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Present and future of aquaculture vaccines against fish bacterial diseases

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Abstract. The following aspects are described for each of the main bacterial diseases in which vaccination is employed: (i) the biochemical, antigenic and genetic heterogeneity of the etiological agents; (ii) their geographical distribution and host range; (iii) the effectiveness and problems of current commercial vaccines; and (iv) the new vaccination approaches using recombinant DNA technology or other strategies different to the classic bacterins. In addition, economic aspects and future trends related to fish vaccination are also addressed.

Key words. Vaccines – Bacteria – Fish – Aquaculture.

Présent et futur des vaccins en aquaculture contre les maladies bactériennes des poissons

Résumé. *Les aspects suivants sont décrits pour chacune des principales maladies bactériennes pour lesquelles on emploie la vaccination : (i) l'hétérogénéité biochimique, antigénique et génétique des agents étiologiques ; (ii) leur distribution géographique et la gamme de leurs hôtes ; et (iii) l'efficacité et les problèmes des vaccins commerciaux actuels, et, (iv) les nouvelles approches concernant la vaccination qui font appel à la technologie de l'ADN recombinant ou à des stratégies autres que les bactérines classiques. En outre, les aspects économiques et les tendances futures liées à la vaccination des poissons sont également abordés.*

Most-clés. *Vaccins – Bactéries – Poissons – Aquaculture.*

I – Introduction

It is well known that the appearance and development of a fish disease process is the result of the interaction between pathogen, host, and environment. Therefore, only multidisciplinary studies involving knowledge of the characteristics of the potential pathogenic microorganisms for fish, aspects of the biology of the fish hosts, as well as a better understanding of the environmental factors affecting them, will allow the application of adequate measures to prevent and control the main diseases limiting the production of freshwater and marine fishes. Regarding the infectious fish diseases caused by bacteria, although pathogenic species have been described in the majority of the existing taxonomic groups, only a relatively small number are responsible for important economic losses in the extensive cultures worldwide.

Vaccination is becoming an increasingly important part of aquaculture, since it is considered a cost effective method of controlling different threatening diseases. The term vaccination strategy has been defined to include the decision as to which diseases to vaccinate against, as well as the vaccine type, vaccination method, the timing of vaccination and the use of revaccination.

One important consideration for development and commercialisation of vaccines includes the application methods and procedures that can be integrated into the normal production protocols of the target fish species that are relevant to the typical ecology and epidemiology of the disease (i.e. seasonal occurrence, fish size, host and geographic range of the disease).

Before attempting any vaccination strategy, it is important to determine when the immune system is both morphologically and functionally mature. Salmonids, characterized by production

of large yolk-filled eggs followed by a long egg incubation period, are much more immunologically developed at the time of hatching compared to strictly marine species, such as turbot, halibut, sea bream, sea bass or sole, which have a short egg phase after which the immature and vulnerable larvae hatch into the marine environment. In addition, it is important to take into account that the time from hatching to first feeding is much shorter in strictly marine fishes compared with salmonids.

Data on the earliest time to vaccinate marine species are scarce. In most marine species, at the time of hatching, the lymphoid system is still developing and will not become functionally mature until 70-100 days post-hatch, and, therefore, non-specific defence mechanisms constitute the most important part of the defence mechanisms of the larval fish. Therefore, in general, if the fish are vaccinated at a very early age, the protection period will be limited and they will need revaccination after a period of approximately 1 month. In addition, if the disease also occurs during the on-growing period, a third vaccination must be given when fish reach 30-50 g.

One interesting topic to be investigated is the "maternal immunity", since the presence and the significance of the transfer mechanism for immunoglobulins from mother to offspring is still controversial. Immunization of pre-spawning females may have potential as a means of protecting fish against pathogens which affect the early life stages, such as *Flavobacterium psychrophilum*, *Edwardsiella ictaluri*, or *Photobacterium damselae* subsp. *piscicida*.

II – Primary factors affecting the efficacy of vaccination

1. Type of vaccine formulation

A. Bacterins

Most bacterial vaccines used in aquaculture to date have been inactivated vaccines obtained from a broth culture of a specific strain(s) subjected to subsequent formalin inactivation (Newman, 1993; Toranzo *et al.*, 1997). The best results are obtained with those bacterins that include both bacterial cells and extracellular products. Whereas with some vaccines acceptable levels of protection are achieved with aqueous formulations administered by injection or immersion, for other bacterins, such as those devised for salmonids against *Aeromonas salmonicida* subsp. *salmonicida*, an acceptable level of protection can only be achieved by immunization with oil-adjuvanted bacterins delivered by injection.

B. Live attenuated vaccines

These should potentially have many advantages in aquaculture. Vaccination with a live vaccine is in reality an infection and, if the vaccine strain is shed by vaccinated fish, an effective dissemination of the antigen in the population would take place over an extended period of time. Live vaccines also have the advantage that they stimulate the cellular branch of the immune system.

Some live vaccines have been tested experimentally: *Aeromonas salmonicida*, *Edwardsiella tarda*, *E. ictaluri, Ph. damselae* subsp. *piscicida*. However, problems concerning safety, persistence in the fish and in the environment, reversion to virulence, risk of spreading to non-target animals including wild fish, among others, must be resolved before the use of these live attenuated strains can be allowed in the field. At present, only an *E. ictaluri* attenuated live vaccine has been licensed in the USA to be used by bath in 9-day old fish to prevent ESC of catfish (Klesius and Shoemaker, 1998).

C. DNA vaccines

DNA vaccines have theoretical advantages over conventional vaccines: in mammals, the specific immune response after DNA vaccination encompasses antibodies, T-helper cells and

cytotoxic cells. However, before DNA vaccines are applied in commercial enterprises in aquaculture, safety for the fish, environment and consumer have to be addressed. As the DNA-sequence encodes only a single microbial gene, there should be no possibility of reversion to virulence, which is a critical factor in relation to environmental safety in aquaculture.

2. Monovalent and polyvalent vaccines

The ideal vaccine formulation is a polyvalent vaccine which protects simultaneously against the majority of the diseases to which a particular fish species is susceptible. In addition, these polyvalent vaccines must cover all the main serotypes of each pathogen existing in a particular geographical area. Examples of the efficacy of polyvalent vaccines are those used in salmonids and turbot in which polyvalent vaccines give similar or superior protection than the respective monovalent vaccines. However, care must be taken in the formulation of polyvalent vaccines because the problem of antigen competition can occur, especially when these vaccines are administered by injection.

3. Route and strategy of administration

Fish are commonly immunized by three procedures: intraperitoneal injection (ip), immersion in a diluted vaccine solution (short or long bath), or oral administration of the vaccine. Although these methods have different advantages and disadvantages with respect to the level of protection, side effects, practicality and cost-efficiency, it is widely accepted that only the injection and immersion routes give enough protection to be used as the primary route of fish immunization in commercial production. For oral vaccination, research has been focused on protecting the antigens from digestion and decomposition during passage through the stomach and anterior part of the gut. However, promising results have been obtained using encapsulation of antigens in alginate or polylactic glycolic acid microparticles. From the economic stand point, oral vaccination is the ideal route to be employed in a vaccination program which requires one or more booster immunizations.

III – Current status in the development of vaccination strategies to prevent bacterial diseases

1. Vibriosis

Within the genus *Vibrio*, the species causing the most economically serious diseases in marine culture are *Vibrio anguillarum*, *V. ordalii*, *V. salmonicida* and *V. vulnificus* biotype 2.

Vibrio anguillarum is the responsible agent of the classical vibriosis which affects salmonid and non-salmonid fish with a world wide distribution. Although up to a total of 23 O serotypes (O1-O23, European serotype designation) are known to occur among *V. anguillarum* isolates (Sørensen and Larsen, 1986; Pedersen *et al.*, 1999), only serotypes O1, O2 and, to a lesser extent, serotype O3, have been associated with mortalities in farmed and feral fish throughout the world (Tajima *et al.*, 1985; Toranzo and Barja, 1990, 1993a; Larsen *et al.*, 1994; Toranzo *et al.*, 1997). The remaining serotypes are considered to be environmental strains and are isolated only on rare occasions as being responsible for vibriosis in fish. Whereas serotypes O1 and 02 have a wide distribution, serotype O3 affects mainly eel and ayu.

In contrast to serotype O1, which is antigenically homogeneous, serotypes O2 and O3 display antigenic heterogeneity with the existence of two subgroups being demonstrated within each serotype, which are named, respectively, O2a and O2b, and O3A and O3B (Olsen and Larsen, 1993; Santos *et al.*, 1995).

Although there a great number of commercial *V. anguillarum* vaccines have been developed for use mainly by bath or injection (Newman, 1993; Toranzo *et al.*, 1997), the majority of them include in their formulations only serotype O1, or a mixture of serotypes O1 and O2a. To our knowledge, only one licensed bacterin (GAVA-3), developed by our research group and marketed by Hipra Laboratories (Spain), covers the three antigenic entities of *V. anguillarum* responsible for most epizootics (O1, O2a and O2b). However, different polyvalent oil-adjuvanted vaccines, including different combinations of *V. anguillarum* with other pathogens, such as *V. ordalii*, *V. salmonicida, Aeromonas salmonicida*, *Moritella viscosa* and infectious pancreatic necrosis virus, are also available on the market to be used for salmonids by the ip route (Toranzo *et al.*, 1997; Greger and Goodrich, 1999). In the case of strictly marine fishes, such as turbot (*Scophthalmus maximus*) or sea bass (*Dicentrarchus labrax*), *V. anguillarum* vaccines are being employed by bath in 1-2 g fish, with two immersions in the vaccine bath being necessary after a monthly interval.

The species *Vibrio ordalii* has been established to accommodate strains formerly classified as *V. anguillarum* biotype 2 (Schiewe and Crosa, 1981) which only affects salmonids. In contrast to *V. anguillarum*, *V. ordalii* is antigenically homogeneous with no serotypes being detected. Although some cross-reactions exist between *V. ordalii* and *V. anguillarum* serotype O2, both species do not have identical antigenic properties (Mutharia *et al.,* 1992). In fact, commercial bacterins including *V. anguillarum* serotype O1 and *V. ordalii* as antigens elicit very poor protection against infections by *V. anguillarum* serotype O2 (Toranzo *et al*., 1997).

Vibrio salmonicida is the etiological agent of the "cold water diseases" or "Hitra diseases" which affect only salmonids and cod (*Gadus morhua*) cultured in Canada and Nordic countries of Europe (mainly Norway and the UK) (Egidius *et al*.,1986; Sørum *et al.*, 1990). This pathogen is biochemically and antigenically homogeneous with a hydrophobic protein, called VS-P1, present in the surface layer, being the dominant antigen in all the strains (Espelid *et al.*, 1987; Hjelmeland *et al.*, 1988). As stated above, salmonids in Nordic countries are systematically vaccinated with polyvalent bacterins containing at least two pathogenic vibrios, *V. anguillarum* and *V. salmonicida* (Toranzo *et al.*, 1997).

Vibrio vulnificus comprises two biotypes. Whereas biotype 1 is an opportunistic human pathogen causing disease generally associated with handling or ingestion of raw shellfish, the strains of biotype 2 are virulent for eels (Tison *et al.*, 1982; Biosca *et al.*, 1991; Dalsgaard *et al.*, 1998). Although these strains can belong to distinct serotypes, only serovar E behaves as a primary pathogen for eels (*Anguilla anguilla* and *A. japonica*). In addition, biotype 2 may also cause, on some occasions, infection in humans and, thus, represents a potential health hazard for fish farmers (Amaro and Biosca, 1996). Although until recently no vaccines had been manufactured to prevent the vibriosis caused by *V. vulnificus*, a specific bacterin named Vulnivaccine, was developed by the University of Valencia (Spain) which proved to be effective for eels under field conditions (Fouz *et al.*, 2001). However, a triple exposure to the vaccine in a short space of time (approximately 1 month) by prolonged immersion was needed to ensure an acceptable level of protection. Since no cross-protection between serotypes exists, vaccinated eels with *V. vulnificus* serovar E can be infected by other less frequent serovars of the pathogen which act as secondary pathogens (Fouz and Amaro, 2003).

2. Winter ulcer

"Winter ulcer" is a disease affecting sea-farmed Atlantic salmon (*Salmo salar*) reared at cold temperatures and, therefore, occurs in Norway, Iceland and Scotland mainly during the winter season (Lunder *et al.*, 1995; Benediktsdóttir *et al.*, 1998; Bruno *et al.*, 1998b). The etiological agent is *Moritella viscosa* (formerly *Vibrio viscosus)* (Benediktsdóttir *et al.*, 2000). An inactivated oil-adjuvanted vaccine against *M. viscosa* has been shown to give protection in Atlantic salmon (Greger and Goodrich, 1999). Today, *M. viscosa* has been incorporated in the oil-based multivalent vaccines employed routinely in the salmon industry of the affected countries.

3. Pasteurellosis

Pasteurellosis, currently described also as photobacteriosis, is caused by the halophilic bacterium *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*), which causes economic losses in the marine culture of yellowtail (*Seriola quinqueradiata*) in Japan, gilthead sea bream (*Sparus aurata*), sea bass and sole (*Solea* spp.) in the Mediterranean countries of Europe and hybrid striped bass (*Morone saxatilis* x *M. chrysops*) in the USA (Toranzo *et al.*, 1991a; Magariños *et al.*, 1996, 2003; Romalde and Magariños, 1997; Romalde *et al.*, 1999a; Zorrilla *et al.*, 1999).

Severe mortalities occur usually when water temperatures are above 18-20ºC. Below this temperature, fish can harbour the pathogen as a subclinical infection for long time periods (Magariños *et al.*, 2001). Regardless of the geographic origin and source of isolation, all strains of this pathogen are biochemically and serologically homogeneous (Magariños *et al.*, 1992a,b, 1996; Bakopoulos *et al.*, 1997). However, DNA fingerprinting methods, such as ribotyping (Magariños *et al.*, 1997), AFLP (Thyssen *et al.*, 2000; Kvitt *et al.*, 2002) and RAPD (Magariños *et al.*, 2000), have proved to be valuable epidemiological tools since they allowed two clear separate clonal lineages to be detected within *Ph. damselae* subsp. *piscicida*, the European and Japanese isolates.

In recent years, several commercial vaccines against *Ph. damselae* subsp. *piscicida* have been made available on the market but their efficacy is dependent on fish species, fish size, vaccine formulation and use of immunostimulants (Romalde and Magariños, 1997). However, only the licensed bacterin (DI vaccine) patented by the University of Santiago (Spain) and commercialized by Hipra (Spain) demonstrated their effectiveness in gilthead sea bream larvae of only 50 days old. Therefore, bearing in mind that the majority of the pasteurellosis outbreaks occur from larval stages to fingerlings of 10-30 g, a vaccination program which comprises a first dip immunization at the larval stage (average 0.05 g) and a booster vaccination when fish reach a size of about 1-2 g is highly recommended in order to avoid the high economic losses caused by this disease (Magariños *et al.*, 1999).

Recently, different stable attenuated siderophore deficient and *aro*-A deletion mutant strains have been constructed using an allelic replacement technique, which in experimental trials proved to be useful candidates as live vaccines for striped bass hybrids (Hawke *et al.*, 2002).

4. Furunculosis

Aeromonas salmonicida subsp. *salmonicida* is the causative agent of the so-called "typical" furunculosis, which causes economically devastating losses in cultivated salmonids in fresh and marine waters. It also affects a variety of non-salmonid fish, and shows a widespread distribution (Toranzo *et al.*, 1991b; Toranzo and Barja, 1992; Austin *et al.*, 1996; Bernoth, 1997; Ellis, 1997; Hiney and Oliver, 1999). *Aeromonas salmonicida* subsp. *salmonicida* can be defined as biochemically, antigenically and genetically homogeneous with no biotypes, serotypes or genotypes being detected (Toranzo *et al.*, 1991b; Austin and Austin, 1999; Hiney and Oliver, 1999). The "atypical" strains of *A. salmonicida* are included within three subspecies, *masoucida*, *achromogenes* and *smithia*, and they cause ulcerative diseases in a variety of fish species, such as goldfish (*Carassius auratus*), carps (*Cyprinus* spp.), eels, marine flat fish and salmonids, mainly in Europe and Japan.

Although many furunculosis bacterins have been developed and commercialized since 1980, to be used in salmonids by injection, immersion or the oral route (Newman, 1993; Midtlyng, 1997), their efficacy has been questioned because of the lack of repetitive results and/or the short protection period. The best results in terms of protection have been reported in salmonids with the mineral oil-adjuvanted vaccines. However, these bacterins have been shown to posses several adverse side-effects, such as the induced formation of internal granulomatous lesions

adherent to the viscera and a reduction in weight gain (Ellis, 1997). To avoid these drawbacks, new non-mineral oil-adjuvanted vaccines have been recently developed and are now on the market. Polyvalent vaccines for salmonids, incorporating different *Vibrio* species and *A. salmonicida* as antigens, are also available and they seem to be more effective than monovalent furunculosis bacterins. However, in the case of turbot, the protection covered by the furunculosis vaccines is very short (about 3 months) even by the ip route. In addition, revaccination experiments in turbot by bath have been unsuccessful. Currently, new vaccines and/or immunization strategies are being investigated in order to achieve long-term protection of turbot against furunculosis.

Different approaches have been used to develop live attenuated vaccines against furunculosis (Munn, 1994). Although A-layer and O-antigen deficient *A. salmonicida* vaccines were effective in providing high levels of fish protection (Thornton *et al.*, 1991, 1994), concern exists about possible reversion to virulence for these incompletely attenuated vaccine strains. However, recombinant DNA technology allowed the construction of highly attenuated and stable *aro*A auxotrophic mutant strains, using an allelic replacement technique, which were employed experimentally as safe live vaccines with a high level of success (Vaughan *et al.*, 1993), but their approval for use in the field has not yet been forthcoming.

5. Motile Aeromonas septicaemia

Motile aeromonads of the *Aeromonas hydrophila* complex cause a haemorrhagic septicaemia in numerous species of cultured and wild freshwater fish, such as rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), Coho salmon (*Oncorhynchus kisutch*), eel, carp, channel catfish (*Ictalurus punctatus*), tilapia (*Oreochromis* spp.), ayu (*Plecoglossus altivelis*), and goldfish (Santos *et al.*, 1988; Cahil, 1990; Thune *et al.*, 1993; Joseph and Carnahan, 1994; Austin *et al.*, 1996; Aoki, 1999; Austin and Austin, 1999; Nielsen *et al.*, 2001). Although, classically, three species, *A. hydrophila*, *A. sobria* and *A. cavieae* were included within the motile *Aeromonas*, further taxonomic data including genetic studies allowed the identification of at least 10 new motile *Aeromonas* species. However, *A. hydrophila* is still regarded as the predominant fish pathogen, although its importance may have been overestimated in the past.

Although motile *Aeromonas* species are typically recognized as opportunistic pathogens or secondary invaders, there have been reported cases of *A. hydrophila* acting as a primary fish pathogen. Outbreaks of *Aeromonas* septicaemia are usually associated with a change in environmental conditions. Stressors, including overcrowding, high temperature, a sudden change of temperature, handling, transfer of fish, low dissolved oxygen, poor nutritional status and fungal or parasitic damage of the epidermis, contribute to physiological changes and heighten susceptibility to infection.

Although experimental vaccination to prevent infections by *A. hydrophila* in different fish species has been examined (Newman, 1993; Aoki, 1999), the development of an appropriate commercial vaccine is hampered by the great phenotypic and serological heterogeneity existing within the group of mesophilic motile *Aeromonas* species. In fact, almost 100 serotypes have been reported to exist within the motile *Aeromonas* group (Shimada and Kosako, 1991; Janda *et al.*, 1996; Nielsen *et al.*, 2001) Prophylactic measures, such as good hygiene, avoidance of overcrowding, and excessive handling, are the best methods of prevention.

6. Yersiniosis

Yersinia ruckeri is the causal agent of yersiniosis or enteric red mouth (ERM) disease, which produces important economic losses in salmonid pisciculture all over the world. Moreover, sporadic isolations of this bacterium have also been documented in cultured non-salmonid fish in either fresh or sea water (Romalde, 1992; Furones *et al.*, 1993; Toranzo and Barja, 1993a; Stevenson, 1997; Horne and Barnes, 1999). *Y. ruckeri* was also recovered from wild fish, birds

and mammals, which can act as potential vectors of the disease (Willumsen, 1989), and it has been demonstrated that this pathogen can persist in the environment (seawater and sediments) in a dormant but infective state (Romalde *et al.*, 1994).

Classically, *Y. ruckeri* has been divided into two biotypes and five serovars. Biotype 1 corresponded with serovar I and included the non-sorbitol fermenting strains. Biotype 2 comprised the remaining serovars (II, III, V and VI) and contained the sorbitol-fermenting isolates. Further studies resulted in the acceptation of a new different serotyping scheme (Romalde *et al.*, 1993), consisting of four O-serotypes: serotype O1 with two subgroups, O1a (former serovar I) and O1b (formar serovar III); serotype O2 (former serovar II) with three subgroups O2a, O2b and O2c; and the serotypes O3 and O4, which correspond respectively to former serovars V and VI in order to follow a logical chronological numerical order. However, for vaccination purposes, two groups (O1a and O2b) cause most epizootic outbreaks in cultured salmonids.

Although commercial ERM vaccines have been extensively used for decades, with generally high efficacy (Newman, 1993; Stevenson, 1997), they do not eliminate the carrier state, since the apparently healthy vaccinated fish act as a vehicle by which ERM could be spread into non-endemic areas of the disease. Most of the commercial vaccines are based only on the Hagerman strain that belongs to serotype O1a. However, it has been demonstrated that not all antigenic variants of *Y. ruckeri* can be effectively cross-protected by this serotype (Romalde, 1992; Stevenson, 1997). Therefore, the inclusion of at least the predominant serovars (O1a and O2b) in the commercial vaccines is encouraged.

7. Enteric septicaemia of catfish, ESC (*Edwardsiella ictaluri*)

Edwardsiella ictaluri is the enterobacterium responsible for enteric septicemia of catfish, with channel catfish being the most susceptible fish species among the ictalurids. This disease constitutes the greatest disease problem affecting the catfish industry in the United States. In fact, commercial catfish production accounts for 85-90% of the total fin fish aquaculture production in this country (Plumb, 1994a, 1999). The bacterium is considered to be biochemically and serologically homogeneous.

The first attempts to develop vaccines against *E. ictaluri* focused on the use of killed bacterins and they have delivered equivocal results, because the evaluation of vaccination efficacy in many of the studies is rendered difficult by the failure to control natural exposure to *E. ictaluri*, resulting in positive antibody titres in non-vaccinated fish (Plumb, 1994a, 1999; Thune *et al.*, 1997). The first commercial bacterins for *E. ictaluri* were licensed to be used by immersion or oral routes. The best strategy devised for preventing ESC is an initial immersion of fry or fingerlings, followed by an oral booster 1-2 months later (Thune *et al.*, 1994; Shoemaker and Klesius, 1997). Although the percentage protection only ranged between 10 and 30%, vaccinated fish grew faster and showed a lower feed conversion rate. Therefore, the farmers will have to analyze the benefit to cost ratio to determine if this vaccination strategy is feasible.

Since *E. ictaluri* is an intracellular pathogen for channel catfish, it is not unusual that killed vaccines have not been very successful. Recently, an attenuated O-antigen deficient *E. ictaluri* strain has been developed which was safe and provided high long-lasting acquired immunity (for at least 4 months) following a single bath immersion in 9-14 day old channel catfish without booster vaccination (Klesius and Shoemaker, 1998). This modified live *E. ictaluri* vaccine has been produced since 2000, by Intervet Inc., under the trade name AQUAVAC-ESCO, and constitutes the first licensed bacterial live vaccine in aquaculture formulated with an attenuated pathogenic strain.

8. Marine flexibacteriosis

Flexibacter maritimus (formerly, *Cytophaga marina* and *Flexibacter marinus*) is the causative

agent of flexibacteriosis in marine fish (Wakabayashi *et al.*, 1986; Bernardet and Grimont, 1989). Several other names, such as "gliding bacterial diseases of sea fish", "eroded mouth syndrome", and "black patch necrosis", have been used to designate the disease caused by this bacterium. In addition, on the basis of recent phylogenetic, chemotaxonomic and phenotypic studies it was proposed that *Flexibacter maritimus* should be transferred to the new genus *Tenacibaculum*, as *Tenacibaculum maritimum* (Sukui *et al.*, 2001).

Marine flexibacteriosis is widely distributed in cultured and wild fish in Europe, Japan and North America (McVicar and White, 1979, 1982; Wakabayashi *et al.*, 1986; Devesa *et al.*, 1989; Pazos *et al.,* 1993; Chen *et al.*, 1995; Ostland *et al.*, 1999; Santos *et al.*, 1999). In Europe, the disease has been reported in sole, gilthead sea bream, sea bass, turbot, and Atlantic and Coho salmons. In Japan, *F. maritimus* has been isolated from red sea bream (*Pagrus major*), black sea bream (*Acanthopagrus schlegeli*) and flounder (*Paralichthys olivaceus*). In North America, marine flexibacteriosis has been described in Atlantic salmon, white sea bass (*Atractoscion nobilis*), Pacific sardine (*Sardinops sagax*) and northern anchovy (*Engraulis mordax*).

Although the bacterium is biochemically homogeneous, at least two major "O" serogroups can be detected, which seem to be related to the host species (Ostland *et al.*, 1999; Avendaño *et al.*, 2003). However, this antigenic heterogeneity would warrant further investigation to clarify the value of serotyping as an epidemiological marker in this fish pathogen.

Although, until recently, no vaccines were available to prevent the disease (Bernardet, 1997), a flexibacteriosis vaccine ("FM 95") was patented by the University of Santiago (Spain) and is the only bacterin currently on the market to prevent mortalities caused by *F. maritimus* in turbot (Santos *et al.*, 1999). Since this disease affects all sizes of turbot, the vaccine is applied by bath when the fish reach 1-2 g, and later by injection when the fish attain 20-30 g. Whereas the percentage of protection by bath is about 50%, when the vaccine is administered by ip injection the protection increases to more than 85%. Divalent formulations to prevent simultaneously turbot flexibacteriosis and vibriosis or flexibacteriosis and streptococcosis are also available.

Currently, a flexibacteriosis bacterin specific for cultured sole has been developed by our research group which conferred relative percentage survival (RPS) values higher than 90% in laboratory trials performed by ip injection (Romalde *et al*., 2003).

9. Cold water disease or rainbow trout fry syndrome (RTFS)

Flavobacterium psychrophilum (syn., *Cytophaga psychrophila* and *Flexibacter psychrophilus*) has been known as the causative agent of bacterial cold water disease (BCWD) or peduncle disease in salmonids since 1948. The same bacterium has been shown to be the agent involved in the rainbow trout fry syndrome (RTFS) since the decade of the 1980s. The disease has been reported in the USA, Europe, Japan, Tasmania and Chile. Although farmed salmonids (especially Coho salmon and rainbow trout) reared in fresh water are particularly susceptible, the pathogen has been isolated from non-salmonid fish such as eels and cyprinids in Europe, and ayu in Japan (Bernardet and Kerouault, 1989; Lehmann *et al.*, 1991; Toranzo and Barja, 1993b; Bernardet, 1997; Dalsgaard, 1993; Wakabayashi *et al.*, 1994; Iida and Mizokami, 1996; Cipriano *et al.*, 1996; Lorenzen *et al.*, 1997; Lorenzen and Olesen, 1997). The disease usually occurs in very young fish in which the pathogen provokes an acute septicaemia with spleen hypertrophy. In fingerlings, external lesions may also appear. The severity of the disease occurs typically when water temperatures are between 4 and 13ºC.

Although classically three main serotypes were defined for this bacterium (Lorenzen and Olesen, 1997), recent ELISA assays established a total of at least five serogroups, although no correlation was apparent between serotypes and the geographical origin of strains, the species of host fish or the virulence of the isolates (Faruk *et al.*, 2002).

Few vaccination attempts for preventing the disease caused by *F. psychrophilum* have been

published (Obach and Baudin-Laurencin, 1991; Bernardet, 1997; Rahman *et al.*, 2000). This has been due, in part, to the difficulties in culturing this gliding bacterium, as well as to the lack of an experimental challenge model giving well-controlled and quantitatively reproducible effects (García *et al.*, 2000). Recent vaccination experiments performed with young rainbow trout demonstrated that only significant protection was achieved using oil-adjuvanted ip vaccines (LaFrentz *et al.*, 2002). However, this route is impracticable for the early life fish stages in which *F. psychrophilum* infections usually occur. In addition, no cross protection among serotypes was obtained. Therefore, it is important to consider the inclusion of all the serotypes of *F. psychrophilum* occurring in a particular geographical area in RTFS vaccines. Although no commercial vaccines against this disease are available, some countries are using autogenous bacterins made from single farm isolates.

10. Columnaris disease or saddleback disease

"Columnaris" disease is caused by the chromogenic gliding bacterium *Flavobacterium columnare* (syn., *Chondrococcus columnaris*, *Cytophaga columnaris*, *Flexibacter columnaris*) (Bernardet and Grimont, 1989). This disease exists worldwide in fresh and brackish waters especially in the USA, Europe and Asia, and affects mainly ictalurids, eels, salmonids, cyprinids, centrarchids, and ornamental fish such as golden shiner and goldfish (Song *et al.*, 1988; Bernardet, 1989; Wakabayashi, 1993; Plumb, 1994b; Syamsudin and Plumb, 1996). Columnaris disease usually occurs when the water temperature exceeds 15ºC. The disease can be easily complicated by dual infections in which another bacterial or protozoan parasite can be involved.

Although this pathogen is biochemically homogeneous, the strains are not antigenically identical, since four major serological groups and several minor ones were shown by reciprocal absorption, and this can complicate serological typing.

Several vaccination experiments against *F. columnare* have been performed on several fish species using different routes of administration (i.e. injection, bath, oral) but the results in field trials were inconsistent, possibly due to the intimate association of stress with the disease process. No commercial vaccines are available (Newman, 1993; Bernardet, 1997).

11. Pseudomonadiasis

Among the *Pseudomonas* species recovered from diseased fish (*P. chlororaphis*, *P. anguilliseptica*, *P. fluorescens*, *P. putida*, *P. plecoglossicida*), *Pseudomonas anguilliseptica* is considered the most significant pathogen for cultured fish (Toranzo and Barja, 1993a; Austin and Austin, 1999).

Pseudomonas anguilliseptica was originally described in 1972 as the etiological agent of "Sekiten-bio" or "red spot disease", which caused massive mortalities in pond-cultured Japanese eel in Japan (Wakabayashi and Egusa, 1972). Since then, this bacterium has been recorded in European eel reared in Taiwan, Scotland and Denmark (Kuo and Kou, 1978; Stewart *et al.*, 1983). The pathogen was subsequently isolated from other fish species, such as black sea bream and ayu in Japan (Nakai *et al.*, 1985), salmonids in Finland (Wiklund and Bylund, 1990), wild herring (*Clupea harengus membras*) in the Baltic sea (Lönnström *et al.*, 1994), and from 1995 it was considered to be the agent responsible for the "winter disease syndrome" characteristic of gilthead sea bream cultured in the Mediterranean area (Berthe *et al.*, 1995; Doménech *et al.*, 1999). Very recently, *P. anguilliseptica* was also recovered as an emerging pathogen of turbot cultured in Spain (Romalde *et al.*, 2001; López-Romalde *et al.*, 2003a).

Regarding the serological characteristics, recent studies have indicated the existence of two major O serotypes related to the fish host, one characteristic of the eel isolates and another

typical of the gilthead sea bream and turbot isolates (Romalde *et al.*, 2001; López-Romalde *et al.*, 2003b). In addition, genetic characterization studies employing RAPD techniques revealed the presence of two genetic groups which were coincident with the two serological groups (López-Romalde *et al.*, 2003a). All this information is very useful for developing an adequate vaccine against this disease.

Recent research efforts by our group in collaboration with the Hipra Veterinary Laboratory (Spain) led to the development of aqueous and non-mineral oil-adjuvanted bacterins (including both major serotypes detected), which proved to be effective in experimental trials in gilthead sea bream and turbot (Romalde *et al*., 2003).

12. Streptococcosis

Streptococcal infection of fish is considered to be a re-emerging pathology affecting a variety of wild and cultured fish around the world (Kitao, 1993; Bercovier *et al.*, 1997; Romalde and Toranzo, 1999, 2002). Classification of Gram-positive cocci based on DNA-DNA hybridization coupled with 16S sequencing has shown that at least six different defined species are considered of significance as fish pathogens: *Lactococcus garvieae* (syn. *Enterococcus seriolicida*), *Lactococccus piscium*, *Streptococcus iniae* (syn*. S. shiloi*), *Streptococcus agalactiae* (syn. *S. difficile*), *Streptococcus parauberis*, and *Vagococcus salmoninarum*. Therefore, streptococcosis of fish should be regarded as a complex of similar diseases caused by different genera and species capable of inducing central nervous system damage characterized by suppurative exophthalmia ("pop-eye") and meningoencephalitis. Whereas "warm water" streptococcosis (causing mortalities at temperatures above 15ºC) typically involves *L. garvieae*, *S. iniae*, *S. agalactiae* and *S. parauberis*, "cold water" streptococcosis (occurring at temperatures below 15ºC) is caused by *L. piscium* and *V. salmoninarum*. It is important to mention that the aetiological agents of "warm water" streptococcosis are considered also as potential zoonotic agents capable of causing disease in humans.

Among these fish streptococci, *L. garvieae*, *S. iniae* and *S. parauberis* can be regarded as the main etiological agents causing diseases in aquaculture.

Lactococcus garvieae is capable of infecting saltwater fish species, such as yellowtail in Japan and fresh water species like rainbow trout, mainly in Italy, Spain, France and, to a lesser extent, in the UK and Australia (Kusuda *et al.*, 1991; Eldar *et al.*, 1996, 1999a; Bercovier *et al.*, 1997; Eldar and Ghitino, 1999; Ravelo *et al.*, 2001, 2003). The existence of two serogroups associated with the presence (serotype KG^-) or absence (KG^+) of a capsule (Yoshida *et al.*, 1996) has been demonstrated in this pathogen. Only the capsulated strains are pathogenic for fish.

Streptococcus iniae is the main etiological agent of streptococcosis in tilapia and striped bass hybrids in America and Israel and rainbow trout in Israel. However, it was isolated from marine fish, such as yellowtail and flounder in Japan, European sea bass and red drum (*Sciaenops ocellatus*) in Israel, and barramundi (*Latex calcarifer*) in Australia (Perera *et al.*, 1994; Eldar *et al.*, 1995, 1999b; Bromage *et al.*, 1999; Eldar and Ghitino, 1999; Nguyen and Kanei, 1999). Although for several years this pathogen was considered as a serologically homogeneous species, a new serotype (denominated serotype II) with different antigenic determinants in its capsule has emerged in recent years in Israel and the USA (Bachrach *et al*., 2001; Barnes *et al*., 2003).

Streptococcus parauberis is endemic for turbot cultured in Spain (Toranzo *et al.*, 1994, 1995a; Doménech *et al.*, 1996). This pathogen constitutes a biochemically and antigenically homogeneous group which has facilitated the development of a vaccine formulation (Toranzo *et al*., 1995b).

Several attempts have been made to develop appropriate vaccination programs for fish

streptococcosis. However, considerable variability in the protection was observed depending on the fish and bacterial species, as well as the route of administration. All the streptococcosis vaccines rendered good levels of protection only when they were administered by intraperitoneal injection. However, whereas *L. garvieae* and *S. iniae* experimental vaccines conferred high protection in rainbow trout for only 3-6 months (Bercovier *et al.*, 1997; Eldar *et al.*, 1997), the *L. garvieae* and *S. parauberis* bacterins displayed high levels of long-term protection in yellowtail and turbot, respectively (Toranzo *et al.*, 1995b; Romalde *et al.*, 1999b; Ooyama *et al.*, 1999).

Precaution must be taken in the antigenic formulation of rainbow trout lactococcosis vaccines because several failures were recently shown in both licensed and autogenous vaccines (which caused heavy losses on the farms concerned). The antigenic composition of these bacterins corresponded to avirulent non-capsulated strains of *L. garvieae* which gave little protection against a natural infection of virulent capsulated strains. In the case of *S. iniae* vaccines, they must be based on the inclusion of both serotypes detected for the pathogen, since it was demonstrated that vaccines formulated only with serotype I do not protect fish against infection caused by serotype II (Bachrach *et al*., 2001).

13. Bacterial kidney disease

Bacterial kidney disease (BKD), caused by the Gram-positive diplobacillus *Renibacterium salmoninarum*, is a chronic systemic disease of salmonids which causes mortality in cultured fish in fresh and marine environments (Sanders and Fryer, 1980; Evelyn, 1993; Evenden *et al.*, 1993; Fryer and Lannan, 1993; Toranzo and Barja, 1993a; Kaattari and Piganelli, 1997; Wiens and Kaattari, 1999). The pathogen has been also found in wild fish populations. The disease has been reported to occur in North America, Japan, Western Europe and Chile.

Renibacterium salmoninarum isolates are biochemically and antigenically homogeneous (Bruno and Munro, 1986; Kaattari and Piganelli, 1997). The main common antigen is the heat-stable p57 protein which is present on the cell surface and is also released into fish sera and tissues during the infection (Wiens and Kaattari, 1999).

Although vaccination trials using classical bacterins, recombinant vaccines or attenuated live vaccines have been reported, and there is evidence that under some conditions *Renibacterium* elicits an immune response in fish (Newman, 1993; Kaattari and Piganelli, 1997; Griffiths *et al.*, 1998; Daly *et al.*, 2001), the protective ability of a vaccine in field conditions is questionable because of the intracellular nature and vertical transmission of the pathogen, as well as the possible immunosupressive role of the protein p57 (Wood and Kaattari, 1996). Although a whole cell *R. salmoninarum* bacterin in which the p57 protein was eliminated ($p57^-$ vaccine) failed to protect salmonids reliably by the ip route, promising results were obtained when this vaccine was administered by the oral route (Piganelli *et al.*, 1999).

Recently, a commercial aqueous live vaccine developed by Novartis, S.A, has been licensed under the name of "Renogen" for BKD prevention (Salonius *et al*., 2003). This vaccine is constituted by live cells of *Arthrobacter davidanieli* (proposed nomenclature), a non-pathogenic environmental bacterium which express an extracellular polysaccharide with antigenic homology to that of *R. salmoninarum*. In field trials, "Renogen" conferred significant long-term protection on Atlantic salmon against BKD, with RPS values of 79% 24 months after vaccination.

14. Piscirickettsiosis

Piscirickettsiosis is a septicaemic condition of salmonids (Fryer and Lannan, 1996; Almendras and Fuentealba, 1997; Lannan *et al.*, 1999; Larenas *et al.*, 1999). The causative agent of the disease is *Piscirickettsia salmonis* (Fryer *et al.*, 1992), a non-motile Gram-negative, obligate

intracellular bacterium. The disease was described for the first time in 1989 affecting Coho salmon cultured in Chile (Bravo and Campos, 1989; Branson and Nieto, 1991; Cvitanich *et al.*, 1991) where mortalities between 30-90% were reported. From 1992, the disease was also described in Ireland, Norway, Scotland, and both the west and east coasts of Canada (Rodger and Drinan, 1993; Grant *et al.*, 1996; Olsen *et al.*, 1997; Palmer *et al.*, 1997; Jones *et al.*, 1998).

Although at present there are some commercialized vaccines available in Chile against *P. salmonis*, the efficacy of these bacterins is questioned because of the lack of enough protection data under experimental and field conditions (Smith *et al.*, 1997; Larenas *et al.*, 1999). Recently, a monovalent recombinant subunit vaccine for *P. salmonis* has been constructed which elicited a high protection in Coho salmon in laboratory trials (Kuzyk *et al.*, 2001). In addition, the live vaccine "Renogen" devised to prevent bacterial kidney disease was also demonstrated to be effective in reducing mortality from *P. salmonis* in Pacific salmon with significant long-term protection under both laboratory and field conditions (Salonius *et al*., 2003).

Salmonids have not been the only target fish of *Rickettsia*-like organisms (RLOs), and several reports have been published describing rickettsial infections as being responsible for epizootic outbreaks in non-salmonid fresh water and marine fishes, such as different species of tilapia in Taiwan and Hawaii, imported blue-eyed plecostomus (*Panaque suttoni*) in the USA and juvenile sea bass in Europe (Comps *et al.*, 1996; Lannan *et al.*, 1999; Steiropoulos *et al*., 2002; Mauel *et al*., 2003). Although in the majority of the cases no comparison between these *Rickettsia*-like organisms and the *P. salmonis* isolates have been conducted, recent immunohistochemistry studies (Steiropoulos *et al.*, 2002) demonstrated antigenic similarities between the RLOs from European sea bass and *P. salmonis*.

15. Mycobacteriosis (fish tuberculosis)

Mycobacteriosis in fish (or fish tuberculosis) is a subacute to chronic wasting disease known to affect nearly 200 freshwater and saltwater species. Although *Mycobacterium marinum* is considered the primary causative agent of fish mycobacteriosis, a great number of *Mycobacterium* species associated with tubercle granulomas in cultured, aquarium and wild fish populations have been described: *M. marinum*, *M. fortuitum*, *M. chelonae*, *M. smegmatis*, *M. abscessus*, *M. neonarum, M. simiae*, *M. scrofulaceum*, *M. poriferae* and *M. triplex*-like (Hedrick *et al.*, 1987; Bragg *et al.*, 1990; Lansdell *et al.*, 1993; Colorni *et al.*, 1996; Bruno *et al.*, 1998a; Chinabut, 1999; Talaat *et al.*, 1999; Diamant *et al.*, 2000; Herbst *et al.*, 2001; Rhodes *et al.*, 2001; dos Santos *et al.*, 2002). All these species can also cause disease in humans.

Mycobacteriosis has been documented in cultured fish such as Pacific and Atlantic salmon, pejerrey (*Odonthestes bonariensis*), snakehead fish (*Chana striatus*), turbot, tilapia, European sea bass and red drum, but, since 1990, mycobacteriosis caused by *M. marinum* has represented a significant threat especially for sea bass cultured in the Mediterranean and on the Red Sea coasts of Israel (Colorni, 1992; Colorni *et al.*, 1993, 1996; Diamant *et al.*, 2000). Recently, this disease has also been considered a matter of concern for turbot culture in Europe (dos Santos *et al.*, 2002).

At present no vaccines are available to prevent this disease in fish.

IV – Economic aspects related to vaccination

All sustainable industries producing live stock intensively rely on effective vaccination programs. However, vaccines are not "cure all" remedies but are an integral part of comprehensive health management programs. Most producers do not realise the true economic value of vaccines. However, fish vaccines are normally more cost effective than other investments related to growing fish commercially. When producers have to choose between vaccines, they must bear in mind that small improvements in vaccine efficacy far outweigh any economic benefits related to large differences in vaccine price.

The following parameters of economic importance must be considered in the implementation of vaccination strategies against particular diseases:

(i) Expected/historical mortality of fish due to the diseases
(ii) Degree/duration of disease protection provided by vaccines
(iii) Expected/historical drug cost needed to treat against the disease(s) when not vaccinated.

In addition, there are other economic aspects (potential benefits and/or costs) that must be taken into account when vaccinating fish:

1. Potential benefits

(i) Increased appetite and growth in vaccinated compared to non-vaccinated fish because of the better food conversion rates in vaccinated fish.
(ii) Potential of growing vaccinated fish at higher densities because disease is not a limiting factor in the population.
(iii) Reduction of drug use and, therefore, the incidence of appearance of bacterial drug resistance, as well as drug residues in the final product.
(iv) Improvement of industry image for the sanitary quality of the fish produced, as well as from the environmental safety stand point.

2. Potential costs

(i) Post-vaccination mortality as a result of:
- Fish are latent carriers of pathogen which "emerges".
- Fish are weakened by improper handling or rearing practices.

(ii) Decrease in growth caused by side effects such as those produced by some adjuvanted vaccines.

V – The general key rules of fish vaccination

(i) Do not let vaccines solve your husbandry problems. Events or practices such as overstocking, undue stress, or poor water quality can cause breakdowns in vaccine protection.
(ii) Only vaccinate healthy fish. The performance of vaccines is very dependent on the health status of the fish at the time of vaccination. Vaccines cannot be expected to give good or long-term protection if the fish are sick, in poor condition, or they are carriers of pathogens when vaccinated.
(iii) Allow sufficient time for immunity to develop. Immunity takes time to develop and thus vaccinated fish are not immediately protected. Thus, vaccinated fish must be maintained during this time in the less stressful conditions as possible. The time of the development of immunity is dependent mainly upon the surrounding water temperature (i.e. at 10ºC it takes 15-20 days).
(iv) Strictly follow the recommendations of vaccine usage when immunizing fish. Do not try to shorten the recommended time of exposure to the vaccines; do not modify the dilution or dose recommended; do not overload the net when fish are vaccinated by dip immersion; do ensure that the water used to dilute the vaccine is a similar temperature to that in which the fish are being held; do not use the vaccine after the expiry data.
(v) Do not expect vaccines to eradicate disease. If vaccines against a particular disease are used routinely on the farm, evidence of this disease will largely disappear. However, this does not mean that the organism which causes the disease has been eradicated. In fact, it is still present and capable of infecting susceptible unvaccinated fish.

VI – Future prospects

(i) To achieve progress in fish vaccinology, an increase in the co-operation between basic and applied science (i.e., between the immunologist/microbiologist and the vaccinologist) is needed.

(ii) Since there is not always correlation between the major antigens expressed *in vitro* and those expressed *in vivo*, the development of more effective vaccines for the diseases in aquaculture should rely in the identification of the important immunogens expressed by the pathogens *in vivo*, and the selection of *in vitro* conditions that maximize their expression.

(iii) Improvement in oral immunization with biodegradable microparticle-based vaccines to be used for booster vaccination.

(iv) Development of new non-mineral oil adjuvants lacking side effects.

(v) Development of polyvalent vaccines and standardization of a vaccination calendar appropriate for each economically important fish species.

(vi) Investigation of the mechanisms of immunoglobulin transfer from pre-spawning females to offspring as a useful way of protecting fish against pathogens which affect early life stages.

Note

Since the preparation of this paper, new emerging or re-emerging pathogens, including *Francisella philomiragia* subsp. *noatunensis*, *Edwardsiella tarda*, and *Streptococcus phocae*, have gained importance in different cultured fish throughout the world. In addition, new biotypes or serotypes of known pathogens, such as *Yersinia ruckeri* and *Tenacibaculum maritimum*, have caused important outbreaks in specific areas or fish species. Therefore, studies have been performed or are in progress to formulate vaccines to prevent these new pathologies.

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