MOLECULAR DETECTION OF MYCOBACTERIA IN FILLETS OF NILE TILAPIA (*Oreochromis niloticus*) FOR HUMAN CONSUMPTION

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ABSTRACT

Fifty fillets of Nile tilapia (*Oreochromis niloticus*) from a fish market in a municipality of Sao Paulo State, Brazil, were analyzed for the prevalence of *Mycobacterium* spp. After fish collection, the fillets were individually stored and identified in hermetic sterile plastic bags, placed in a thermal box with ice and taken to the laboratory. Muscle samples (20 mg) were obtained and evaluated by standard PCR (Polymerase Chain Reaction). The results showed the presence of *Mycobacterium* spp. in 100% of the samples (50/50). Sanger sequencing revealed the presence of *Mycobacterium gordonae* in all samples. Even though *M. gordonae* is generally viewed as having low pathogenic potential in humans, the literature reports infection in immunosuppressed patients, such as in lungs, soft tissues, cornea, peritoneal cavity and, less frequently, disseminated disease, and bacteremia. Thus, the considerably high number of this organism found in this study in a popular dish like tilapia fillets is of great concern. The changes in feeding habits of the population, such as the increase of consumption of raw fish must draw attention of fish farmers and surveillance committees given that suggestive macroscopic lesions are usually absent in the products. Moreover, it is necessary to assess the potential role of *Mycobacterium gordonae* as infection agent when ingested through food for both immunosuppressed and immunocompetent persons.

Keywords: *Mycobacterium* spp., *Mycobacterium gordonae,* Nile tilapia, bacteriosis.

DETECÇÃO MOLECULAR DE MICOBACTERIA EM FILES DE TILAPIA DO NILO (*Oreochromis niloticus*) PARA CONSUMO HUMANO

RESUMO

Cinquenta filés de tilápia do Nilo (*Oreochromis niloticus*) de um mercado de peixes de um município do Estado de São Paulo, Brasil, foram analisados quanto à prevalência de *Mycobacterium* spp. Após a coleta dos peixes, os filés foram armazenados individualmente e identificados em sacos plásticos estéreis herméticos, colocados em caixa térmica com gelo e levados ao laboratório. Amostras do músculo esquelético (20 mg) foram obtidas e avaliadas por PCR (Polymerase Chain Reaction). Os resultados mostraram a presença de *Mycobacterium* spp. em 100% das amostras (50/50). O sequenciamento Sanger revelou a presença de *Mycobacterium gordonae* em todas as amostras. Embora o *M. gordonae* seja de baixo potencial patogênico em humanos, a literatura relata infecção em pacientes imunossuprimidos, como pulmões, tecidos moles, córnea, cavidade peritoneal e, menos frequentemente, bacteremia.

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Assim, o número consideravelmente elevado deste organismo encontrado neste estudo em um prato popular como o filé de tilápia é de grande preocupação. As mudanças nos hábitos alimentares da população, como o aumento do consumo de pescado cru, devem chamar a atenção dos piscicultores e dos comitês de fiscalização, visto que lesões macroscópicas sugestivas geralmente estão ausentes nos produtos. Além disso, é necessário avaliar o papel potencial do *Mycobacterium gordonae* como agente infeccioso quando ingerido através de alimentos, tanto para pessoas imunossuprimidas como para imunocompetentes.

Palabras-chave: *Mycobacterium* spp., *Mycobacterium gordonae*, tilápia do Nilo, bacteriose.

DETECCIÓN MOLECULAR DE MICOBACTERIAS EM FILETES DE TILAPIA DEL NILO (*Oreochromis niloticus*) FOR HUMAN CONSUMPTION

RESUMEN

Se analizó la prevalencia de *Mycobacterium* spp. en cincuenta filetes de tilapia del Nilo (*Oreochromis niloticus*) de un mercado de pescado en un municipio del estado de Sao Paulo, Brasil. Luego de la recolección del pescado, los filetes fueron almacenados individualmente e identificados en bolsas plásticas herméticas estériles, colocadas en una caja térmica con hielo y llevadas al laboratorio. Se obtuvieron muestras de músculo (20 mg) y se evaluaron mediante PCR (reacción en cadena de la polimerasa). Los resultados mostraron la presencia de *Mycobacterium* spp. en el 100% de las muestras (50/50). La secuenciación de Sanger reveló la presencia de *Mycobacterium gordonae* en todas las muestras. Aunque generalmente se considera que *M. gordonae* tiene un bajo potencial patógeno en humanos, la literatura informa sobre infecciones en pacientes inmunodeprimidos, como en pulmones, tejidos blandos, córnea, cavidad peritoneal y, con menor frecuencia, enfermedades diseminadas y bacteriemia. Por lo tanto, la cantidad considerablemente alta de este organismo encontrada en este estudio en un plato popular como los filetes de tilapia es motivo de gran preocupación. Los cambios en los hábitos alimentarios de la población, como el aumento del consumo de pescado crudo, deben llamar la atención de los piscicultores y de los comités de vigilancia, dado que las lesiones macroscópicas sugerentes generalmente están ausentes en los productos. Además, es necesario evaluar el papel potencial de *Mycobacterium gordonae* como agente infeccioso cuando se ingiere a través de los alimentos tanto en personas inmunodeprimidas como inmunocompetentes.

Palabras clave: *Mycobacterium* spp., *Mycobacterium gordonae*, tilapia del Nilo, bacteriosis.

INTRODUCTION

Non-tuberculous mycobacteria (NTM) are present in all types of natural environments like soil, water and aerosols, as well as in potable water distribution systems throughout the world as persistent residents of the microbiota (1). Currently, these organisms have received attention as potential causative agents of infections in humans (2). They can affect a wide variety of animals, including pecilotherms, with several species of mycobacteria causing diseases in fish, reptiles and amphibians. *Mycobacterium marinum*, *M. fortuitum* e *M. chelonae* are the most commonly associated with diseases in fish (3).

However, in recent years, a wide range of *Mycobacterium* spp have been isolated from fish - *M. abscessus*, *M. kansasii*, *M. peregrinum*, *M. septicum*, *M. neoaurum*, *M. shottsii*, *M. pseudoshottsii*, *M. scrofulaceum*, *M. simiae* - showing that mycobacteriosis are among the most common diseases in both commercial and wild fish worldwide (3, 4).

In humans, the occurrence of mycobacteriosis is usually associated with aquatic activity such as swimming, fishing, navigation or aquarism (5). Several other forms of transmission were reported, like handling of aquariums or tanks with contaminated fish, bites of dolphin in captive, injuries from fish-fin hitting, and boat engine handling, among others (5, 6).

NTM can be transmitted to humans by ingestion of contaminated food. Bacteria of this genus have been isolated from beef, pork and lamb (7), dairy products (8), water (9), vegetables, fruits, herbs (10), oysters (11) and fish (12, 13).

Among the NTM, *M. gordonae* is present in all types of environments as the third most frequent *Mycobacterium* spp after *Mycobacterium tuberculosis* and the *Mycobacterium avium* complex (14). However, surprisingly, this bacterium is responsible for a few significant infections in humans, and is generally viewed as having low pathogenic potential (14).

M. gordonae is commonly found in water as a normally non-pathogenic colonizing agent and when recovered in culture, is considered to be clinically insignificant or a contaminant (15). Reports of infections in humans are usually associated with immunodeficient patients involving the lungs, soft tissues, cornea and peritoneal cavity (16, 17, 18, 19, 20). Less frequently, *M. gordonae* can cause disseminated disease and bacteremia (18, 21, 22).

Most of bacteria responsible for foodborne diseases do not cause any macroscopic changes in fish fillets, making the detection more difficult by traditional methods of inspection. Therefore, given the low availability of data from national surveys concerning the prevalence of pathogenic bacteria in fish, and the importance of Nile tilapia in the international economy, the aim of the present study was to verify the presence and prevalence of *Mycobacterium* spp. in fillets of Nile tilapia (*Oreochromis niloticus*) used for human consumption.

MATERIAL AND METHODS

Sampling

Considering the endemic and / or unknown nature of most diseases of aquatic animals in Brazil, the prevalence used was 10%, in a discretionary manner, as recommended by the Ministry of Fisheries and Aquaculture (23).

The “EpiTools” tool (“freeware”) of the company “AusVet Animal Health Services” was used to calculate the sampling, in which it is possible to determine the size of the sampling in order to identify the presence of diseases using pooled samples (http://epitools.ausvet.com.au/content.php?page=PPFreedom). A 95% confidence interval was selected for the study of proportion. With these data, in order to achieve reliable results, a minimum of 32 samples would be necessary, but since prevalence is unknown and can vary according to pathogen, 50 samples were collected in order to obtain a greater safety margin for interpretation of the results.

The material obtained did not have information (labeling) that allowed to characterize its origin in a precise way. Thus, 50 frozen fillets of Nile tilapia (*O. niloticus*) were sampled in a single collection, in the form of convenience sampling, in a fish market in a Sao Paulo State municipality. The fillets were packed, identified in individual hermetic sterile plastic bags, and stored in a thermal box with ice to preserve the temperature during transport to the laboratory.

DNA extraction

For DNA extraction, 20 mg of the muscle tissue of each fillet were weighed. The samples were then subjected to extraction using the Relia Prep™ gDNA Tissue kit (Promega™), according to the manufacturer's recommendations. Afterwards, the samples were quantified and

evaluated for their purity in the Nanodrop 2000™ spectrophotometer at 260/280 and 260/230 ratios. The DNA obtained was maintained at -20° C.

Molecular tests

Conventional PCR technique was used for *Mycobacterium* spp DNA search, since this methodology allows the subsequent sequencing to confirm the species.

Mycobacterium spp.

For the detection and identification of Mycobacterium spp, a pair of primers that recognize several species of this bacteria affecting fish, humans, and found in the environment, were selected according to Chen et al. (24). These authors designed the primers for the 16S ribosomal region, which is conserved for these microorganisms. For the PCR reaction, a mix of 5.3 μL of water free of nucleic acids, 10 μL of GoTaq Green 1x (Promega™), 0.6 μL of the *primer* Myc16SrRNAF (10 pmol / μL), 0.6 μL of the *primer* Myc16SrRNAR (10 pmol / μL), 0.5 μL of DMSO and 3 μL of extracted DNA, were used. Nucleic acid free water and a sample of naturally infected Nile tilapia were used as negative and positive control, respectively.

PCR was undertaken according to Chen et al. (24), initial denaturation at 94° C for 5 minutes, followed by 40 cycles at 94° C for 1 minute, 50° C for 1 minute, 72° C for 1 minute and final extension at 72° C for 6 minutes. The amplification was visualized by 1.5% agarose gel under ultraviolet light.

Sequencing

Amplified samples for *Mycobacterium* spp. were purified with Machery-Nagel™ PCR Clean-up, Gel Extraction kit, following the manufacturer's recommendations for confirmation of the pathogen. The purity ratio (260/280 and 260/230) and quantification of the purified samples were undertaken by Nanodrop 2000™ spectrophotometer. Only samples with a purity ratio higher than 1.7 were accepted. Quantification was performed according to the ABI 3500™ sequencer manufacturer's recommendations (Applied Biosystems), which estimates the minimum concentration based on the size of the amplified product.

The purified and quantified samples were submitted to Sanger sequencing using the ABI Big Dye Terminator Chemistry kit (Applied Biosystems™), followed by capillary electrophoresis (Applied Biosystems, ABI 3500™). The quality of the electropherograms was assessed by Sequencing Analysis software version 5.4, aligned in ClustalW in the Molecular Evolutionary Genetics Analysis program version 5.1. Sequences **were** submitted to BLAST (Basic Local Alignment Search Tool) to confirm identity using the program Geneious 7.0.6.

Statistical analysis

The present work is a descriptive study related to detection of *Mycobacterium* spp. with importance for Public Health. Thus, data were evaluated according to their prevalence.

RESULTS

The samples of Nile tilapia fillets analyzed did not show any macroscopic alterations suggesting infectious or parasitic processes.

Regarding the application of PCR technique for *Mycobacterium* spp., all samples were positive, amplifying a product of 240 bp, expected size for this microorganism. Sanger

sequencing confirmed *Mycobacterium gordonae* (93% identity) in all samples (accession number: MF472702).

DISCUSSION

Few studies available in the literature aimed at investigating the occurrence of mycobacteria in samples of ready-to-eat Nile tilapia (*Oreochromis niloticus*) from clinically healthy fish populations. Most of the studies involved fish populations that showed clinical signs of mycobacteriosis from aquaculture and fish incubators (25, 26, 27).

The present study aimed at analyzing fillets obtained from healthy individuals of Nile tilapia destined for human consumption. All samples (100%) were positive for *M. gordonae*. This result may be related to the breeding system with high population density in tanks that this fish species is commonly submitted. High density fish populations in farms causes loss of water quality increasing organic pollution, which leads to a higher occurrence of mycobacteria. This factor was associated with the frequent occurrence of several species of mycobacteria in freshwater aquariums and breeding tanks (28, 29, 30). According to Falkinham III (31), mycobacterial adaptation may occur due to resistance to anaerobic conditions. The gradual reduction in oxygen concentration in cultures does not affect the survival of these organisms because of their ability to enter in a latency stage (32).

The persistence of mycobacteria in human-created environments (e.g. potable water distribution systems) is due to their ability to live in environments with very low levels of nutrients, nutritional diversity, biofilm formation, and resistance to disinfectants. The use of disinfectants in drinking water systems or the introduction of hydrocarbon pollutants into soils and water seems to act as selective agents, leading to the proliferation and final dominance of mycobacteria in these habitats. It is likely that human activities (such as disinfection and pollution) are causing selection of mycobacteria. The aging population and the increase in prevalence of immunodeficient individuals can predispose these groups to infection and disease (1).

According to Mrlik et al. (4), breeding systems with high population density of fish act as organically polluted environments where an abundant number of mycobacteria containing a wide range of species of this genus are expected. In that study, at least six species of mycobacteria from pond water were isolated (*M. gordonae*, *M. chimaera*, *M. intracellulare*, *M. kumamotonense*, *M. montefiorense* e *M. nebraskense*).

Pavlik & Falkinham (33) propose that the route of contamination of fish by such mycobacteria may be due to leaching, since such bacteria are also present in the soil and thus would be carried to the breeding ponds. Once in the ponds, they would come in contact with fish through gills and skin.

According to Eckburg et al. (15), *M. gordonae* is commonly found in water, being considered a colonizer normally nonpathogenic to humans, which, when recovered in culture, is considered clinically insignificant or a contaminant. It is generally difficult to discern true mycobacterial infection from colonization due to this organism since *M. gordonae* is ubiquitous and commonly an innocuous saprophyte. However, several case reports indicate the occurrence of infections, which are usually associated with immunodeficient patients and involve lungs, soft tissues, cornea and peritoneal cavity (19, 20), causing, to a lesser extent, disseminated disease and bacteremia (18, 21, 22). Risk factors for colonization of the respiratory tract or lung disease by *M. gordonae* have not been defined so far. Abnormal lung architecture and host immunity may play important roles in the development of the disease caused by this species of mycobacteria (20).

Although most of reported cases of infection have occurred in immunocompromised patients, several studies have demonstrated infection in immunocompetent patients, mostly

associated with lung diseases, including pulmonary infection (34), pulmonary nodule, necrotic granulomatous pneumonia (35) and pulmonary disease (36, 37).

In Brazil, the official control of fishes and their products involves the analysis of indicators of freshness, control of histamine (in forming species), biotoxins and parasites (38). However, due to the fact that most bacterial pathogens do not cause any macroscopic changes in fish fillets, their detection by traditional methods of inspection becomes impractical. Regarding this, it is impossible to ignore the growth and development of Brazilian aquaculture, and the importance of this activity as a source of food. However, growth, without the application of good practices, can momentarily increase productivity in the sector; however, the risk of disease also increases (39).

Most diseases of aquatic animals in Brazil are endemic and / or unknown (23). Despite a number of diseases caused by bacteria and viruses have been registered, the knowledge of these agents is still restricted. Some may even be mandatory reporting diseases (39).

CONCLUSIONS

Due to the evidence of *M. gordonae* as a possible infectious agent in humans, additional studies are necessary to assess the real risk presented by this pathogen when ingested by food in both immunosuppressed and immunocompetent persons. The changing dietary habits of the population must be taken into consideration, given that different forms of preparation and consumption of fish, including raw fish, can expose the consumer to risk.

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