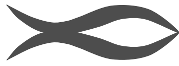


www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 14: 575-580 (2014) DOI: 10.4194/1303-2712-v14_2_29

SHORT PAPER

Identification of Mycobacterium Agent Isolated from Tissues of Nile tilapia (*Oreochromis niloticus*)

Maurilio Lara-Flores[1], Gabriel Aguirre-Guzman[2,*], Sara Berenice Balan-Zetina[1], Karina Yamile Sonda-Santos[1], Ana Antonia Zapata[1]

[1] Instituto de Ecología, Pesquerías y Oceanografía del Golfo de México (EPOMEX), Universidad Autónoma de Campeche. Av. Agustín Melgar y Juan de la Barrera S/N, Col. Buenavista, Campeche, México.
[2] Facultad de Medicina Veterinaria y Zootecnia. Universidad Autónoma de Tamaulipas. Km 5 Carr. Cd. Victoria-Mante, Ciudad Victoria, Tamaulipas, México..

* Corresponding Author: Tel.: +52. 834 3181800; Fax: +52. 834 3181800;
E-mail: gaguirre_guzman@hotmail.com

Received 4 December 2013
Accepted 10 April 2014

Abstract

Nile tilapia culture in Campeche, Mexico experiences sporadic and significant fish mortalities, where mycobacteriosis signs of disease are detected. Thirty fishes(30 ± 4 g) from different farms were monitored after a disease outbreak.Nile tilapia (*Oreochromisniloticus*) samples (60%) of spleen, liver and stomach display multiples granulomas with a thick capsule, walled off by epithelioid cells and robust acid-fast rods on the necrotic center. A great number of acid-fast bacilli where detected with Ziehl-Neelsen stain protocol. The bacteriological analysis of kidney displayed a 90% (26 organisms) of positive development in Lowenstein-Jensen medium with yellow or orange colonies when exposed to light and acid-fast rods.A 562-bp fragment confirms the positive result of the presence of the *Mycobacterium* spp.by PCR analysis;where phylogenetic analysis suggests that *Mycobacteriumfortuitum* and *M. marinum* were the principal mycobacterial species associated with tilapia mortality in Campeche, Mexico.

Keywords:

Introduction

The main objective of aquaculture activities is the productions of aquatic organisms for human use or enhancement propose. One of the most significant threats to successful aquaculture is the infectious diseases. Commercial fish farming is an important industry, where *Oreochromis* sp, *Sarotherodon*sp, and other cichlid hybrids (typically names as Tilapia) represent the third main group used in freshwater aquaculture and which show a world production around to 2,790,350 metric ton. Tilapia culture in Mexico has a mean production near 75,000 metric tons from 1988-2007; where most of the tilapia farms are located in Veracruz, Michoacán, Jalisco, Sinaloa, Tabasco, and Tamaulipas. Also, some States as Chihuahua, Campeche, Durango, Mexico, Michoacán, Morelos, and Puebla have suitable aquatic conditions and agreeable characteristics for aquaculture production.

The fish culture facilities the bacteria growth, spread, and produce diseases; which has a direct effect on production, feed efficiency and growth performance (Walker and Winton, 2010). In resent year, the studies associates with fish diseases have found new emerging diseases, and their corresponding pathogen, which may generate economic losses in commercial aquaculture. Some bacteria are easily transported to other regions through multiple aquaculture activities as transporting of broodstock, egg, fingerlings, juvenile, frozen products, equipment and material, and handling of wastewaters (Walker and Winton, 2010).

Mycobacterium spp. has been reported to affect different fishes species from aquaculture activities as Atlantic salmon, ornamental fish, rainbow trout, sea bass, striped bass, seabream, snakehead, tilapia, and turbot; where *M. abscessus, M. avium, M. chelonae*, *M. fortuitum, M. gordonae,M. haemophilum*, *M. marinum,M. peregrinum, M. piscium, M. salmoniphilum, M. simiae, M. shottsii, M. szulgai* and *M. triplex* are main species reported (Ferreira, *et al.*, 2006; Gauthier and Rhodes, 2006; Zanoni, *et al.*, 2008; Sonda-Santos and Lara-Flores, 2012). The horizontal transmission of *Mycobacterium* spp. in aquaculture fishes is poorly understood. Wolf and Smith (1999) and Ranzani-Paiva, *et al.*(2004) suggest that potential routesof infection include the ingestion of contaminated detritus, food or tissues, and through skin or gill tissue lesions, where the bacterial cells remain viable for years (Ferreira, *et al.*, 2006).

Mycobacteriosis or fish tuberculosis in fish

© Published by Central Fisheries Research Institute (CFRI) Trabzon, Turkey
in cooperation with Japan International Cooperation Agency (JICA), Japan

described as a systematic, chronic and progressive disease with clinical signs that include skin discoloration, in appetance, lethargy, abnormal swimming, cutaneous ulcerations or erosions, ascites, reduced growth, exophthalmia, grey or white nodules in internal organs, and hypertrophy of spleen, kidney and liver (Ferreira, *et al*., 2006; Gauthier and Rhodes, 2009). Significant consequences of mycobacteriosis include mortality, morbidity, and effects of subclinical infection such as decreased feed efficiency, decreased growth rates, and decreased marketability (Wolf and Smith, 2009). Factors that may predispose fish to mycobacterial infection include dietary or environmental contamination, stress, inadequate nutrition and poor water quality.

The detection of mycobacterial species that affect aquaculture fish displays a great importance for the complicated of their taxonomic analysis based on traditional techniques (e.g., biochemical test, drug susceptibility, enzymatic activity, growth, and morphology). Over the last two decades, the molecular techniques have been extensively applied as agree, modern, and standard protocols to diagnosis of fish mycobacteria, characterizing bacteria, taxonomic and/or phylogenetic relationships studies (Heckert, *et al*., 2001). The aim of this study was identifies mycobacterial species associated with massive tilapia (*Oreochromis niloticus*) mortality detected in Campeche (2008).

Materials and Methods

Fish farmsculturing tilapia were visited in Champotón River, Campeche, México. These farms cultured Nile,Brasilian, andStirling tilapia (*O. niloticus*), and hybrid tilapia (*O. niloticus* x *O. mossambicus*), and. Thirty fishes (average weight: 30±4 g) were monitored assuming a prevalence of 10% (Office International des Epizootiesor OIE, 2010). The fish samples were select from freshwater cages with the use of a hand net. Each fish was sacrificed in the farm by severing the spinal cord with a sharp blade and examined (Choich, *et al*., 2004). Examination consisted of recording visual gross lesions as well as sampling aseptically blood and tissues (gill, gut, kidney, and liver) for bacteriology and histology in the EPOMEX Institute of Autonomy University of Campeche, and molecular analysis in the Biotechnology Institute of National Autonomous University of Mexico.

Bacterial isolation was attempted from kidney and blood samples. A sterile standard loop (10-µL) were inserted into collected samples, which were streaked on the surface of a sterile tubes containing 10 mL of Lowenstein-Jensen medium (LJ, DIFCO™ 244420) using a zigzagging method and cultured at 35°C for 48 to 120 h. Single colonies were re-cultured on petri dish with JL medium to obtain pure isolates. Colony morphology was recorded and cultures were subjected to Ziehl-Neelsen (ZN, HYCEL™ 64293) staining and examined microscopically (Ranzani-Paiva, *et al*., 2004; Lara-Flores, *et al*., 2013). The presence of bacilli acid-alcohol-resistant was confirmed as positive for mycobacterium cell, which were then used for the molecular analysis.

Tissue samples of gill, gut, kidney, and liver were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 3-5 µm, and stained with Haematoxylin-Eosin (HE) and Ziehl-Neelsen. In the samples that were positive in bacteriology analyses where sections of tissue were examined under light microscopy for granulomas and acid-fast rods detection (Zanoni, *et al*., 2008).

Total DNA was obtained from different Mycobacterim-like strain tilapia fish using according to a Sonda-Santos and Lara-Flores (2012) protocol, which use a T_{39} = GCGAACGGGTGAGTAACACG and T_{13} = GCACACAGGCCACAAGGGA primers that specifically amplified a 924 DNA fragment and a 562-bp fragments with *Ban*l restriction (Talaat, *et al*., 1997; Sonda-Santos and Lara-Flores, 2012). The PCR reactions (50 µL) containing 0.2 mM of each dexoynucleotide, 20 pmol of each primer, 25 mM $MgCl_2$, 10 mM Tris-HCl, 1.25 U of Taq DNA Polymerase (Promega) and 40 ng of template DNA. The PCR reaction was carried out in a PCR programmed thermocycler (Multigene, Mini TC-020-24)) under the following conditions: 94°C for 3 min followed by 94°C for 45 sec (denaturation); 50°C for 75 sec (annealing), and 72°C for 30 sec (extension). After 35 cycles, the reactions were cooled down to 4ºC. A 924 bp band was considered as a positive result which was visualized by electrophoresis using 5 µL of completed PCR reaction on a 2.0% agarose gel stained with ethidiumbormide, visualized under UV light, and analyzed with imaging system software from Quantitive One (Bio-Rad, 2006).

The products of PCR were sequenced by the Institute of Biotechnology (IBT-UNAM) using the specific primers (T_{39} and T_{13}). The sequencing were evaluated with maximum-parsimony and maximum-likelihood analysis and visualized using MEGA Blast 5 nucleotide sequence software (Tortoli, 2012) and then compared with the published sequences in GenBak using BLAST software (http://www.ncbi.nlm.gov/blast/) from National Center for Biotechnology Information. The position numbering corresponds with that of the *Escherichia coli* sequence (GenBank reference sequence).

Results

The clinical examination of fish display various external signs of diseases as body deformity, necrotic and hemorrhagic areas in fin and gill, exophthalmia, and ulcers in head, mouth and skin. Where histological examination carried out on the spleen, liver and stomach of these fishes revealed multiples granulomas of different size with a thick capsule, walled off by epithelioid cells and robust acid-fast

rods on the necrotic center (Figure 1a). A great number of acid-fast bacilli where detected with Ziehl-Neelsen stain protocol in the 60% of the samples (Figure 1b).

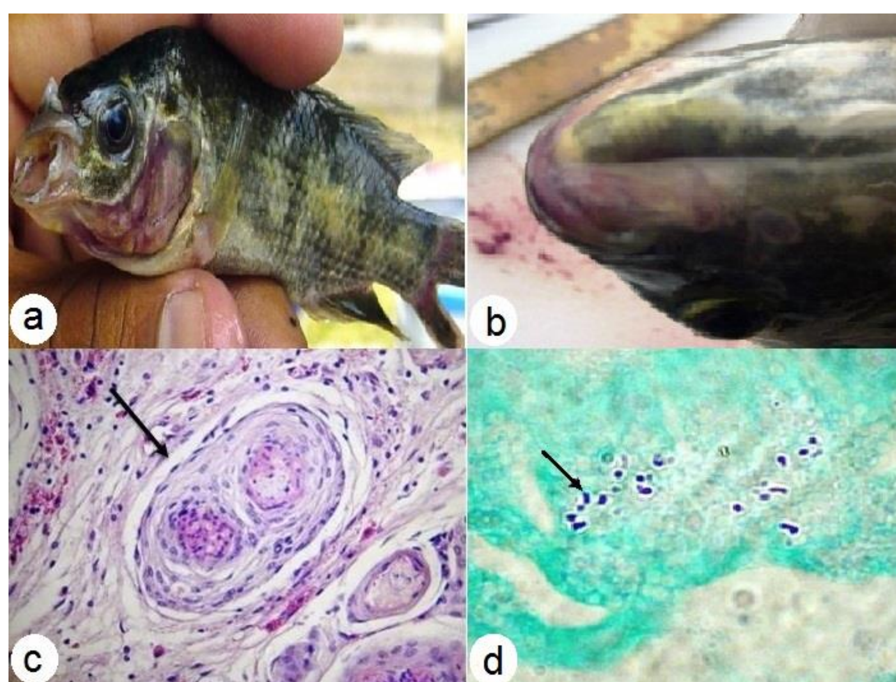
The bacteriological analysis of kidney displayed a 90% (26 organisms) of positive development in LJ with yellow colonies when exposed to light and acid-fast rods. Isolates from affected tissues of tilapia were submitted for genetic evaluation by PCR analysis with positive result at the presence of the *Mycobacterium* spp. with a 562-bp band which is able with *Ban*I restriction (data published in Sonda-Santos and Lara-Flores, 2012). A complementary molecular approach was used to assist in *Mycobacterium* identification. MEGA 5 analysis of the PCR sequence of the strains revealed a high relation to *Mycobacterium* sp. This analysis show a 100% identity with *M. marinum* (strains 15, 26), 98% with *M. marinum* (strain 63), 97% with *M. marinum* (strains 21, 24), 98% with *Mycobacterium* sp. (strain 32), 97% with *M. chelonae* (strain 33), and 87% with *M. fortuitum* (strain 19). The maximum parsimony phylogenetic tree show two groups of *Mycobacterium* sp., one group with relation to *M. paratuberculosis*(strains 15, 21, 24, 26, 33, 36) and other with *M. fortuitum* (strain 19, 32) (Figure 2). The strains 33 and 63, and 15 and 26 show a closely phylogenetic relation. The maximum-likelihood analysis show that all mycobacterium isolate as *M. fortuitum* (Figure 3).

Discussion

The increasing commercial importance of freshwater fish production has stimulated the need for a complete understanding of the pathologies which result in mortality among fish (Zanoni, *et al*., 2008). In Mexico, tilapia is one of the principal fish product obtained from aquaculture. The production areas have shown a sudden and significant increase in mortality 2003-2006, which had a direct effect on fish farms. The detection of traditional and new pathogens (bacteria, fungi, virus, etc.) on fish is priority for the aquatic health management, which helps to increase the knowledge about their presence and biogeographically distribution on aquaculture and fisheries production and help with the generate tools for diseases control.

Traditionally, the presences of external signs of diseases associated to presence of acid-fast bacilli in histological samples are used for compatible diagnosis of mycobacteriosis in fish, where bacteriology culture is not always successful performed (Ranzani-Paiva, *et al*., 2004).. The clinical signs of diseases clearly suggested the possible presence of mycobacterium infection according to Wolf and Smith (1999), Ranzani-Paiva, *et al*. (2004), Gauthier and Rhodes (2009), and Shukla, *et al*.(2013). In general, the presence of granulomas and acid-fast rods (Fig 1a,b) in the infected fishes showed the same structure as those described in others species with mycobacterial infections (Heckert, *et al*., 2001; dos Santos, *et al*., 2002; Shukla, *et al*., 2013). The primary pathogens of mycobacteriosis in fish that report those signs of diseases are *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. marinum*, *M. triplex* (Rhodes, *et al*., 2005; Ferreira, *et al*., 2006).

The bacteriological analysis of kidney displayed a 90% (26 organisms) of positive development in LJ



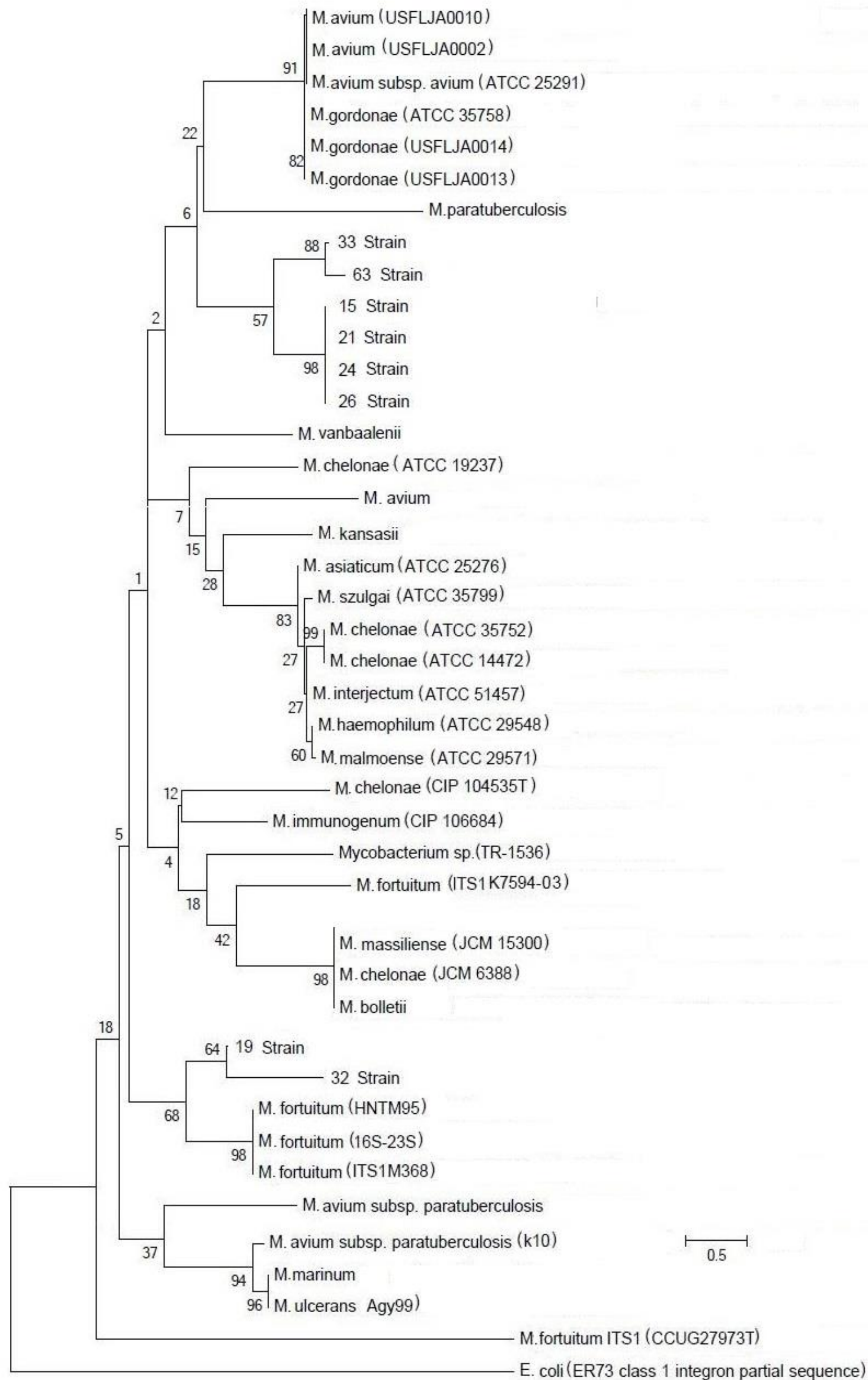


Figure 2. Maximum parsimony phylogenetic tree relating sequence-based identification of *Mycobacterium* strain collected in Nile tilapia (*Oreochromis niloticus*).

with yellow colonies when exposed to light and acid-fast rods. Actually, the specific identification of mycobacterium is very important. However, traditional techniques for identifying mycobacteria based on growth characteristics and biochemical tests have advantages and disadvantages as time-consuming, easy, cost-effective, high dependence of technical experience (Shukla, *et al*., 2013). The PCR identification of possibly *Mycobacterium* strains could be of extreme diagnostic value. Isolates from affected tissues of tilapia were submitted for genetic evaluation by PCR analysis with positive result at the presence of the *Mycobacterium* spp. with a 562-bp band which is able with *Ban*I restriction (data published on Santos-Sondas and Lara-Flores, 2012)(Talaat, *et al*., 1997; Sonda-Santos and Lara-

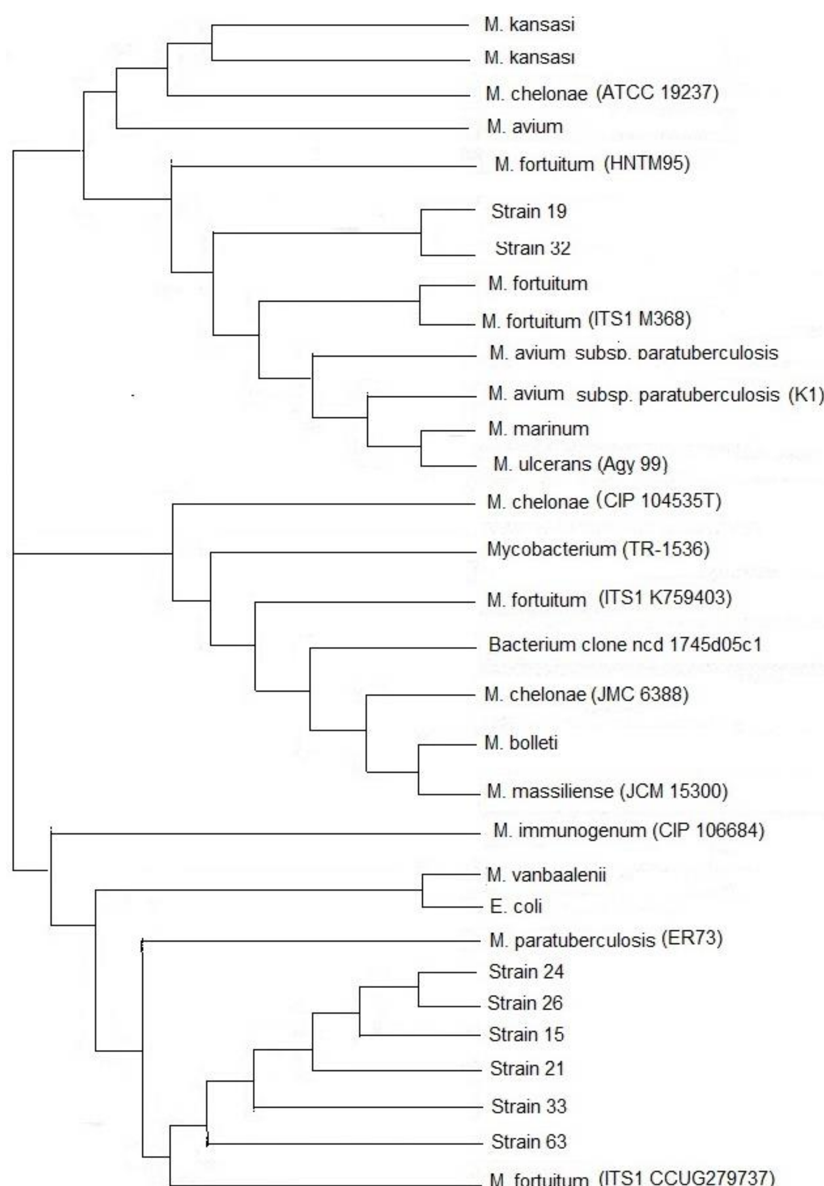


Figure 3. Maximum likelihood phylogenetic tree relating sequence-based identification of *Mycobacterium* strain collected in Nile tilapia (*Oreochromis niloticus*).

Flores, 2012; Shukla, *et al*., 2013).

In this study, In this study, show different Mycobacterium species in tilapia tissues (Table 1) and also show a complementary molecular approach was used to assist in *Mycobacterium* identification. MEGA 5 analysis of the PCR sequence of the strains revealed a high relation to *Mycobacterium* spp. This analysis show a 100% identity with *M. marinum* (strains 15, 26), 98% with *M. marinum* (strain 63), 97% with *M. marinum* (strains 21, 24), 98% with *Mycobacterium* sp. (strain 32), 97% with *M. chelonae* (strain 33), and 87% with *M. fortuitum* (strain 19). The maximum parsimony phylogenetic tree show two groups of *Mycobacterium* sp., one group with relation to *M. paratuberculosis* (strains 15, 21, 24, 26, 33, 36) and other with *M. fortuitum* (strain 19, 32)(Figure 2). The strains 33 and 63, and 15 and 26 show a closely phylogenetic relation. The maximum-likelihood analysis show that all mycobacterium isolate as *M. fortuitum*(Figure 3). These results demonstrate the identification of species usually reported as pathogen of fish for these molecular protocols (Wolf and Smith, 1999; Heckert, *et al*., 2001; Zanoni, *et al*., 2008). However, other studies with new DNA sequence data (16S rRNA gene) help in the identification of several additional *Mycobacterium* species that infect fishes.

The occurrence of mycobacterial infections in fish may be considered a significant problem in Campeche, Mexico. The results suggest that *M. fortuitum* and *M. marinum*were the principal mycobacterial species associated to Tilapia mortality in 2008. Mycobacteriosis is an infectious disease of various saquacultured finfishes, which have been described as causing chromic mortality with significant economic impact and have zoonotic potential. Further analyses we carried out in order to

Table 1. Mycobacterium species isolated from Nile tilapia (*Oreochromis niloticus*)

No. of Sample and source	Mycobacterium species		
	M. marinum	*M. fortuitum*	*M. paratuberculosis*
15 (spleen)	X		X
19 (liver)		X	X
21 (liver)	X		
24 (lesion on skin)	X		X
26 (liver)	X		X
32 (lesion on skin)		X	
33 (spleen)			X
63 (lesion on skin)	X		X

determinate the prevalence of the infection in this and others farms, and to identify its source, since this could be affect the development of the aquaculture in the region. The different sources of the juvenile tilapia are also a significant problem for detecting the origin source of mycobacteria, because those farms display any health certificate or control. It is necessary use different studies techniques to understand host defense mechanisms, prophylactic strategies, pathogenesis, transmission, virulence of those bacterias to prevent and/or eliminate an emergency disease outbreak in the farms. This work also show that traditional protocols are helpful in the identification of mycobacteria, but molecular tools are important for the complete detection of the etiological agent of fish disease.

Acknowledgments

This work was supported by grants from the Mexican Council of Science and Technology or CONACYT [Project FOMIX-CAMPECHE (CAMP-2004-C02-1)].

References

Choich, J., Salierno, J.D., Silbergeld, E.K. and Kane, A.S. 2004. Altered brain activity in brevetoxin-exposed bluegill, *Lepomismacrochirus*, visualized using *in vivo* C^{14} 2-deoxyglucose labeling. Env. Res., 94:192–197. doi: 10.1016/j.envres.2003.07.009

dos Santos, N.M.S., do Vale, A., Sousa, M.J. and Silva, M.T. 2002.Mycobacterial infection in farmed turbot *Scophthalmusmaximus*, Dis.Aquat.Org., 52: 87-91. doi: 10.3354/dao052087

Ferreira, R., de Sauza Fonseca, L., MunizAfonso, A., Gomes da Silva, M., Saad, M.H. and Lilenbaum, W. 2006. A report of mycobacteriosis caused by *Mycobacterium marinum* in bullfrogs (*Ranacates beiana*). Vet. J., 171: 177–180. doi: 10.1016/j.tvjl.2004.08.018

Gauthier, D.T. and Rhodes, M.W. 2009.Mycobacteriosis in fishes: A review. Vet. J., 180: 33–47. doi: 10.1016/j.tvjl.2008.05.012

Heckert, R.A., Elankumaran M.A. and Baya A. 2001.Detection of a new *Mycobacterium* species in wild striped bass in the Chesapeake Bay. J. Clin. Microbiol., 39: 710–715. doi: 10.1128/JCM.39.2.710-715.2001

Lara-Flores, M., Balan-Zetina, S., Zapata, A. and Sonda-Santos, K. 2013.Determination and prevalence of *Mycobacterium* spp., in Nile tilapia (*Oreochromis niloticus*) cultured in México. Rev. MVZ Córdoba, 18: 3273-3281.

OIE. 2010. Manual of Diagnostic Tests for Aquatic Animals. Fifth edition, World Organisation for Animal Health, Paris

Ranzani-Paiva, M.J.T., Massatoshi, C., Cocuzza, A. and Risaffi, V. 2004.Effects of an experimental challenge with *Mycobacterium marinum*on the blood parameters of Nile Tilapia, *Oreochromis niloticus* (Linnaeus, 1757).Braz. Arch. Boil. Techn., 47:945-953.

Rhodes, M.W., Kator, H., McNabb, A., Deshayes, C., Reyrat, J.M., Brown-Elliott, B.A., Wallace, Jr.R., Trott, K.A., Parker, J.M., Lifland, B., Osterhout, G., Kaattari, I., Reece, K., Vogelbein, W. and Ottinger, C.A. 2005.*Mycobacterium pseudoshottsii* sp. nov., a slowly growing chromogenic species isolated from Chesapeake Bay striped bass (*Moronesaxatilis*). International J. Syst. Evol. Microbiol., 55:1139–1147. doi: 10.1099/ijs.0.63343-0

Shukla, S., Sharma, R. and Shukla, S.K. 2013. Detection and identification of globally distributed mycobacterial fish pathogens in some ornamental fish in India. Folia Microbiol., 58: 429-436. doi: 10.1007/s12223-013-0225-y

Sonda-Santos, K.Y. and Lara-Flores, M. 2012. Detection of *Mycobacterium* spp. by polymerase chain reaction in Nile tilapia (*Oreochromis niloticus*) in Campeche, Mexico. Afr. J. Microbiol. Res., 6: 2785-2787. doi: 10.1016/S0378-1135(97)00120-X

Talaat, A.M., Reimschuessel, R. and Trucksis, M. 1997.Identification of mycobacteria infecting fish to the species level using polymerase chain reaction and restriction enzyme analysis.Vet. Microbiol., 58: 229-237. doi: 10.1016/S0378-1135(97)00120-X

Tortoli, E. 2012. Phylogeny of the genus *Mycobacterium*: Many doubts, few certainties. Infect. Gen. Evol., 12: 827–831. doi: 10.1016/j.meegid.2011.05.025

Walker, P.J. and Winton, J.R. 2010. Emerging viral diseases of fish and shrimp. Vet. Res., 41:51. doi: 10.1051/vetres/2010022.

Wolf, J.C. and Smith, S.A. 1999. Comparative severity of experimentally induced mycobacteriosis in striped bass *Morones axatilis* and hybrid tilapia *Oreochromis* spp. Dis.Aquat. Org., 38: 191-200. doi: 10.3354/dao038191

Zanoni, R.G., Florio, D., Fioravanti, M.L., Rossi, M. and Prearo, M. 2008.Occurrence of *Mycobacterium* spp. in ornamental fish in Italy. J. Fish Dis., 31:433–441. doi: 10.1111/j.1365-2761.2008.00924.x