Implementation and analysis of diagnostic techniques for *Mycobacterium spp.* and *Francisella spp.* in granulomatous disease of fish in breeding and wild aquaculture of São Paulo/Brazil

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Abstract— *Mycobacterium spp. and Francisella spp. bacteria have serious implications for Animal Health, Public Health and Agribusiness and yet, in Brazil, there is little knowledge about the best diagnostic techniques to detect and characterize them. Therefore, the occurrence of these bacteria was verified in 519 fish from fish farms (active collection), wild freshwater animals from the State of São Paulo (active collection), and in materials filed in our laboratory (passive collection), using the techniques in situ hybridization (IHS), immunohistochemistry (IHC), optical microscopy (MO) (H&E and (ZN) Ziehl Neelsen or Fite-Faraco), and negative staining for transmission electron microscopy (TEM). Histologically, granulomas were observed in 135 fish. By the ZN Faraco technique, Mycobacterium spp was found in 54 animals. By Immunohistochemistry and in situ Hybridization, 46 fish were found infected with Mycobacterium spp, 40 with Francisella spp and 30 with both bacteria. In one of the animals the presence of granulomas was found, although not caused by Mycobaterium spp or Francisella spp. TEM also showed the presence of other bacteria, protozoa, and viruses. The aim of this study was to evaluate the best diagnostic techniques for Mycobaterium spp or Francisella spp in fish fragments.*

Keywords— Francisella spp; Mycobacterium spp, fish disease.

I. INTRODUCTION

There are several etiological agents of acute, chronic, granulomatous, systemic, or focal diseases in animals worldwide. In aquaculture, in fish, reptiles, amphibians and crustaceans, bacteria have caused great losses in production, due to the death of the infected animals or the bad aspect of the sick ones, that makes commercialization unfeasible [1]. In addition, according to Miller and Neely [1], and Wang et al. [2], many of the bacteria pathogenic to aquatic animals are zoonotic, affecting the public health. The growing expansion of consumption in national and international markets since 1990 for various fish, especially tilapia, led to new cultivation strategies, focused especially on the increase of the stocking density and the use of formulated feeds, characterized by a marked reduction in natural food. Therefore, it is necessary to know the bacterial agents, their pathogenesis, which includes the parasite-host-environment relationship, so that new effective protocols can be used, to avoid the dispersion and circulation of these bacterial agents [3].

When tracking the first responses to bacterial infection, the immediate immune response, the knowledge comes that the macrophages residing in the tissue are the cells that first contact the infecting bacteria. In the case of *Mycobacteria* [4], the macrophages residing in the first response, phagocytize and eradicate mycobacteria, suggesting that, in order to establish a successful infection, the mycobacteria must escape from the initially infected resident macrophages, or cause macrophage apoptotic death and go to the monocytes thus permitting growth. Some cytosolic pathogens, to prevent antimicrobial autophagy, invoke specific mechanisms, such as altering its surface when recruiting proteins from the host, as is the case of

Francisella, a cytosolic pathogen that rapidly breaks down its phagosome and resides and successfully proliferates in the cytosol, for an extended period of time, without triggering an autophagy response [5].

1.1 *Mycobacterium* Spp.

Mycobacterium spp. is a pathogen capable of causing serious and costly diseases in many invertebrates such as crustaceans [6] and vertebrates, such as humans (tuberculosis, leprosy, Buruli ulcer), livestock (bovine tuberculosis) and ectothermic animals (reptiles, amphibians and fish) [7, 8, 9, 10, 11, 12, 13]. In recent years, due to the decrease in fishing activities, there has been an increasing interest in fish farming, and this increase in farms has favored the development of diseases such as mycobacteriosis and franciselosis [14].

Mycobacterium marinum, *M. fortuitum* and *M. chelonae* are the main agents of the disease, called mycobacteriosis or tuberculosis of wild or captive fish, and they are a part of 120 or more species of mycobacteria [15]. In marine ornamental fish and inland water fish these diseases are relatively common [3, 16, 17, 18]. It is a zoonosis because some species of fish mycobacteria are potentially capable of infecting humans [19, 20].

In fish, the severity of the infection varies from chronic, with no major tissue changes, in which a few fish die, to conditions of severe and acute infections, with high mortality [5].

In humans, M. marinum causes skin lesions such as pool granulomas [21, 22]. This disease is associated with aquatic activities such as swimming, fishing, managing aquariums, sailing, fish bites, fin wounds, and cleaning tanks and aquariums [21]. M. fortuitum can cause severe injuries such as lung diseases [23, 24].

Microscopically, we generally observe the formation of granulomas. Epithelioid granulomas, or immune granulomas, are characteristic of insoluble particles, typically microorganisms that can induce an immune response. Their center can be filled with caseous necrosis. Macrophages phagocytize such agents and present antigens to T lymphocytes. Their role is to prevent the spread of these agents, and they reveal them to the giant Langhans cell. Macroscopically we have a variety of lesions such as external ulcers, exophthalmos, weight loss or can even occur without symptoms, and this happens when the infection is acute. They mainly appear in the spleen, kidney and liver, in the form of whitish gray areas that can coalesce. As a result of all that, the granuloma is composed of macrophages, epithelioid cells, giant cells, and it is surrounded by T lymphocytes and, in some cases, plasmocytes. The older ones develop a fibroblast capsule and connective tissue. The mechanism of granuloma formation has not been fully clarified [4].

1.2 *Francisella Spp.*

Recently, the bacterium of the genus *Francisella spp*, an emerging pathogen and infectious agent extremely virulent for several animal species, has been found in marine and freshwater fish, amphibians, reptiles and even mollusks. It has been associated with massive tilapia mortalities in commercial farms in Taiwan, Hawaii and Costa Rica [25, 26, 27, 28], and, with breeding casualties between 5% and 80%, with an average of 50%. In 2005, this bacteriosis, initially confused with the disease caused by Piscirikettsia bacteria (common cause of septicemia in salmonids), decimated the tilapia stocks of one of the main producers and exporters of fresh fillets to the United States, Aqua Corporation in Costa Rica [27].

The *Francisella* genus, of the *Francisellaceae* family, is composed of non-mobile, gram-negative, strictly aerobic bacteria, and facultative intracellular coccobacilli [29] and comprises three widely known species, *F. tularensis, F philomiragia*, and *F. novicida*. Some authors consider the species *F. novicida* as a subspecies of *F. tularensis*, since it is being divided into three subspecies, *F. tularensis spp tularensis, holorctica* and *mediasiatica* [30, 31, 32]. The species *F. philomiragia* is divided into *F. noatunensis* spp *noatunensis* and *F. noatunensis spp orientalis* [33, 34]. Some authors do not consider it as a zoonotic factor and, therefore, apparently without the risk of people becoming contaminated [31]. Others consider *F. tularensis spp tularensis*, found mainly in North America, as the most virulent for animals and humans [30, 35]. Considering the apparent link between *F. tularensis* and aquatic environments, fish and amphibians have been considered likely reservoirs [36].

Clinical signs, not specific to this bacteriosis, include loss of appetite, pallor, lethargic behavior, and erratic swimming. Focal hemorrhagic areas, loss of scales, erosion of the epidermis, exophthalmos, renomegaly and splenomegaly can be observed [28].

Internally, a more specific sign of the disease is observed, which is the presence of many white nodules in the gills, with epithelial hyperplasia and whitish nodules in the spleen, kidney, and gonads. These nodules have occasionally been noticed also in the liver and the heart. The lesions contain a large number of cocobastonetes which accumulate in cellular cytoplasm and, therefore, the presence of focal and diffuse necrotizing vasculitis, particularly in the spleen and kidney, is common, resulting in chronic inflammation and granuloma formation [25].

The primary target cells for *Francisella spp* in vertebrates are phagocytes [37], epithelial cells and dendritic cells [38, 39]. Phagocytes are important in the initial control of infections by internalizing pathogens and in the formation of phagolysosomes that eventually degrade this content. Meanwhile, intracellular bacteria, including members of the genus *Francisella*, have developed resistance to this phagolysosome degradation. Golovliov et al. [20], Clemens et al. [5], Santic et al. [40] and Birkbeck et al. [41], noticed that the tilapia affected in fish farms in South America, showed intramuscular lesions, significantly affecting the processing of the carcasses. Up to 30% of the fish fillets from the affected stocks showed dark granulomatous lesions.

Other granulomatous diseases in fish can be caused by *Nocardia spp, Rhodococcus spp., Renibacterium salmoninarum, Citrobacter freundii, Photobacterium damselae, Vibrio spp., Seriola liquifaciens, Edwardsiella tarda, Piscirickettsia spp, and Flavobacterium spp.* [42].

The aim of this study was to investigate these diseases of fish and to evaluate the best diagnostic technique.

II. MATERIALS AND METHODS

From a total of 519 fish, both male and female, which were randomly collected from aquaculture farms (active collection), wild freshwater animals from the State of São Paulo (active collection) and from materials filed in our laboratory (passive collection), and with the use of *in situ* hybridization (IHS), immunohistochemistry (IHC), optical microscopy (MO) (H&E and Ziehl Neelsen or Fite-Faraco), and negative staining for transmission electron microscopy (TEM), we managed to identify the bacteria when they were present. Samples of spleen, hepatopancreas, kidney and gills were fixed in 10% neutral buffered formalin or frozen. The sampled fishes varied in length from 10 cm to 52 cm. Granulomas were macroscopically and histologically observed in the samples from 135 fish (1 and 1a).

We follow all the Ethical principles in Animal Experimentation adopted by the Brazilian Society of Science in Laboratory Animals(SBCAL / COBEA), by the National Council for the Control of Animal Experimentation (CONCEA) and the Brazilian Guideline for the Care and Use of Animals for Scientific and Didactic Purposes (DBCA): Protocol nº 169/20 about the Project: "Investigação de agentes bacterianos (*Mycobacterium* spp. e *Francisella* spp.) causadores de doenças granulomatosas em pisciculturas comerciais de água doce e marinha no sudeste do Brasil".

(https://www.sbcal.org.br/conteudo/view?ID_CONTEUDO=65;https://www.gov.br/mcti/pt-br; https://www.fc.unesp.br/Home/Pesquisa/diretriz-brasileira-para-o-cuidado-e-a-utilizacao-de-animais-para-fins-cientificos-e-didaticos.pdf); https://www.gov.br/mcti/pt-br) [43].

2.1 H-E Technique

Serial sections were prepared from the fixed material: fragments embedded paraffin. 5µm sections were cut using a microtome and adhered to the glass slides and stained by hematoxylin-eosin.

2.2 Fite- Faraco Ziehl-Neelsen technique (Z-N)

(We used Fite-Faraco staining protocol since the classic staining protocol of Ziehl Neelsen may result in false negatives). Serial sections were prepared from the fixed material: fragments embedded paraffin. 5µm sections were cut using a microtome and adhered to the glass slides. The sections will be de-paraffinize in a solution composed of two parts of xylol and one part of peanut oil (or almond oil) for 15 minutes. The sections are then washed in tap water to remove the remaining xylene / oil mixture. Filter on carbol fuchsin solution, DO NOT HEAT, for 20 min. Wash in running tap water. Differentiation will be done by means of 10% sulfuric acid for 2 minutes. Wash well in running tap water, rinse distilled water. Counterstain in 0.25% methylene blue for 20 seconds. Wash and blot dry. DO NOT DEHYDRATE IN ALCOHOL. Clear in xylene. Repeat the blotting-xylene treatment until section is clear. Mount in a DPX type mountan [44].

For transmission electron microscopy (TEM), a negative staining technique was used. The samples were suspended in 0.1M phosphate buffer pH 7.0 and placed in contact with metal grids previously coated with collodion and carbon film drained with filter paper. They were negatively contrasted with ammonium molybdate to 2% and pH 5.0 and observed using a Philips EM 208 TEM [45, 46].

2.3 Immunohistochemistry Technique

Sections of the organ fragments were submitted to Tris HCl 10% for melanin removal. 5 µm thick, were deparaffinized and rehydrated. Antigen retrieval was performed at room temperature, by applying 100 µl of Proteinase K (Dako - S3020) for each cut for 5 minutes, followed by a wash with distilled water. The blocking of endogenous peroxidase, aimed at the minimization of unspecific reactions, was obtained with 200 µl of hydrogen peroxide, 10 volumes at 3% in distilled water for 20 minutes. Sections were then rinsed with distilled water, followed by a wash with phosphate buffer (0.1 M PBS). Thereafter, 100 µl of primary antibody policlonal for anti-*Mycobacterium marinum* e anti-*Francisella* (Sinapse Biotecnologia Ltda), were diluted at 1:500. The Dako background reducing components (Code S3022) were applied at room temperature for each cut and incubated in a humid chamber for 18 hours in a refrigerator (2-8ºC). After this period, two washes with phosphate buffer (0.1 M PBS) for 1 minute were performed. The visualization system used was the LSAB® + System-HRP (Dako-code K0690), following the protocol recommended by the manufacturer. The incubation time was 20 minutes, at room temperature, for each of the reagents, alternated with two washes with phosphate buffer (0.1 M PBS) for 1 minute. The substrate chromogen system used was the Liquid DAB+ Substrate Chromogen System (Dako - code International. with 5 min incubation at room temperature, followed by a rinsing in distilled running water. The counterstaining was performed with hematoxylin. Negative checks were due to the lack of addition of antibody fragments of healthy fish gills, liver, spleen or kidney [47].

In situ* hybridization** The organ fragments were collected and fixed in 10% formalin for 36-48 hours, dehydrated with an increasing concentration of alcohols (70º, 80º, 95º and absolute), diaphanized with xylene and placed in a paraffin bath in a stove at 58ºC overnight. The 4 µm thick cuts were placed on marked slides and kept at room temperature. Prior to use, they were deparaffinized with xylene and rehydrated in decreasing concentration of alcohols (absolute, 95º, 80°, 70°) and distilled water. For antigen recovery, a pretreatment was performed, using a hot bath at 96º C and diluted buffer (Dako S1699) for 40 min. When the slides with cuts were cooled, the endogenous peroxidase was stopped at room temperature for 20 min. and, next, the enzymatic digestion of the tissues, with proteinase k (Dako) at room temperature for 5-15 min, was performed. The specific biotinylated probes were mixed, (Francisella spp***. [48]: Primers FLB16S180f: 5'-GCG-GATTAA- AGG-TGG-CCT-TTG-C-3' (forward primer) e FLB16S465r: 5'-CCT-GCA-AGC-TAT-TAA-CTC-ACAGG-3' (reverse primer). Modification of 5´ biotin (Invitrogen) e ***Micobacterium spp*** [49]: Primers specifically amplified: fragments 924-bp, T-39 (5´-GCGAACGGGTGAGTAACACG-3´) e T-13 (5´-TGCACACAGGCCACAAGGGA-3´ Modification of 5´ biotin (Invitrogen), including specific target DNA on the organ fragments, and a cover glass was laid on them. Samples from these fragments and probes were denatured and hybridized overnight (18 hours) in a Dako hybridization system (denaturation at 96ºC and hybridization at 37ºC). After stringency, a wash with TBST (Tris-buffered saline / Tween) was performed. The visualization system used was the primary streptavidin in a diluting buffer (Dako – Kit cod. K0690) for 30 minutes in a humid chamber, Biotinyl Tyramide reagent for 15 min, at room temperature and then the secondary streptavidin for 15 min. All the procedures were alternated with two washes of TBST buffer for 5 minutes. The substrate-chromogen system used was the Liquid DAB+ Substrate Chromogen System (Dako - code. K3468), and incubation was performed for 5 minutes at room temperature, followed by a wash in distilled running water. Counter-staining was performed with hematoxylin [50].

III. RESULTS

Of the 519 fishes examined, 54 were positives to *Mycobacterium spp* when stained using the Fite-Faraco Z-N technique (figure 2). In the H&E staining, animals presented numerous granulomas (figure 3a and 3b) of numerous sizes, with caseous necrosis in the center, eosinophilic cells and surrounded by inflammatory cells and fibroblasts. It was observed, thus, lymphocytes, neutrophils and heterophiles. Some macrophages alone or in groups, were filled with golden-yellow substance (melanomacrophage) next to the granulomatous or degenerative lesions. The most severe changes were observed in the

kidney that showed convoluted tubules in vacuolar degeneration or necrosis. Glomeruli were also visualized in degenerating, necrotic or deformed, hypo- or hyperplastic and presenting increased Bowman's space and nephrocalcinosis.

By immunohistochemistry and *in situ* hybridization, we found that 46 animals presented *Mycobacterium* spp, 40 presented *Francisella* spp, and 30 presented both bacteria (Figs 4, 5, 6 and 7). By TEM we observed the presence of mycobacteria in 28 fish and *Francisella* in 23 fish, out of the 79 fish in the active collection. Among these 79 fish, 17 presented both bacteria and one of the animals presented a granuloma, not caused by *Mycobaterium* spp or *Francisella* spp. TEM also showed the presence of other bacteria, protozoa, and viruses. (fig 8 and 9).

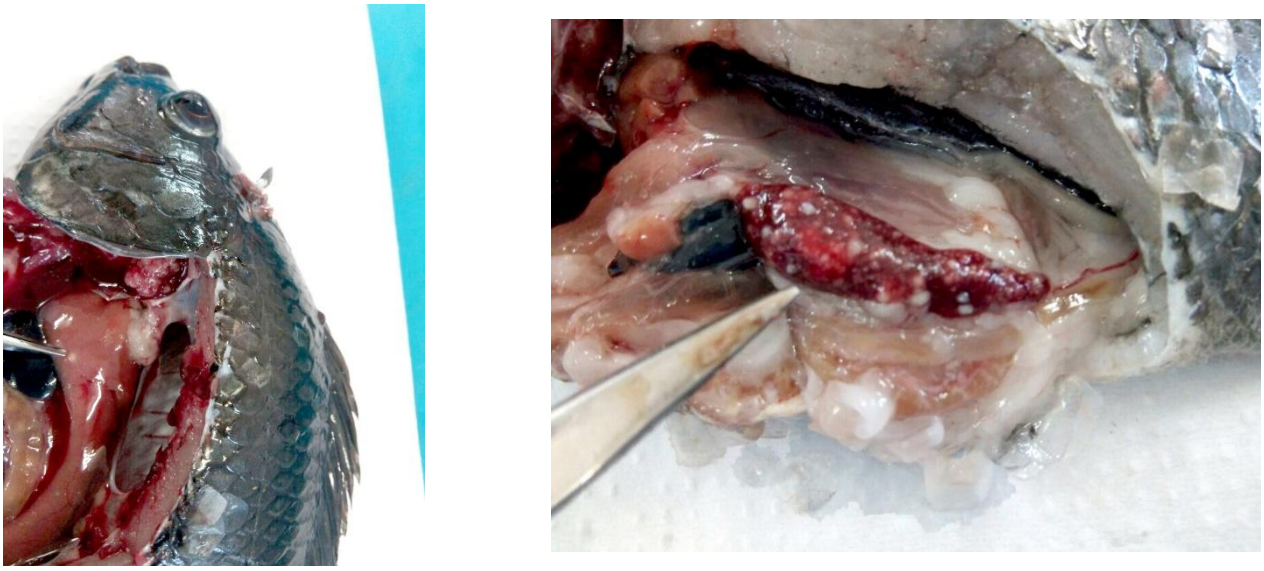

FIGURE 1: Presence of macroscopic granulomas indicated at the end of the forceps

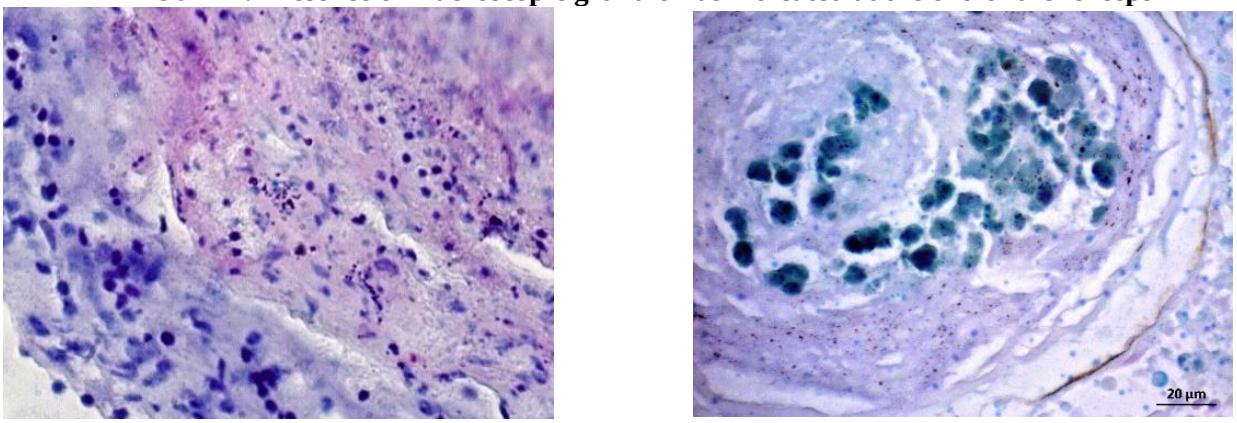

FIGURE 2: Hepatopancreas photomicrograph showing numerous red mycobacteria (small dots) in a granuloma using Fite-Faraco Ziehl Neelsen staining. X400, X200

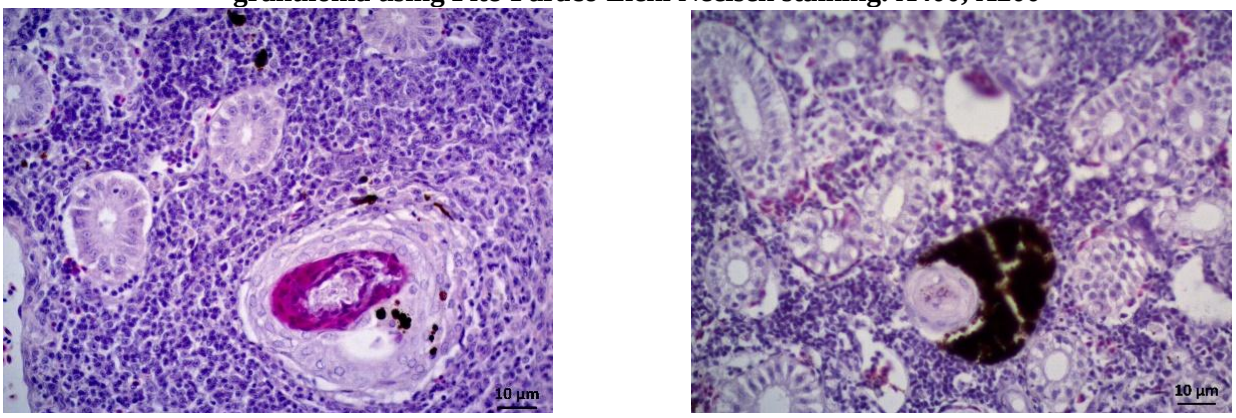

FIGURE 3: In the H&E staining, animals presented kidney granulomas (figure 3(a)) of numerous sizes. In figure 3b a melanomacrophage surrounding or encompassing a kidney granuloma X400

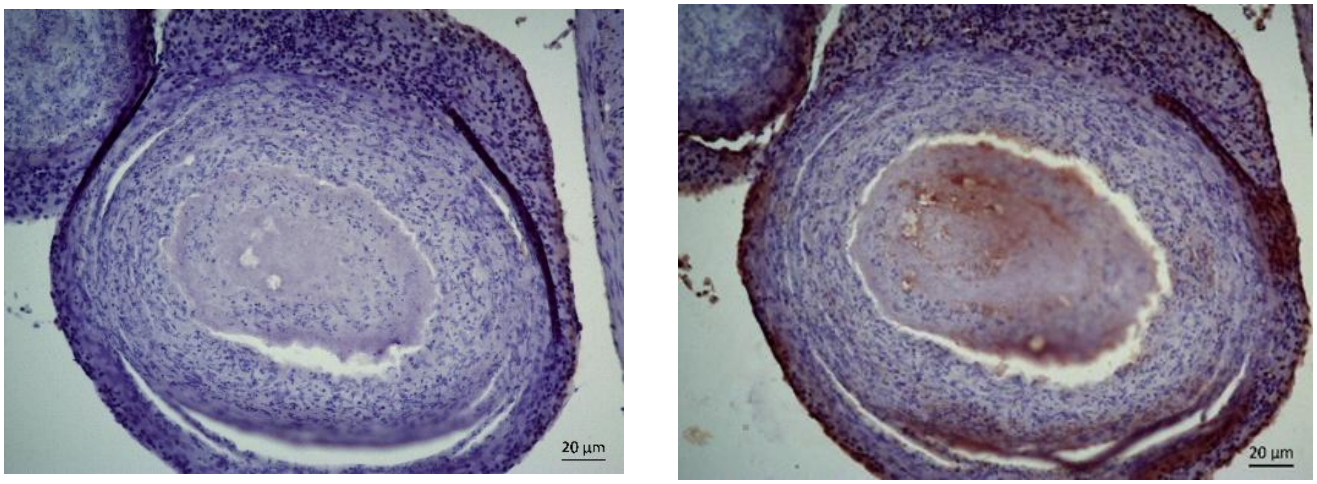

FIGURE 4: Slide photomicrograph by immunohistochemistry and negative for Mycobacterium spp. X200 and Figure 4a Positive immunohistochemistry for *Francisella* spp spp. X200

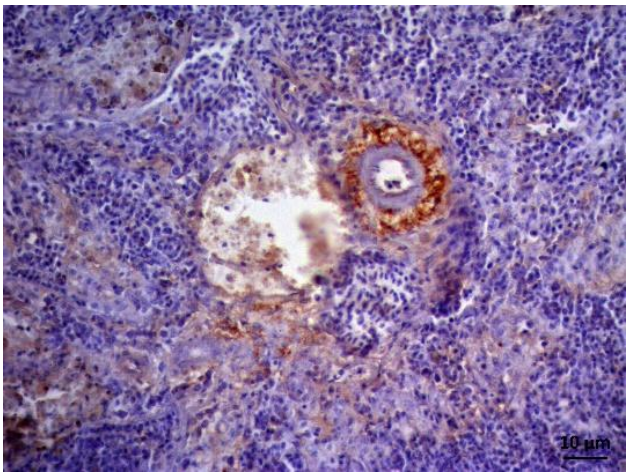

FIGURE 5. Photomicrograph of immunohistochemistry positive for *Mycobacterium* spp in kidney X630.

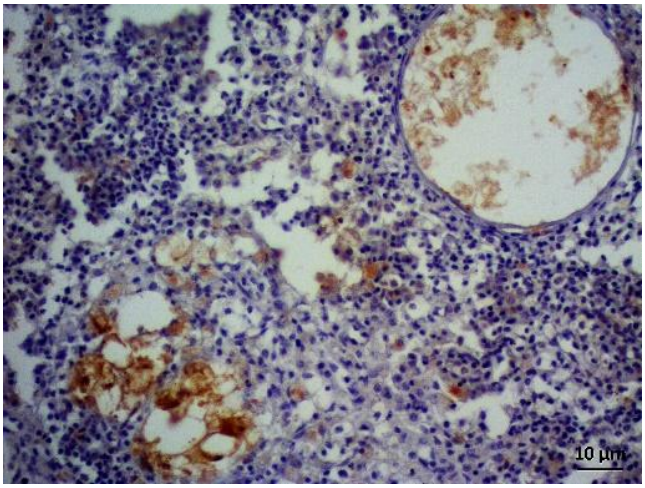

FIGURE 6. Photomicrograph of positive spleen for *Francisella spp* with the *in-situ* hybridization technique X 400

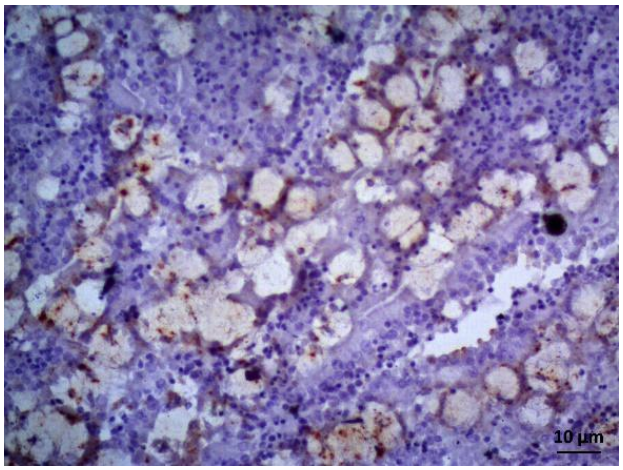

FIGURE 7. Photomicrograph of positive kidney for *Mycobacterium* spp with the *in-situ* hybridization technique X 400

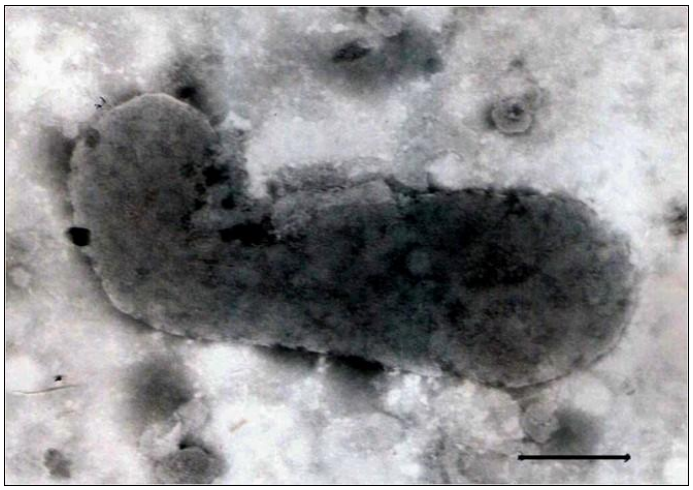

FIGURE 8. Electron micrography of *Mycobacterium spp.*, contrasted by ammonium molybdate in the negative staining technique. Bar: 100 nm

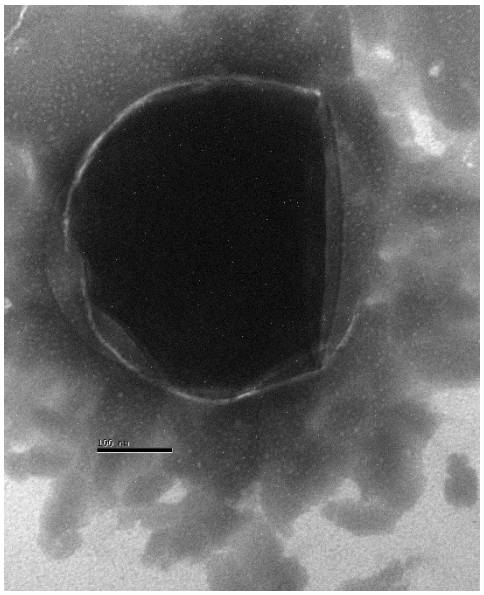

FIGURE 9. Electron micrography of *Francisella* spp., Contrasted by ammonium molybdate in the negative staining technique. Bar: 100 nm

IV. DISCUSSION

Brazil has enormous potential for animal and fish farming production, given its vast land, water sources, and favourable weather conditions. In Brazil, fish are typically bred in lakes, rivers, and the sea, to be used as food. Those used to carry on this study, though, were not intended for consumption.

High concentrations of fish can favour the onset of epizootic disease outbreaks, caused by *Mycobacteria spp* or *Francisella spp*, although in natural environmental conditions spontaneous disease outbreaks can also occur [19, 51]. Therefore, *Mycobacterium* spp and *Francisella* spp have serious implications for Animal Health, Public Health, and Agribusiness, and, still, in Brazil, there is little knowledge about the best diagnostic techniques to detect and characterize them.

This study was first focused at the diagnosis of bacterial diseases in fish, the behavioural changes of the affected animals, the clinical signs and the mortality rate and, if possible, the bacterial isolation through molecular and conventional microbiological methods, considering the characteristic disease and the peculiarity of each agent.

The culture and identification of *Mycobacteria* spp or *Francisella* spp, however, is hampered by its fastidious nature, co-infection with other fast-growing bacteria, low levels of microorganisms in tissues and/or submission of inadequate samples to the diagnostic centre [52, 53].

Several fish bacterial diseases can present in a similar way to francisellosis and mycobacteriosis, commonly characterized by granulomas in multiple organs, usually macroscopically visible.

In the present study, out of the 519 fish examined, 135 were found positive for granulomatous inflammatory disease, caused by unknown organisms, through routine histopathological analysis (H&E). Histopathological examinations are important for early diagnosis of infection in fish. Granulomas (fig 7.8) are suggestive of mycobacteriosis or francisellosis but are not pathognomonic of the diseases. The genera Nocardia, Edwardsiella, Photobacterium, Piscirickettsia, Renibacterium and streptococci are etiologic agents also inducing granulomatous diseases in wild and cultured fish species worldwide [42, 43].

Thus, histopathological diagnoses in tissue fragments, using routine hematoxylin-eosin staining, are particularly important because they allow a morphological evaluation. In 54 animals, a Fite-Faraco Z-N technique revealed the presence of Mycobacterium, eosinophilic small pleomorphic bacteria within vacuolated macrophages located in areas of moderate to severe chronic inflammatory cell infiltration or in the necrotic central area of granuloma. However, the use of the histochemistry, Fite-Faraco Ziehl Neelsen, was complicated. Some results generated doubts and, therefore, for the final diagnosis it was not considered the most conclusive. The same difficulty in this histochemical diagnosis was mentioned by Toenshoff et al. [53], who had observed that mycobacteria can be refractive to culture and are not always readily observable in histological preparations, even when stained by Ziehl-Neelson [54].

The difference in sensitivity between Z-N and IHC may be explained by the Z-N technique detecting only "perfectly preserved" organisms, whereas IHC detects mycobacterial antigens in fragments of living or dead organisms, even with "defective" cell walls [55].

Electron microscopy (TEM) corroborated the results obtained by the IHC and HS, by allowing the visualization of the microorganism and becoming more efficient when associated with antibodies linked to the colloidal gold technique. The main advantage of TEM is the ability to analyze the interior of the sample, the subcellular ultrastructure. There are several drawbacks to the TEM technique. Many materials require extensive sample preparation to produce a sample thin enough to be transparent to electrons, making TEM analysis a relatively time-consuming process, with a low volume of samples. The sample structure can also change during the preparation process and the field of view is relatively small, increasing the possibility that the analyzed region may not be characteristic of the entire sample.

***In situ* hybridization (IHS)** is a technique with which specific nucleotide sequences are identified in cells or histological sections. These can be DNA or RNA, endogenous, bacterial, or viral. This research technique is being translated into a diagnostic practice, mainly in the areas of genes expression, infection, cytogenetics of interphase, and rapid diagnosis [56].

The great advantage of the *in-situ* hybridization reaction, furthermore, is the possibility of precisely locating a specific gene or its transcripts in a paraffinized or frozen tissue. With PCR, mRNA or DNA can be detected in tissue extracts, however we cannot observe the distribution of transcripts or the DNA of a type of cell population, or in the areas of an adult and/or developing tissue [56].

Immunohistochemistry (IHC) is the technique that combines morphology (it has a histological style remarkably like the one of conventional histology) with antigen-antibody reactions marked with a chromogen. Between IHC and IHS, the latter still has the advantage of being ten times less expensive, that is, monoclonal or polyclonal biotinylated antibody is ten times more expensive than a biotinylated probe.

Another great advantage of these techniques is the possibility of retrospective studies because Hybridization and Immunohistochemistry are performed in histological sections, fixed in formalin and paraffinized, therefore, like those present in archival materials or museums [52], also reported that the current availability of highly specific and sensitive molecular diagnostic techniques facilitated the detection, description, and characterization of many previously unidentified or misdiagnosed pathogens from archived samples.

Therefore, the use of molecular techniques in the diagnosis of fish diseases by infectious agents, mainly bacteria and viruses, provided a significant improvement in the efficiency and quality of the diagnosis of these pathologies.

The benefits resulting from the association of these more refined technologies with conventional Clinic and Pathology are unquestionable, and, as a result, our knowledge about some diseases is admirably increasing. Consequently, it is always necessary to associate the findings by molecular techniques with the isolated clinical history and the macroscopic and microscopic findings [57, 58].

V. CONCLUSIONS

In general, molecular techniques are more accurate, sensitive, and specific, and may result in positive predictive values and negative predictive values remarkably high, depending on the prevalence of the diseases researched in the population studied. The results indicate that both assays, alone or in combination, constitute sensitive tools for initial, rapid diagnosis of mycobacteriosis or francisellosis in fish.

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