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Lactococcosis in Reared Fish in Brazil and Control Strategies

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Introduction

Lactococcus garvieae is a Gram positive bacterium responsible for diseases in humans, domestic animals, and fish [1]. This bacterium is an emerging pathogen that could pose a worldwide problem for fish farming, particularly in intensive culture systems [2]. Disease outbreaks caused by *L. garvieae* have been described in freshwater and marine fish species in America, Europe, Asia and Oceania [3], although *L. garvieae* has also been isolated from tropical fish such as Nile tilapia *Oreochromis niloticus* (L.) and spotted sorubim, *Pseudoplatystoma corruscans* (Spix & Agassiz) in Brazil [4]. The authors are however not aware of any clinical information concerning these cases and, to the best of our knowledge, there are no reports of *L. garvieae* outbreaks in other cultured tropical fish species in Brazil.

High water temperature as well as poor water quality in ponds increases the susceptibility towards *L. garvieae* infections. Infections are characterised by the development of hyperacute haemorrhagic septicaemia. The major diagnostic clinical symptoms are anorexia, melanosis, lethargy, loss of orientation, erratic swimming, exophthalmia (uni- or bilateral), ascites, rectal prolapse, as well as haemorrhages in the periorbital and intraocular area, the base of fins, the perianal region, the opercula and the buccal region [3,5]. Since 2010, outbreaks of septicaemia and meningoencephalitis have been observed in several commercial farms of barred sorubim, *Pseudoplatystoma reticulatum* (L.) and hybrid sorubim (*P. corruscans x P. reticulatum*) in Brazil, being a problem to be solved.

Diagnostic-isolation and identification of *Lactococcus garvieae*

In the summer of 2011 outbreaks of septicaemia and meningoencephalitis occurred in two fish farms in Mato Grosso do Sul, Brazil, one of which cultivated barred sorubim and a hybrid sorubim. In both cases the fish were reared in ponds. Five fish showing clinical signs of the disease were sampled from each farm and stored at 4°C prior to transport to a laboratory for bacteriological analysis. Were sampled aseptically by Fukushima et al. [6] swabs of brain and kidney tissue from each fish, streaked onto 5% sheep blood agar, and incubated at 28°C for 72 h. Pure colonies were subjected to Gram staining, followed by catalase and oxidase tests. Isolates were characterized phenotypically, using the API 20 Strep kit (BioMerieux, France). Strains were stored at -80°C in brain/heart infusion broth with 15% glycerol, until use. For the purpose of molecular analysis, the isolates were thawed, streaked onto 5% sheep blood agar, and incubated at 28°C for 24 h. Total bacterial DNA of the strains was extracted using the DNeasy kit (Qiagen), following the manufacturer's instructions. To confirm the identification of bacteria, a *L. garvieae*-specific PCR was performed using primers pLG-1 (5′ CAT AAC AAT GAG AAT CGC 3′) and pLG-2 (5′ GCA CCC TCG CGG GTT G 3′) according to the method described by Mata et al. [7], with some modifications.

Amplification and sequencing of the 16S rRNA gene were performed by Fukushima et al. [6] for three isolates (BR-LG1, BR-LG2, and BR-LG3) that had been randomly selected from the total number of strains isolated. 16S rRNA was amplified by PCR with the universal primers C70 (50-AGA GTT TGA TYMTGG C-30) and B37 (50-TAC GGY TAC CTT GTT ACG A-30) according to the method described in Fox et al. [8]. Sequences were compared to sequences from the NCBI database, using the BLASTn algorithm. The limit fixed for identification of a bacterial species was 98% nucleotide identity for the 16S rRNA gene. The phylogenetic relationships of the isolates were determined by comparative 16S rRNA gene sequence analysis. Sequences of the isolates were aligned in BioEdit using CLUSTAL W [9] with sequences of the following bacterial species: L. garvieae ATCC 49156 (GenBank accession number NC015930.1), *L.garvieae* (X54262.1), *L. lactis subsp. cremoris* (AB181302), *L.lactis subsp. lactis* (NC002662), *Globicatella sanguis* (S50214.1), *Vagococcus fluvialis* (X54258.1), *Enterococcus faecalis* (AF039902.1), *Enterococcus hirae* (AF061011.1), *Enterococcus durans* (AF061000.1), *Enterococcus faecium* (AF039901.1), and *Leuconostoc mesenteroides* (M23035.1). Genetic distances matrix was obtained using Kimura's two-parameter model [10], and an evolutionary tree was created using the neighbour-joining method [11] with Mega5 [12].

To fulfil Koch's postulate, hybrid sorubim fingerlings were experimentally infected with a randomly selected *L. garvieae* strain (BR-LG3), isolated from the second disease outbreak event. Fish (of average weight 10.27 g) were maintained in 57 L aquaria, supplied with flow-through dechlorinated water (2 L h^{-1}) at a temperature ranging from 28° to 30°C, and equipped with a supplementary aeration system. Three fish were randomly collected prior to the challenge assays and submitted to bacteriological analysis and to *L. garvieae*-specific PCR, to ensure that they were free of bacterial infection. Each experimental group (n=6) was kept in a separate glass aquarium. To carry out experimental infection, the isolate BR-LG3 was thawed, streaked onto 5% sheep blood agar, and incubated at 28°C for 24 h. Colonies selected for the challenge assay were inoculated in BHI broth and incubated at 30°C for 18 h under low agitation (150 rpm) until reaching a concentration of 10^7 cfu ml^{-1}. Prior to the challenges, the fish were anesthetized by immersion in a bath containing 10 mg/L of benzocaine (Sigma-Aldrich). Five experimental groups were used in the challenge assay. Members of groups I and II were infected by means of intraperitoneal inoculation with 0.1 mL of *L. garvieae* at a final dosage

of 5.5×10^6 cfu per fish. Fish from of groups III and IV were subjected to intraperitoneal injection of 0.1 mL of sterile BHI. The fifth group was kept under the same conditions, to serve as an experimental control. Fish were monitored four times a day during the 21-day experimental period. Bacteriological analysis was carried out on all dead fish. At the end of the experiments, all surviving fish were euthanized by benzocaine overdose and submitted to the same examination, to check for macroscopic lesions and to determine if these fish were asymptomatic carriers.

Outbreaks in the two farms indicated high mortality rates and similar clinical presentations. The main clinically-verified symptoms were anorexia, lethargy, melanosis, erratic swimming, and skin lesions. During the first outbreak at the barred sorubim farm, juveniles were found to be the most susceptible life stage. In the hybrid sorubim farm, the majority of mortalities were recorded in fingerlings. There was no epidemiological connection between the two cases. The principal predisposing factor for both outbreaks was the increase in water temperature. Five brain isolates from diseased fish were evaluated. These were characterized as Gram-positive cocci that were catalase-negative, oxidase-negative, and non-haemolytic. The API 20 Strep test indicated similar biochemical profiles, but the identifications from this kit were inconclusive. Positive results in *L. garvieae*-specific PCR assay were verified for the five strains, with the amplification of a 1.100 bp fragment. Blast analysis of 16S rRNA sequences of Brazilian isolates presented a 99% similarity in sequences from the *L. garvieae* Lg2 [13] and ATCC49156 strains, the latter having been previously isolated from diseased fish [14]. In the phylogenetic tree, Brazilian strains were grouped in the same cluster as *L. garvieae* isolates ATCC49156 and X54262, indicating 100% of bootstrap percentage. The disease was successfully reproduced in hybrid sorubim. The main verified clinical symptoms were anorexia, lethargy, exophthalmia, and skin darkness. Groups III and IV presented mortality rates of 66% (four fish of each group). The bacterium was recovered from several organs of diseased fish: brain, kidney, spleen, and liver. The two remaining fish from these groups presented clinical signs of disease that were, however, recovered before the end of the experimental period. Positive results, in terms of brain and kidney bacteriology, were obtained for these animals. Neither clinical signs nor mortalities were observed in fish from the control groups during the experimental period. The study of Fukushima et al. [6] represented the first record, from Brazil, of *L.garvieae* infection in commercial farms cultivating tropical catfish barred sorubim and hybrid sorubim. This pathogen is a classical etiologic agent, responsible for diseases in cold-water fish [3]. An increasing number of cases have however recently been reported from tropical fish, such as sorubim Pseudoplatystoma corruscans, and Nile tilapia Oreochromis niloticus, as well as other aquatic animals [4,15]. *L. garvieae* has previously been misidentified in microbiology laboratories, due to its similarity with members of the genus Enterococcus, and because of the previous reliance on phenotypic methods [16]. We were unable to identify the isolates when using biochemical assays, indicating the importance of molecular methods for the correct diagnosis of this pathogen in aquatic animals.

Control Strategies

Despite the increasing number of studies on the diagnosis of bacterial agents in recent years, there are still few alternatives available to control bacterial infections in fish in Brazil. Regarding methods of disease control, the lack of studies is even greater, since there are only two antimicrobials legalized in Brazil, Florfenicol and Oxytetracycline, according to the Compendium of Veterinary Products, and a single commercial vaccine for prevention of *Streptococcus agalactiae* [17]. More studies for strategic control of these diseases are required for the generation and availability of products certified by the competent Brazilian official public agencies, which are still scarce in the country.

In other countries the most used substances in the control of lactococcosis in rainbow trout are erythromycin, oxytetracycline, amoxiline and doxycycline. Among these, only oxytetracycline is allowed in Brazil, and its results are inconstant around the world, such as Japan [18] and Turkey [19]. Antibiotic therapy is not an effective control measure for *L. garvieae* infection [13,20], and losses can exceed 80% of total production [3]. Application of chemotherapeutic agents is effective under experimental conditions, but is ultimately an unsustainable strategy in the control of lactococcosis due to the development and spread of antibiotic resistance [21].

Vaccination of susceptible populations is the best measure to control lactococcosis, and several studies have been conducted to develop an appropriate vaccination strategy against this disease. In addition, the immunogenicity of susceptible species has been investigated, and variations in protective responses have been observed among different species of fish, which has important implications for the formulation of a vaccine and the vaccination route [22-26]. On economic, environmental and ethical grounds, disease prevention by vaccination is the most appropriate method for pathogen control that is currently available to the aquaculture sector [27,28]. For the disease to become manageable on fish farms in the foreseeable future, studies are required to ensure the development of appropriate vaccination protocols for each species of fish against the diseases to which they are susceptible.

Nowadays, vaccination is considered the best option to control lactococcosis, due to the poor efficiency of chemo-therapeutic agents under field conditions and the risks associated with the spread of antibiotic resistance [21]. The alternatives investigated in the control of lactococcosis have been intraperitoneal injection of *L. garvieae* bacteriophages in farmed fish [29], utilization of Aeromonas as a probiotic to stimulate innate immunity, increasing the number of leukocytes, improving phagocytic activity and the respiratory activity of leukocytes [30], and the most effective alternative, that is vaccination with formalin inactivated *L. garvieae* strains [3]. Fukushima et al. [6] concluded that administration of the oil-adjuvanted vaccine increased the level of specific antibodies and improved the agglutination ability and survival probability of sorubim exposed to live *L. garvieae*. These results also demonstrated that the oil-adjuvanted vaccine enhanced the potency and duration of protection against L. garvieae infection and, in the future, could offer an appropriate strategy to prevent these infections in Brazilian sorubim farms. Field trials will be necessary to accurately determine the efficacy of the *L. garvieae* vaccine under the conditions in which it will be applied.

Final Considerations

L. garvieae is an emerging pathogen that infects both barred sorubim and hybrid sorubim in Brazil. We recommend that future studies should focus on the genetic diversity of *L. garvieae* as well as the development of prophylactics, to control this disease in Brazilian farms. Bacterial diseases are common in aquatic systems, but adequate management practices and early diagnosis can prevent mortality. Given the economic and health importance of this pathogen, it is necessary to have a better sanitary monitoring in these breeding places,

especially in the detection of the pathogen. In addition, it is fundamental to adopt strategies to control the disease in Brazil, especially through the vaccination of fish, according to what is already practiced in other producing countries. The use of vaccines, while representing more cost to producers, may be an economically advantageous solution, especially considering that, in addition to reducing mortality, fish will be able to better express their growth potential. We emphasize the need of higher number of studies for generation and availability of preventives and curatives products, which are still scarce in the country, certified by the competent Brazilian official public agencies, as alternative to control these bacteriosis.

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