.3 kDa HSP named ChaPs.  | 2006 [51] |
|  |  |  |
| ChaPs synthetic peptides and recombinant protein | Characterization of ChaPs immunogenic epitopes for the design and formulation of piscirickettsiosis vaccines | 2009 [126] |
|  |  |  |
| *P. salmonis* PS2C field strain with MicroMatrix™. | Bacterium grown in cell culture and incorporated in an oral delivery vehicle containing bioadhesive cationic polysaccharide formulation MicroMatrix™. Oral vaccination leading to anti-*P. salmonis* specific antibodies and >80% end-of-trial protection of fish against lethal pathogen challenge. | 2011 [50] |

**Table 4. Experimental vaccine strategies for *P. salmonis***. RPS = relative percentage survival.

**Figure legends**

**Figure 1. Organism overview for *Piscirickettsia salmonis* strain LF-89 (ATCC VR1361).** The genome sequence for *P. salmonis* LF-89 (ATCC VR1361; ASM153472v1 latest genome assembly version) was downloaded from NCBI (<https://www.ncbi.nlm.nih.gov/>) and uploaded to the Rapid Annotation using Subsystem Technology (RAST; <http://rast.nmpdr.org/>). The annotated genome was browsed in the SEED viewer to provide an overview of the susbsytem features of the organism.