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Comparative Study of Ziehl Neelsen's Technique and the Fite-Faraco Technique in the Histopathological Diagnosis of Mycobacteriosis in Fish

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Authors' contributions

This work was carried out in collaboration among all authors. Authors LAR and VFP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MCK managed the analyses of the study and authors AFFM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Mycobacteria are widespread in the natural environment, both in terrestrial and aquatic environments, and some species can be zoonotic. In aquatic organisms, especially in fish and crustaceans, they are diagnosed with certain frequency. Taking into account that mycobacteria affecting fish, as well as the species causing leprosy, have less mycolic acid, the authors used the Fite-Faraco (FF) technique in this work and compared it with the classic Ziehl Neelsen (ZN) staining. In the Laboratory of Immunology and Pathology of Aquatic Organisms (LIPOA), the authors chose from their tissue archive tissue material belonging to 10 specimens of fish of different species, belonging to LIPOA, which were reported cases over a period of 15 years (from 2002 to 2017). The

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specimens had in common the presence of lesions classified as granulomatous inflammation, but with the ZN technique it was not possible to obtain a definitive diagnosis of mycobacteriosis. The authors observed that in tissues from fish infected with Mycobacteria, the FF technique was more useful, since the presence of bacilli can be observed, where previously the ZN technique was used and no bacilli were observed.

Keywords: Mycolic acids; fish; fite-faraco; histopathology; mycobacteriosis; ziehl neelsen.

1. INTRODUCTION

Mycobacteriosis is a generic term describing diseases caused by a group of bacteria known as mycobacteria, which are widespread in the natural environment, both in terrestrial and aquatic environments. A small fraction of mycobacterial species causes diseases in animals and humans, turning into a zoonosis [1,2].

In aquatic organisms, especially in fish and crustaceans, mycobacteria are diagnosed with certain frequency [3,4].

The genus Mycobacterium contains many species of bacteria that cause diseases in animals, most incidences of mycobacterial diseases in fish occur by *M. marinum*, *M. fortuitum* and *M. chelonae*. Among them, *M. marinum* has been reported in tropical freshwater and marine fish, and *M. fortuitum* in tropical or temperate water fish. *M. chelonae* has been associated with diseases in Pacific Salmon [5]. A recently described mycobacterial species, *Mycobacterium shottsii*, is the type most commonly associated with the current mycobacterial outbreak in *Morone saxatilis* in Chesapeake Bay in the United States of America [6].

In humans, tuberculosis produced by *Mycobacterium tuberculosis* and leprosy caused by *Mycobacterium leprae* are the global diseases, old in time and still produced today by this genus of bacteria [7,8]. In addition, in humans, a number of infections called "atypical" or non-tuberculous mycobacterial infections can be found, which are those produced by mycobacteria that affect other species of animals, including those affecting fish [9,10].

Mycolic acids are broad fatty acids found in the cell walls of mycobacteria. The presence of these mycolic acids on the cell wall is a distinct morphological trait of these bacteria. Mycolic acids were first isolated [11] from the *M. tuberculosis* extract [12].

The presence of mycolic acids in these bacteria generates greater resistance to medical treatment, since they hinder the effective activity of hydrophobic antibiotics [13]. Additionally, the mycolic acids allow the bacteria to remain unharmed inside macrophages, effectively hiding themselves from the host's immune system [14,15].

The staining for resistant acid-alcohol bacilli, developed by Paul Ehrlich, is based on detecting the presence of mycolic acids for the identification of *Mycobacterium tuberculosis* [16]. Similarly, the staining of Ziehl Neelsen (ZN), which is universally used in tissue samples for the diagnosis of tuberculosis, is directly related to the presence of this mycolic acid [17]. Some species of Mycobacterium such as *M. leprae* have less mycolic acid, and since tissue samples are processed with organic solvents, they can generate a negative result with Ziehl Neelsen staining, since these solvents eliminate mycolic acid from bacilli [18,19]. In the case of *Mycobacterium leprae*, techniques have been implemented that do not use pure organic solvents such as xylol, adopting mixtures with vegetable oils, the most common being peanuts. The most known and effective technique is that of Fite-Faraco (FF) [20-23].

Taking into account that the mycobacteria that affect fish, also as the species that causes leprosy, have less amount of mycolic acid, the authors used in this work the technique of FF according to Luna [24], and compared it with the classic ZN staining. The authors observed that in tissues from fish infected with Mycobacterium, the FF technique was more useful, since the presence of bacilli can be observed, where previously the ZN technique was used and its result was negative.

We studied granulomatous lesions in different organs of 10 fish of different species and observed two types of granulomas: in four fish, the authors observed granulomas similar to human tuberculosis and tuberculoid leprosy, and in six fish, non-specific "foreign body" type granulomas. All granulomatous lesions were

negative with the ZN technique, and the FF technique was applied in the same tissues, and of the 10 samples corresponding to the specimens restudied, six were positive with the FF technique, thus establishing the diagnosis of mycobacteriosis. The main objective of this work is to recommend the use of the FF technique when a mycobacteriosis is suspected in fish since the ZN technique can give false negative results.

2. MATERIALS AND METHODS

The authors have chosen from their tissue archive tissue material belonging to 10 fish of different species. These cases were reported over a period of 15 years (from 2002 to 2017). The tissues of all specimens were stained with H-E. These specimens had in common lesions classified as granulomatous inflammation; however, no mycobaterium was identified with the ZN technique, which made it impossible to make a definitive diagnosis of mycobacteriosis. The histological preparations were separated to be reviewed and the presence of granulomatous lesions was confirmed in all cases.

New cuts were made in these fabrics, then they were stained with FF for resistant acid-alcohol bacilli. To perform the FF staining, the sections were dewaxed with two 12-minute exchanges in a solution composed of two parts xylol and one-part peanut oil; they were stained with fuchsin carbol for 30 minutes, washed with running water, bleached with 5% sulfuric acid differentiated into 25% ethanol, and then washed again in running water. Slides were contrasted with Harris hematoxylin were dried and finally assembled with Balsam from Canada.

3. RESULTS

The review of the tissues belonging to the studied fish showed granulomatous lesions in the 10 cases. The typical granulomatous lesion described mainly in tuberculosis is composed of a necrotic center (caseous necrosis), surrounded by multinucleated giant cells with nuclei arranged in horseshoes (Langhans cells), vacuolated macrophages (epithelial or Virchow cells) and outside a crown of abundant lymphocytes.

Four of the granulomas found in the 10 fish species studied were similar to those described in human tuberculosis and leprosy. Langhans giant cells with horseshoe nuclei were observed; vacuolated cells, epithelioid or Virchow's, were found in four cases; and caseous necrosis was observed in isolated foci. Abundant peripheral lymphocytes were also observed in these granulomas. In six of the granulomas reviewed, the histological pattern was different from that of human tuberculosis and leprosy.

The giant cells were not the Langhans type, but the observed nuclei were distributed irregularly throughout the cytoplasm ("foreign body" type cells). In granulomas, epithelial or Virchow's cells were also observed. No necrosis was observed, but few lymphocytes were found at the periphery of the lesion. Isolated eosinophilic cells were seen (Figs. 1 and 2).

Mycobacterium with the ZN technique was not observed in any of the 10 granulomatous lesions, but when the same tissues were submitted to the FF technique, of the 10 negative with ZN, six were positive with FF, and several mycobacteria were observed (Table 1) (Figs. 3 and 4).

4. DISCUSSION

The cell wall of mycobacteria has a high concentration of lipids that make them waxy, hydrophobic and impermeable to routine stains such as Gram staining. They are also acid-alcohol resistant, which makes it necessary to use special techniques to observe them [25].

Mycobacterial cell walls contain mycolic acids. These are ß-hydroxycarboxylic acids with chain lengths of up to 90 carbon atoms. The integrity of these acids is related to the carbon chain length of the mycolic acid [26].

The histopathological findings of cases of mycobacteria in fish reported in the literature vary. Some authors consider that granulomas found in fish are substantially different from those observed in tuberculosis and deny the presence of Langhans giant cells, characteristic of mammalian tuberculosis granuloma [27,28]. Other authors have demonstrated granulomas similar to human tuberculosis and tuberculoid leprosy, with caseous necrosis, Langhans giant cells, epithelial cells, and abundant lymphocytes, which are related to cell-mediated immunity during the histopathogenesis of mycobacterium-generated lesions [29-31]. The authors found granulomas similar to those of tuberculosis and human leprosy in four of the 10 tissues reviewed, while in six cases they found granulomas different from those classically referred to in tuberculosis.

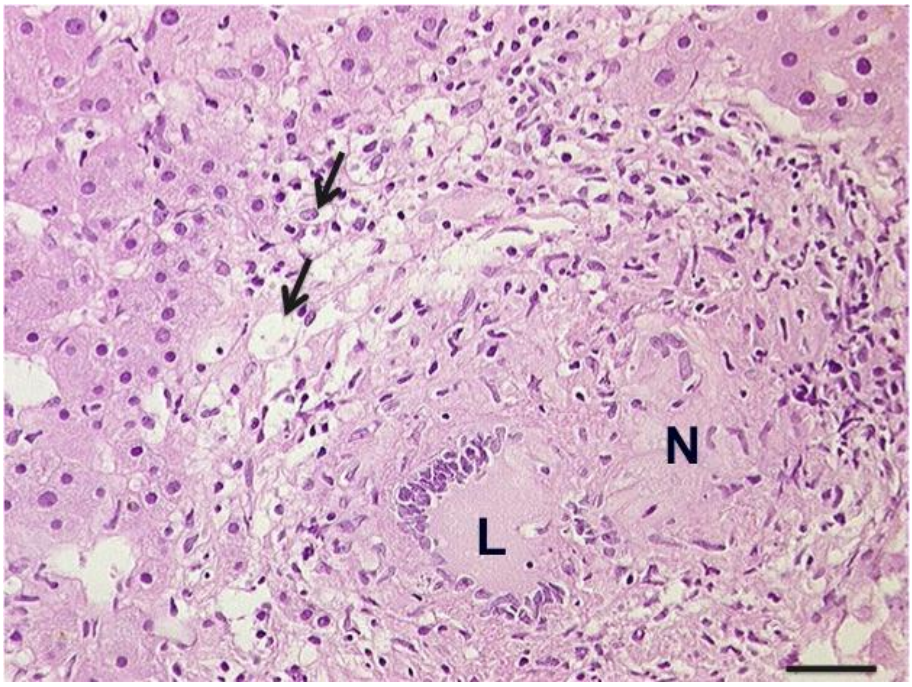

Fig. 1. Granuloma similar to those observed in human tuberculosis with Langhans (L) cells, focal necrosis (N) and Virchow's epitheliolated cells (arrows). H-E, Bar: 50 µ

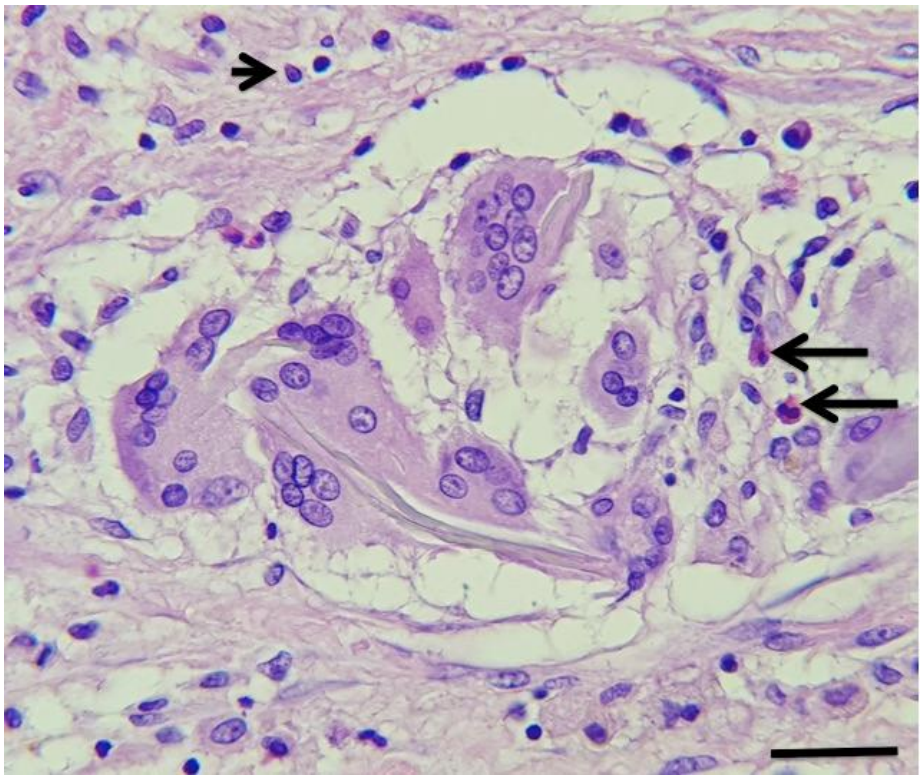

Fig. 2. Non-TB granuloma, with giant foreign body cell, isolated necrosis (N) and abundant lymphocytes (arrow). H-E. Bar: 50 µ

Table 1. Species and tissues studied with H-E, Ziehl-Neelsen and fite-faraco

Species	TS	GL	GT	ZN	FF
Paralichthys orbignyanus	Liver	PR	TG	N	P
Elacatinus Figaro	Whole body	PR	NTG	N	P
Australoheros facetus	Spleen	PR	NTG	N	P
Oncorhynchus mykiss	Spleen and liver	PR	NTG	N	N
Danio rerio	Whole body	PR	TG	N	P
Xiphophorus hellieri	Whole body	PR	NTG	N	N
Lebiste reticulatus	Whole body	PR	TG	N	P
Oreochromis niloticus	Spleen and liver	PR	NTG	N	N
Carasius auratus	Liver	PR	TG	N	P
Salmo trutta	Kidney	PR	NTG	N	N

** TS: Tissue studied; GL: Granulomatous lesion; PR: Present; GT: Granuloma type; TG: Tuberculous-like granuloma; NTG: Nontuberculous granuloma; ZN: Ziehl-Neelsen; FF: Fite-Faraco; P: Positive; N: Negative*

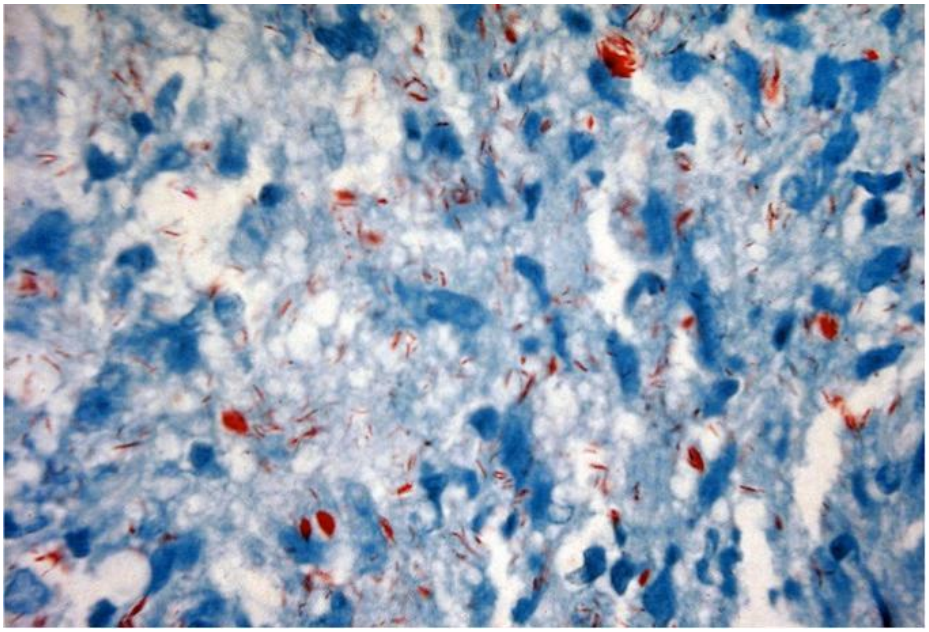

Fig. 3. Acid fast bacilus fite-faraco positive can be observed. Bar: 10 µ

The results show that the FF technique for the diagnosis of mycobacteriosis in fish tissues is more reliable, as it provided positive results in cases where ZN was negative. Some authors show that this high positivity with the FF technique can be attributed to the improvement and precision of the method, which reveals a higher number of bacilli. It is possible that, compared with the routine ZN procedure, the latter is more advantageous for finding leprosy bacilli and atypical or non-tubercular mycobacteria in sections of infected tissues.

Satisfactory demonstration of resistant acid-alcohol bacilli in paraffin-embedded tissue sections is not easily achieved. Some authors have demonstrated the usefulness of FF in the histopathological diagnosis of non-tuberculous Mycobacterium [32,33]. The authors Reja et al. [34] evaluated the diagnosis of *Mycobacterium leprae*, using comparative Polymerase Chain Reaction (PCR), ZN and FF. These authors demonstrated that for ZN, staining the sensitivity was 59.9%; for FF, the sensitivity was 74.6%; and for PCR, the sensitivity was 87.8%, concluding that the combination of staining with FF and the PCR technique provides a rapid and definitive diagnosis in most of the suspected cases of non-TB mycobacteria.

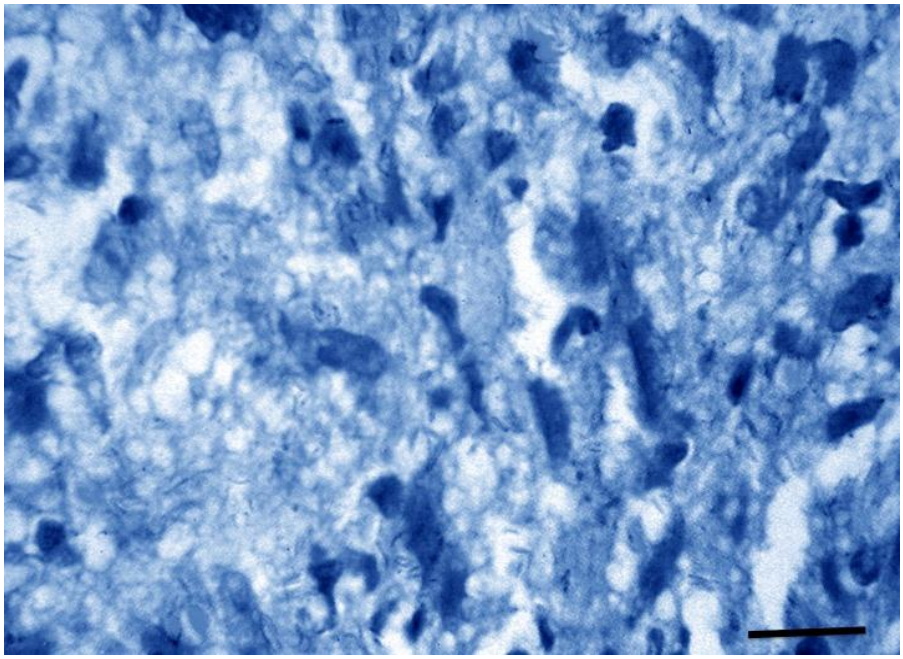

Fig. 4. Tissue sample, exactly similar and same thickness to that of Fig. 3, Ziehl-Neelse negative, Mycobacterium not observed. Bar: 10

FF staining for non-TB Mycobacterium, including that of human leprosy, is related to the fact that the cell wall of mycobacteria has a waxy substance composed of mycolic acids. These are hydroxyl carboxylic acids with chain lengths of up to 90 carbon atoms. The acid strength property is related to the carbon chain length of the mycolic acid. Non-TB mycobacteria, including that of human leprosy and strains affecting fish, have fewer mycolic acids [35]. Dewaxing with the peanut oil / xylene blend helps to protect the most delicate waxy layer from mycobacteria and the residual oil in the sections helps to prevent contraction of this layer. Non-TB Mycobacterium is more easily bleached than TB bacillus and differentiation must be carefully controlled by eliminating alcohol from the hydration and dehydration stages and using 5% sulfuric acid as a bleach in place of the alcoholic solution. Since Harris hematoxylin is used as a contrast in FF staining, the nuclei and bacilli are clearly observed in the cytoplasm. A weak residual red color in the tissues is especially important, and sections that do not present this may not be reliable for diagnosis, so cells and tissue structures are clearly identified when using FF [36,37].

The Ziehl-Neelsen method has been shown to be unsatisfactory for staining acid-resistant organisms in sections of salmonid fish tissue. The phytate-pharmaceutical method of dewaxing sections with a mixture of "light" oil and xylene has been modified using "extra-heavy" mineral oil, discoloration with 3% sulphuric acid and counter-coloration with 0.1% methylene blue. The modified technique Fite-Faraco is the method of choice to stain such organisms.

According to Parisot and Decker [38], the Ziehl-Neelsen method has been shown to be unsatisfactory for staining resistant acid-alcohol organisms in sections of fish tissue. With the Fite-Faraco method, by dewaxing sections with a mixture of vegetable oil and xylol, the lipid layer of the cell membrane is preserved and therefore the authors consider this method to be of choice for staining these organisms.

5. CONCLUSION

The conclusion is that, histopathologically, non-TB mycobacteria, including those affecting fish, produce indistinctly granulomas similar to those of human tuberculosis and tuberculoid leprosy, and granulomas with patterns different from the previous ones. The non-tuberculous or atypical mycobacteria have less and more unstable mycolic acid and tissues when treated with organic solvents almost disappear; therefore, the ZN technique is often negative. On the other hand, the FF technique also uses organic

solvents such as xylol, but because it is mixed with peanut oil, it conserves membrane lipids such as mycolic acid. We believe that it is necessary to use the FF technique when a mycobacteriosis is suspected in fish since the ZN technique can give false negative results.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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