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Review

Lactococcus garvieae in fish: A review

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Abstract

Lactococcus garvieae is the etiological agent of Lactococcosis, an emergent disease which affects many fish species and causes important economic losses both in marine and freshwater aquaculture when water temperature increases over 16 °C in summer months. Normally, it causes a hyperacute and haemorrhagic septicemia. This paper presents a state of the art review of fish Lactococcosis including aspects such as pathogen characterization, pathogenesis, epidemiology, diagnosis and control measures of the disease in farmed fish.

Keywords: *Lactococcus garvieae*; Hemorrhagic septicemia; Fish pathogen; Fish

Résumé

Lactococcus garvieae est l'agent étiologique de la Lactococcose, une maladie émergente qui affecte beaucoup d'espèces poissons et cause importantes pertes économiques en aquaculture marine et continentale quand la température de l'eau augmente au-dessus de 16 °C pendant les mois d'été. Normalement, il cause une septicémie suraiguë et hémorragique. Cette revue présente les dernières connaissances sur la lactococcose d des poissons et contient un entair nombre d'aspects concernant le pathogène, la pathogénie, l'épidémiologie, le diagnostic et le contrôle de la maladie en élivage.

Mots Clés: *Lactococcus garvieae*; Septicémie hémorragique; Pathogènes des poisons; Poissons

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Contents

1. Introduction

Lactococcosis is an emerging pathology affecting a variety of fish species all over the world. The septicemic processes caused by Gram-positive coccus, commonly denominated as Streptococcosis, are not new; they were described for the first time at the end of the 50 s in Japan, where the first cases were diagnosed in the intensive production of rainbow trout [1]. During the last decade we have noted numerous changes in the taxonomy of some implied etiological agents that initially were assigned to the *Streptococcus* genus. The development of new techniques of diagnosis based on genotypic characteristics has made possible this reclassification into new bacterial genera as *Enterococcus*, *Lactococcus*, *Vagococcus* and *Carnobacterium* [2–5].

From an etiological point of view, streptococcal processes can be divided into two groups: warm water infections, caused by those cocci species (*Lactococcus garvieae*, *Streptococcus iniae*, *S. agalactiae* and *S. parauberis*) that are pathogenic for both cultured freshwater and marine fish at water temperature above 15 °C, and coldwater infections, caused by those cocci species (*Vagococcus salmoninarum* and *L. piscium*) that are pathogenic exclusively for salmonid fish at temperature below 15 °C [6].

Lactococcosis is a kind of Streptococcosis caused by *L. garvieae,* which has been particularly devastating in the freshwater culture of salmonid fish and marine-cultured species. The causative agent, *L. garvieae* was first described from an investigation of bovine mastitis in Great Britain [7]. Outbreaks affecting rainbow trout have been reported in several countries, such as Australia, South Africa, Japan, Taiwan, England and countries of the Mediterranean area. The losses produced can exceed approximately 50–80% of the total production [8].

2. Aetiology

The genus *Lactococcus* is included within the family Streptococcaceae. It was described in 1985 after the division of the *Streptococcus* genus, which included a group of agents known as lactic streptococci represented by agents isolated in dairy cattle and milk products [2]. *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *L. garvieae* are the most important species of the *Lactococcus* genus, with clinical significance in humans and animals [9–11]. *L. garvieae*, previously described as *Streptococcus garvieae*, was originally isolated in the United Kingdom from a mastitic udder and was identified as the reference strain (ATCC 43921) for this species [7]. In 1991, it was proposed as a new species, *Enterococcus seriolicida*, in order to bring together a number of Gram-positive isolates recovered from Streptococcosis outbreaks in Japanese yellowtail (*Seriola quinqueradiata*) over the preceding 20 years and *Streptococcus* sp. YT-3 was suggested as the reference strain [12,13].

The first outbreak of Lactococcosis in rainbow trout from Spanish fish farms occurred in 1988 [14]. Initially, the agent was described as an *Enterococcus* sp. until it was definitely identified as *L. garvieae*, which showed biochemical characteristics very similar to *E. seriolicida* [15]. In the following years, *L. garvieae* was isolated from several septicemic processes in fish and phenotypical and molecular taxonomic studies confirmed the same agent as *E. seriolicida.* This species was reclassified as a junior synonym of *L. garvieae* [16–19]. From this time on, Lactococcosis was progressively spread in several countries and aquatic organisms causing significant economic losses. It was isolated as the causative agent of mortality in rainbow trout in Italy [8], Australia and South Africa [20].

L. garvieae was later identified in Taiwan as responsible for outbreaks in giant freshwater prawn (*Macrobrachium rosenbergii*) [21], grey mullet (*Mugil cephalus*) [22] and rainbow trout [23]. Also, this pathogen was isolated in Turkey for the first time in 2001, affecting farmed rainbow trout with a cumulative mortality of approximately 80% [24]. Recently, *L. garvieae* has been identified from outbreaks in rainbow trout in England [25], Portugal [26], France, the Balkans and Israel [27], and from outbreaks in marine species in Korea [28].

The host range of *L. garvieae* is not limited to aquatic species. This agent has also been identified in cows, from subclinical intramammary infections [29], in water buffalos with subclinical mastitis [30], in poultry meat [31], in raw cow's milk [32], in meat products [33], in porcine blood from industrial abattoirs [34] and from cat and

dog tonsils [18]. In addition, the agent has been isolated from humans in several cases, suggesting that *L. garvieae* could be cataloged as a potential zoonotic agent. It has been identified in the USA from urinary tract, blood, skin and pneumonic processes [35] and in Canada from patients with bacterial endocarditis [36]. At a later date, it was isolated in France from an immunosupressed patient, causing septicemia with a liver abscess [37], and from an elderly patient in a case of prosthetic valve endocarditis [38]. It was also reported in a case of a lumbar osteomyelitis secondary to *L. garvieae* that was complicated by endocarditis of an aortic valve prosthesis in a previously well, middle-aged woman in Great Britain [39]. In spite of all these cases, its incidence as a zoonotic agent has not been demonstrated until now.

The identification of *L. garvieae* can be carried out by traditional microbiology, biochemical tests and bacterial growth tests (Table 1). Some of its biochemical characteristics can differ depending on the strain (i.e. capability to hydrolyze

Table 1
Biochemical, cultural and physiological characteristics of *L. garvieae* [40–44]

Character	Reaction	Character	Reaction
Cell morphology	Ovoid cocci	*Production of:*	
Gram	+	Arginine	+
Motility	–	Ornithine	–
Growth on:		Lysine	–
4 °C	+	*Acid from:*	
20 °C	+	Glycerol	–
37 °C	+	Raffinose	–
45 °C	+	Arabinose	–
pH 9.6	+	Sorbitol	+
6.5% NaCl	+	Mannitol	+
Haemolysis	α	Cellobiose	+
Catalase	–	Galactose	+
Oxidase	–	D-glucose	+
TSI	A/A–	Maltose	+
Oxidative/fermentative	F	Trehalose	+
Nitrate reduction	–	D-mannose	+
Citrate	–	Inositol	–
Urea	–	Lactose	(+)
Indol production	–	Ribose	v
Esculin	+	Sucrose	v
VP	+	Adonitol	–
H_2S production	–	Glycogen	–
Arginine dihydrolase	+	Melibiose	–
Pyrrolidonyl arylamidase	+	Melezitose	–
Alkaline phosphatase	–	Starch	–
ß-Glucuronidase	V	Tagatose	v
Leucine arylamidase	+	L-rhamnose	–
Sodium hippurate hydrolisis	–	D-xylose	–
Lancefield group	N	Salicin	+

v: variable reaction/( ): weak or slow reaction.
A/A–: acidification of medium TSI and H_2S not produced.

hipurate) [45]. Generally, it has been described as an α-haemolytic agent [46], although other authors have mentioned it as β-haemolytic [19].

Several studies have been carried out to demonstrate the phenotypic heterogeneity of *L. garvieae*. The researchers have proposed biotyping schemes based on phenotypic characteristics (acidification of tagatose, ribose and sucrose) and have recognized three biotypes of *L. garvieae* [41,47].

Subsequently, a new intraspecies classification was proposed with 13 biotypes, based on acidification of some carbohydrates (tagatose, sucrose, mannitol and cyclodextrin) and the presence of the enzymes pyroglutamic acid arylamidase and *N*-acetyl-β-glucosaminidase, although only six of these biotypes were isolated from fish [48]. Furthermore, random amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) methods have been used for epidemiological characterization of *L. garvieae* isolated from different aquatic species in Japan and Europe. These analyses of RAPD patterns allowed the differentiation of three genogroups, each of which was closely related to the host of origin (rainbow trout, yellowtail and catfish). In addition, within the rainbow trout strains it was possible to demonstrate the existence of three genetically distinct clones associated with the geographical origin of the isolates [48–50].

Recently, 23 strains of *L. garvieae* isolated from different fish species and geographic origin have been phenotypically characterized using conventional and miniaturized systems, employing two different culture media. The results indicated a high level of biochemical homogeneity among the strains and suggested different biotypes should not be established, since they would lack epidemiological or intra-species taxonomic value [46].

On the other hand, *L. garvieae* and *L. lactis* are genealogically quite distinct but phenotypically closely related, making their differentiation on the basis of phenotypic criteria very difficult. Ribosomal RNA gene restriction patterns and rRNA-targeted oligonucleotide probes can be used to distinguish between them [51]. Their different susceptibility to clyndamicin and PCR techniques can also be used [52,53].

3. Morphology and culture

L. garvieae is a facultatively anaerobic, non-motile, non-spore forming, Gram-positive ovoid coccus, occurring in pairs and short chains, and it produces α-haemolysis on blood agar (BA). It grows at 4–45 °C in media containing 6.5% sodium chloride (NaCl) at pH 9.6 with 40% bile and in 0.1% methylene blue-milk. Its optimal growth temperature is 37 °C for 24 h, while at 4 °C it needs between 12 and 15 days [13,15,17]. It also grows rapidly in rich media, such as brain–heart infusion agar (BHIA), trypticase-soy agar (TSA), BA, trypticase-soy broth (TSB), and bile-esculin agar (BEA). However, it does not grow on McConkey agar or *Enterococcus* agar [54].

Several studies have demonstrated that the influence of culture media parameters can affect the growth of pathogens and their production of bacterial enzymes and

toxins [55]. For example, it has been reported that addition of nitrite-N at 1.5 mg l^{-1} in TSB significantly decreased the growth rate of *L. garvieae* and significantly reduced mortality compared to zero nitrite controls when injected into giant freshwater prawns [56]. Also, it has been showed that optimun conditions for *L. garvieae* growth in brain heart infusion broth (BHIB) were pH 7–8 at 25–30 °C [57].

4. Antigenic characteristics and virulence factors

The serological characterization of *L. garvieae* has been carried out in several studies by the slide agglutination technique, identifying an antigen denominated KG from the cellular wall. It has been reported that there are two antigenic types of this pathogen, KG^+ and KG^- type strains; the KG^+ type strain agglutinates with antiserum of KG 7409 strain and the KG^- type strain possesses a specific envelope-like substance, which inhibits agglutination with anti-KG 7409 serum [58]. The KG^- type strain was more virulent than the KG^+ in causing infection in yellowtail [59]. These results were confirmed when the surface morphologies of the KG^- and KG^+ phenotypes were differentiated by scanning electron microscopy. KG^- cells were more hydrophilic than KG^+ cells and were resistant to phagocytosis by yellowtail head kidney phagocytes. The chemiluminescent response of these phagocytic cells was lower with the KG^- phenotype than KG^+. The immune response of yellowtail following injection of the two phenotypes differed with higher agglutinating titers in the KG^+ phenotype compared to the KG^- [60].

Subsequently, 24 isolates of *L. garvieae* from different fish species and geographic origin were studied by slide agglutination tests, using rabbit antisera against representative strains with different origins and by Dot blot assays. These results allowed the establishment of two different groups of isolates, but a correlation between serological group and geographic origin or host source could not be determined [46].

Barnes and Ellis [61] serologically compared 17 geographically distinct strains of *L. garvieae* isolated from diseased fish, using antiserum raised against the pathogen in rainbow trout. Sera raised against capsule deficient isolates did not agglutinate capsulated isolates, whereas all antisera raised against capsulated strains cross reacted with non-capsulated isolates. Antisera raised against capsulated Japanese isolates cross-reacted with other capsulated Japanese isolates, but did not cross-reacted with European capsulated strains. In contrast, antisera against European capsulated isolates cross-reacted with other European isolates regardless of origin but not with Japanese capsulated isolates. Furthermore, agglutination assays performed with several representative lectins showed differences in surface carbohydrate structure between capsulated and non-capsulated isolates, and between Japanese and European capsulated isolates. These results indicate that *L. garvieae* can be distinguished serologically into three different serotypes: a European capsulated serotype, a Japanese capsulated serotype and a non-capsulated serotype from both regions.

Another research group studied 81 isolates of this pathogen from different source and ecosystems comparing genetic similarities (established by rDNA RFLP analysis) with serological data (obtained by Dot blot analysis). The results indicated that in endemic locations the bacterial population presented a clonal structure whereas in sites where lactococcosis is sporadic the population displayed a major genetic heterogeneity [27].

Several virulence experiments have been performed in order to determine the possible correlation between pathogenicity of *L. garvieae* for rainbow trout and the two antigenic profiles (KG^- and KG^+). The results revealed that capsulated strains (KG^-) were more virulent than non capsulated (KG^+) [62], showing LD_{50} values as low as 10^2 bacteria per fish [46].

Profiles of intracellular toxins of *L. garvieae* have been related to the ability to reproduce clinic symptoms and cause death when they are injected intramuscularly in yellowtail [63]. Also, the existence of toxins has been demonstrated in extracellular products with the ability to induce clinical symptoms [64].

5. Pathogenicity and clinical signs of disease

Lactococcosis has been defined as a hyperacute and haemorrhagic septicemia [65], although the evolution of the disease depends on environmental conditions where fish are kept, fundamentally the water temperature and water microbiological quality [15]. The typical clinical signs of the disease observed in salmonids are quite similar to those described for Lactococcosis in other fish species like yellowtail or grey mullet [13,22].

It is important to emphasize that this pathogen causes serious economic losses due to elevated rates of mortality (up to 50%), decreasing of growing rates and the appearance of these fish make them unmarketable.

It has been observed in several experimental tests that incubation period of the disease is very short and the microorganism acts with high virulence. In an experimental infection by intraperitoneal route in yellowtail it caused symptoms 2–3 days post-inoculation [66], while intramuscular infection in grey mullet (*M. cephalus*) produced first symptoms and mortality of 100% 2-day post-inoculation [22]. Also, intraperitoneal experimental infection in rainbow trout caused the first symptoms and deaths 3-day post-inoculation [67].

The gross pathology of Lactococcosis begins with the appearance of a rapid and general anorexia, melanosis, lethargy, loss of orientation and erratic swimming. Typical external signs of affected fish are exophthalmia (uni- or bilateral), the presence of hemorrhages in the periorbital and intraocular area, the base of fins, the perianal region, the opercula and the bucal region. It is also very common to observe fish with swollen abdomens and anal prolapsus [6,15,67,68].

L. garvieae produces lesions in the vascular endothelium that cause blood extravasation, leading to hemorrhages and petechias at the surface of internal organs. Over the external surface, it intensely affects the most irrigated tissues such as perianal or buccal area and fins [15]. Pathogenic activity is mediated by toxins that have ability to reproduce clinical symptoms when they are inoculated in fish [63].

At necropsy, the peritoneal cavity usually presents an accumulation of ascitic fluid, which may be purulent or may contain blood. The main organs affected are the spleen, liver, brain, gut, kidney and heart [26,40,69].

Macroscopic lesions in affected fish are typical of an acute systemic disease with strong congestion in the internal organs, different levels of hemorrhages in the swim bladder, intestine, liver, peritoneum, spleen and kidney [15,70]. Also, enlargement of the spleen, focal areas of necrosis in the liver and spleen, pericarditis, haemorrhagic fluid in the intestine, and yellowish exudate covering the brain surface are typically observed [40,67,71].

Histopathology is reported mainly in the eyes and the capsules of internal organs. Lesions on the ocular area are often observed, consisting of extensive fibroplasias with inflammatory cells infiltration. Also, haemorrhagic panophthalmitis has been observed, with destruction of the anterior and posterior chambers of the eye, affection of the optic nerve papilla and inflammation into retrobulbar fat and striated muscle [6,23]. In the brain, lesions appear in the meninges of the cerebrum and cerebellum, followed by fibroplasias, and macrophage and lymphocyte infiltrations. Affected fish usually present acute meningitis, consisting of an exudate covering the brain surface. The exudate colonies of Gram-positive cocci are widely distributed over the meningeal surface and within the Virchoff's spaces [6,23]. In the heart, lesions are typically represented by fibroplasias, macrophage and lymphocyte infiltration of the in pericardium. In the kidney, the renal tubules have hyaline droplet deposition in the epithelia and hyaline casts in the lumen. Fish suffer a severe peritonitis with fat necrosis. The intestine presents extensive superficial erosions with pseudomembrane-like formation [6,22,23].

6. Epidemiology

There are some factors that must be known before the study of the outbreak and evolution of an infectious disease. The knowledge of these parameters is most important in establishing an effective strategy for prevention, control and eradication of the disease.

6.1. Host

The group of responsible agents of Streptococcosis cause disease in a wide range of aquatic species. At the moment, *L. garvieae* has been isolated as causative agent of disease in rainbow trout [15], yellowtail, tilapia (*Oreochromis* sp.), Japanese eel (*Anguilla japonica*) [13], olive flounder (*Paralichthys olivaceous*) [72], grey mullet [22], catfish [50], wild wrasse (*Coris aygula*) [73], black rockfish (*Sebastes schlegeli*) [74], amberjack (*Seriola dumerili*), kingfish (*Seriola lalandi*) [75] and in giant fresh water prawn (*Macrobrachium rosenbergii*) [21]. Rainbow trout is the most sensitive species and suffers acute disease associated with elevated mortalities compared to other fish species [76]. Other species like common carp (*Cyprinus carpio*) are resistant to the disease [77].

6.2. Age

Since the first reports of the disease, it has been observed that all clinical forms of Lactococcosis showed an absence of clinical symptoms and mortalities in fish weighting under 80 g, although the disease could be reproduced in small fish experimentally infected [15]. Subsequently, in a study of the pathogenicity of *L. garvieae*, younger fish (50 g) suffered a higher mortality than older fish (100 g). Also, the acute period of the disease was reported to be longer in young fish [67]. In fish species like grey mullet, *L. garvieae* can cause outbreaks in all size fish [22]. Recently, it has been observed that Lactococcosis can naturally affect rainbow trout of all sizes from fish farms, from juveniles of 5 g to adults weighing more than 1 kg [23,26].

6.3. Agent

L. garvieae strains show a variable response to virulence and pathogenicity depending on their ability to agglutinate. In the eighties, it was reported that two different serotypes of *L. garvieae* existed, depending on the ability to agglutinate when strains were confronted with antiserum strain KG7409. These results were noted when a specific capsule was present that inhibited agglutination with anti-KG7409 serum [58].

Recently several investigations of *L. garvieae* pathogenicity have demonstrated that capsulated strains (serotype KG^-) are more virulent than non-capsulated strains (serotype KG^+) [46,74,78]. Also, there are several factors of the aquatic environment that influence the appearance of the disease, such as temperature and water quality.

6.4. Temperature

The presence of the agent in the fish farm does not necessarily involve presentation of the clinical process. Water temperature is most important in the development of the disease, which is seasonal. The disease is associated with high water temperature. Most acute outbreaks appear when water temperature is over 18 °C, although acute outbreaks have been described with water temperature of 14–15 °C [15,76]. Experimental studies with rainbow trout maintained at different water temperatures demonstrated mortality of over 85% in a group of fish maintained at 18 °C, while a group at 14 °C suffered no mortality. However, the agent could be isolated in some surviving trout of the second group 25 days post-infection [43].

6.5. Water quality

Bad water quality caused by poor sanitary conditions in fish farms influences evolution of the disease [76]. The disease becomes more pronounced when the aquatic environment is poor, and oxygen deficiency increases virulence and

distribution of the agent [79,80]. Also, excessive ammonium concentration causes an increase in mortality [81].

7. Transmission

The microorganism presumably has come from the external medium when distribution of the agent and the appearance of a Lactococcosis outbreak occur in a fish farm for the first time. The disease can be caused by different infection sources, different methods of introduction and mechanisms of dissemination of the bacteria.

7.1. Infection sources and methods of introduction

The entry of new lots of fish in the fish farm is the most frequent method of introduction of the pathogen. Asymptomatic carriers are the main infection source. They carry *L. garvieae* in their microbiota and can eliminate microorganisms in feces, infecting the rest of the healthy animals in the pond. Also, some fish that have recovered from *L. garvieae* infection continue disseminating the agent for a certain period [76].

L. garvieae has been reported in healthy rainbow trout from a fish farm, which had suffered Lactococcosis outbreaks the previous year. A PCR assay confirmed the existence of asymptomatic carriers that maintain a latent infection. Disease develops when environmental conditions are optimal for the agent [56].

The causative agents of Streptococcosis have been described as part of the intestinal microbiota of some fish species that are used as the main raw material for in dry feed. These feeds can infect fish if thermal treatments are insufficient [82]. These agents can also remain in frozen fish until 6 months [83]. Some of these microorganisms have also been isolated from elements of the aquatic environmental such as mud, sediments and water from fish farms [13,84].

This group of agents can be isolated from several sites not related to aquaculture. The presence of *L. garvieae* has been reported in factories of lactic products and in bovine farms, mainly involving cases of mastitis [7,85]. *L. garvieae* has also been isolated from cat and dog tonsils, although currently it has not been related to transmission to fish [18]. In addition, distribution of the disease in rainbow trout from South Africa has been correlated with the presence of leech species (*Batracobdelloides tricarinata*) and it can be inferred that the leeches could act as a reservoir of the agent or as a vector [86].

7.2. Transmission mechanisms

Transmission of the disease is mainly by horizontal mechanisms. Direct transmission between fish that live in the same ponds is most important, through the water, especially if fish injuries exist, or by the feco-oral route [69]. It has been reported that rainbow trout experimentally infected by the intraperitoneal route eliminated the agent by feces 72 h post-infection [67]. Moreover, carrier status has

been described in both susceptible and non-susceptible fish species, which can also be important in spreading the disease [69]. Also, it is necessary to consider direct routes such as feed or fish that compose the feed in fish farms [83].

8. Diagnosis

8.1. Epidemiological and clinical

Epidemiological and clinical diagnosis is based on the observation of the characteristic symptoms and lesions of the disease. In continental aquaculture farms, it is possible to observe several infection processes that cause a similar symptomatology. Therefore, it is always important to confirm the diagnosis with laboratory methods that allow the etiological agent to be identified.

Epidemiological diagnosis is based on the study of environmental parameters that increase the likelihood of the disease, such as an increase in water temperature or its poor quality. Lactococcosis usually causes acute outbreaks that affect a large number of fish, and can produce mortalities between 10% and 80%. Fish present clinical symptoms such as lethargy, anorexia, melanosis, erratic swimming, uni- or bilateral exophthalmia, hemorrhages in the ocular zone, perianal area, fins and anal prolapsus [6,76].

8.2. Laboratory diagnosis

When Lactococcosis outbreaks occur, samples must be taken from diseased fish recently sacrificed or from dead fish maintained under refrigeration. The most appropriate organs are kidney and brain although the agent can also be isolated from liver, spleen, eye, intestine, or blood [76].

The classical culture media used to isolate pathogenic agents in aquaculture such as TSA, BHIA, BA or nutrient agar are generally useful in growing *L. garvieae* [13,40]. Sometimes, selective media like bile esculin agar, that incorporates inhibitor substances, can be used. The optimal temperature for growing *L. garvieae* is 37 °C, which is necessary for approximately 24 h. This temperature can serve as the first step for differential diagnosis with other fish bacterial pathogens that are unable to grow at high temperature [15].

Classical biochemical tests are the usual methods of identification of this agent, based on its biochemical characteristics. Miniaturized systems of diagnostic API-20 Strep and API-32 Strep consisting of a gallery of different biochemical tests that allow establishment of phenotypic characterization of the agent can also be used. However, different results have been obtained from the same test depending on the culture medium used to isolate the bacterial inoculum and results do not always agree with those obtained with classical biochemical tests [46].

Lactococcus lactis subs. *lactis* is the species most closely related to *L. garvieae*. Both species coincide in the majority of phenotypic characteristics; and the clyndamicin test or PCR techniques are the most useful methods to differentiate one species from the other [35,53].

8.3. Serological techniques

The most used serological techniques for *L. garvieae* diagnosis are slide agglutination using as antigen a pure culture of *L. garvieae* [58,87], fluorescent antibody staining [88], and recently a rapid flow cytometry-based method has been developed, which is useful for detecting the agent in mixed cultures [89].

8.4. Histological techniques

Samples for histopathological diagnosis of *L. garvieae* are obtained from several internal organs such as kidney, liver or encephalon. They must be fixed in a buffered formalin solution (10%) and then must be processed to produce histological sections in paraffin. They must be stained using haematoxillin and eosin technique [90].

8.5. Molecular diagnosis

In recent years, several protocols have been developed for molecular diagnosis of many of the main fish pathogens, demonstrating the usefulness of these methods for fish disease diagnosis.

A PCR-based protocol was developed for *L. garvieae* identification. The authors designed a set of primers, pLG-1 (5′-CATAACAATGAGAATCGC-3′) and pLG-2 (5′-GCACCCTCGCGGGTTG-3′) from the 16S rDNA sequence (EMBL accession no. X54262). The primers were used in a PCR protocol that included a denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min, ending with a 10 min extension step at 72 °C. The specificity of the technique was tested by the analysis of a collection of *L. garvieae* isolated from different geographical origins as well as representatives of other bacterial species responsible for Streptococcosis outbreaks. As results, only *L. garvieae* isolates, regardless of their origin, amplified a fragment of 1100 bp in size, which indicated that the developed protocol was specific for the agent [53].

The utility of this technique for the identification of *L. garvieae* was demonstrated by the analysis of plasma samples from diseased fish. However, the assay failed to detect the pathogen from environmental samples of water from ponds with an active Lactococcosis process [53]. Consequently, another research group developed a similar PCR assay to detect *L. garvieae* based on a set of primers designed from a dihydropteroate synthase gene. These primers amplified a 709 bp fragment from pure cultures of *L. garvieae* and from kidney homogenates of diseased yellowtail, but not from other bacterial agents or tissues from healthy fish [91].

Later, an integrated polymerase chain reaction-based procedure was developed for the detection and identification of species and subspecies of the genus *Lactococcus,* including *L. garvieae.* The method was based on specific PCR amplifications that exploited differences in the sequences of the 16S ribosomal RNA genes of each species, followed by restriction enzyme cleavage of the PCR products. The primers

designed could be used simultaneously, providing a simple scheme for screening unknown isolates [92].

Recently, a multiplex PCR-based method has been designed for simultaneous detection of the main agents involved in warm-water streptococcosis in fish (*L. garvieae*, *S. iniae*, *S. difficilis* and *S. parauberis*). It was effective for the specific detection of the four pathogens not only in pure culture but also from inoculated-fish tissue homogenates and naturally infected fish [93]. In addition, a method based on the ubiquitous and highly conserved single-copy chaperonin 60 gene was developed for differentiating various species of Gram-positive cocci, including *L. garvieae* [94].

9. Treatment

Historically, antibiotics have been used as an effective method to control infections produced by microorganisms from the *Streptococcus* genus in fish [95,96]. However, the indiscriminate use of these substances has led to an increase in antibiotic resistances.

Although some of these substances have demonstrated activity in vitro against *L. garvieae*, they are usually ineffective when used under field conditions, probably because of rapid anorexia in the animals and the appearance of resistant strains [65]. The antibiotics most often used to control Lactococcosis in rainbow trout outbreaks have been erythromycin, oxytetracycline, amoxicillin and low-level doxycycline [97]. The reference strains of *L. garvieae* are sensitive to erithromycin, with a minimum inhibitory concentration (MIC) of 0.12 μg/ml [52]. Also, some sensitivity to ionophores antibiotics has been described. Narasin is the most effective, although its effectiveness has been demonstrated only in vitro [98].

The study of *L. garvieae* strains from different geographic origin showed that all of them were sensitive to enrofloxacyn and nitrofurantoin, and were resistant to oxolinic acid and sulphametoxazol-trimethoprim. However, the results differed with regard to erythromycin, chloramphenicol, oxytetracycline and ampicillin [46].

Recently, outbreaks in Turkey demonstrated that the strains of *L. garvieae* isolated were sensitive to erythromycin, ofloxacin, ampicillin and chloramphenicol, but were resistant to penicillin and clyndamycin [24].

In Japan, oxytetracycline, lincomicin and penicillin have been used with irregular results. The most effective product has been tobicillin, which is an ester derived from penicillin G but is more stable, and which presents a higher concentration in the blood of fish. Its efficacy was tested in vitro with experimentally infected yellowtail and the mortality was 0% in the treated group but was 56% in the control group [99]. Kawanishi et al. [75] investigated the drug resistance and PFGE patterns of *L. garvieae* isolates from cultured *Seriola* (yellowtail, amberjack and kingfish) and observed that 44% of isolates were simultaneously resistant to erythromycin, lincomycin and oxytetracycline, and all resistant isolates possessed *ermB* and *tet*(S) genes as the resistant pattern.

In addition, bacteriophages were investigated as a treatment for *L. garvieae* infections of cultured finfish. Park et al. [100] initially recovered a virulent

bacteriophage of *L. garvieae* from a culture of the bacterium isolated from diseased yellowtail. Initial investigations demonstrated that this was a double-strand, DNA phage with a broad range of infectivity to *L. garvieae* strains but very high host bacterial-species specificity, having no ability to infect 22 other species of aquatic bacteria pathogenic to finfish and shellfish. Park et al. [101] subsequently isolated ten bacteriophages of *L. garvieae* from diseased fish, seawater and sediments by a simple enrichment procedure. These phages varied in their ability to infect *L. garvieae* strains thereby providing a basis for phage typing of *L. garvieae*.

Nakai et al. [102] reported on three of their bacteriophages with respect to the characteristics of environmental and biological stability, fate in the yellowtail and efficacy in the treatment of lactococcosis infections of yellowtail. The efficacy of the *L. garvieae* bacteriophage, PLg-16, in preventing mortality was assessed by two disease models: an injection-challenge to assess the efficacy of injected phage in preventing or reducing mortality; and an anal intubation exposure to assess the efficacy of pre-feeding of phages as a means of preventing the occurrence of disease and mortality. The injection-challenge consisted of i.p. injection of *L. garvieae* and the efficacy of the bacteriophage in preventing mortality was assessed by injecting the phage i.p. either simultaneously with the *L. garvieae* challenge or with a delay of 1 h or 24 h post-challenge. The survival of the yellowtail was 100% when phage was injected concurrently with the *L. garvieae* challenge, 80% with a 1 h delay, 50% with a 24 h delay and 10% with no phage intervention. The data demonstrated a statistically significant reduction in mortality of yellowtail as a result of therapeutic injection of the bacteriophage and also demonstrated that the earlier the intervention the greater the benefit. In contrast, the procedure to assess the potential of prior exposure of yellowtail to an *L. garvieae* bacteriophage to protect against *L. garvieae* infections consisted of feeding dry-pelleted rations containing either no phage, viable phage, or viable phage and *L. garvieae*. The fish were fed the specified rations for 30 min and then challenged with *L. garvieae* by anal intubation. The mortality rate of the yellowtail that did not have prior exposure to bacteriophage in the feed was 65%. Receiving both phage and *L. garvieae* reduced the mortality rate to 20% while a diet containing the phage alone reduced the mortality to 10%. The data indicate that prior exposure to the bacteriophage-protected fish against mortality caused by *L. garvieae* and also demonstrated that it is possible to deliver effective concentrations of the phage orally [102].

Recently, a probiotic has been studied to control lactococcosis and streptococcosis in rainbow trout. A culture of *Aeromonas sobria,* isolated from the digestive tract of rainbow trout, was incorporated into the feed and fed to rainbow trout for 14 days at a dose equivalent to 5×10^7 cells g^{-1} of feed. As result, the untreated controls showed mortalities of 75–100% when were challenged intraperitoneally with *L. garvieae* and *Streptococcus iniae* whereas the group treated with the probiotic remained healthy showing mortalities of only 0–6%. The mode of action was based on the stimulation of innate immunity, such as an increased number of leukocytes and the enhancement of the phagocytic and respiratory burst activity [103].

10. Prophylaxis

Sanitary measures are the first barrier to prevent the introduction of pathogens in the fish farm. Reduction of fish manipulation to a minimum, elimination of dead or diseased fish and maintenance of low densities of culture, constitute the most important measures. In addition, periodic cleaning of the tanks and adequate disinfection of all utensils at the fish farm with products such as formalin, quaternary ammonium, chloramines-T, copper sulfate, hydrogen peroxide or potassium permanganate, decrease the distribution of the agent and its effect on fish [104,105]. It is also beneficial to maintain appropriate microbiological quality at the fish farm, checking the sanitary condition of the water and sediments, and periodically disinfecting all production units [106]. The control of feeding by means of chemical and microbiological analysis is very important because *L. garvieae* has been isolated from fish meal and was viable up to 6-month post-freeze [83].

The purpose of control of fish and egg importations to fish farms is to avoid introduction of the agent and to prevent its dissemination. It is also important to require health certificates guaranteeing that fish are free of Streptococcosis, to carry out microbiological analysis and to maintain fish in quarantine for a period [104,105].

Control of physicochemical parameters is important in preventing the action of *L. garvieae*. The agent begins to cause outbreaks when water temperature increases over 15 °C. Also, low oxygen saturation in the culture tanks lead to major virulence as soon as ammonium concentrations in the water reach high levels [80,81]. Finally, it is important to prevent the entrance of wild ichthyophagous fowls that can act as vectors of the agent [105].

10.1. Vaccines against L. garvieae

Although some chemotherapeutic agents have activity against *L. garvieae*, therapeutic measures usually are ineffective under field conditions. Therefore, vaccination of susceptible populations has become the best option to control Lactococcosis.

Autovaccines have also been developed with strains of *L. garvieae* (inactivated with formalin) isolated from the fish farm where the outbreak was occurring [87,107]. Prieta et al. [15] carried out field trials with this type of vaccines in Spanish fish farms and observed mortalities of 1.7% in vaccinated fish compared to 6.5% in non-vaccinated fish, although they were treated with erythromycin.

In other tests carried out in Israel, protection percentages of 80–90% have been obtained in vitro as well as under field conditions by vaccinating fish intraperitoneally with 0.1 ml [65]. In addition, antiserums have been used to induce protection through passive immunity. Serum from sheep immunized against the strain causing of the outbreak had similar results to those obtained with vaccination, although the period of protection was minor [108].

The use of glycans inoculated intraperitoneally or by the oral route to induce innate protection against *L. garvieae*, resulted in a double level of protection in the

treated group compared to controls, attributed to high activity of a nonspecific immune response and phagocytosis [66].

Vaccination protocol consists of vaccinating fish intraperitoneally one month before water temperature increase over 15 °C. It is important to maintain fish in good health conditions, to reduce stress situations at a minimum and to provide a diet that can stimulate the immune system of the fish [70].

In recent years, adjuvanted vaccines have been developed with different mineral oils. They have been tested in laboratory and field trials, anesthetizing fish and inoculating them intraperitoneally. Complete protection is usually reached 3-week post-vaccination with simple bacterins and 4–5-week post-vaccination with oil-adjuvanted vaccines. Protection remains for a period of 3–4 months with bacterins and 4–5 months with adjuvanted vaccines [68,109]. The optimal time for vaccination is when fish weight approximately 50 g and water temperature is around 12–14 °C [76]. Recent studies evaluating the effect of the inclusion of different adjuvants in the vaccine formulation for rainbow trout have demonstrated that the inclusion of a non-mineral oil adjuvant (Aquamun) yielded a good protection four weeks after vaccination and conferred protection for 8-month post-vaccination, obtaining RPS values of 83.3% [110].

Several studies have been carried out in order to assess oral vaccination with capsulated and non-capsulated antigens as alternatives to the traditional methods of vaccination by the intraperitoneal route, and high levels of protection were obtained by capsulated vaccines with alginate microparticles. However, the use of this method for primary immunization did not guarantee complete protection, although good results were obtained using it as a strategy for booster vaccination against fish lactococcosis [111]. In conclusion, intraperitoneal vaccination with oil-adjuvanted vaccines is at present the most effective method to control Lactococcosis.

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