Piscine mycobacteriosis – Involvement of bacterial species and reflection in pathology

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Summary

Piscine mycobacteriosis is a lethal disease with zoonotic potential, found worldwide in both fresh and marine fish. More than 20 strains of *Mycobacterium spp.* are known to persist in fish so far, but the pathogenicity is currently unknown for most of them. However, *M. marinum* is reported as one of the most pathogenic agents for fish and is involved in zoonotic cases. We examined 47 different cases from two zoological gardens, where fish tuberculosis was identified or previously suspected during the last ten years. We collected PCR and sequencing data, which were then compared to previously collected clinical data and pathology. The clinical signs caused by *Mycobacterium spp.* were similar in all the cases, except for cases infected by *M. marinum*, which lacked the presence of skin lesions. Lesions seen in histology caused by *M. marinum* tended to be more acute and severe compared lesions caused by other *Mycobacterium spp.* The majority of *M. marinum* cases have been reported within marine fish. In contrast to previous studies we detected this species to be the predominant bacteria present within freshwater fish. Interestingly, we detected *M. holsaticum* in one of the seawater systems used in this project, being the first report of this *Mycobacterium* species shown to be present in a fish.

Keywords: Fish tuberculosis; *Mycobacterium marinum*; pathology; PCR; sequencing

Mycobakteriose bei Fischen – Eine Studie unter Berücksichtigung der beteiligten Bakterienarten und Pathologie

Die Fischtuberkulose ist eine meist tödlich verlaufende bakterielle Infektionskrankheit mit Zoonose Potential. Die Krankheit tritt weltweit bei Süss- und Salzwasserfischen auf. Bisher wurden mehr als 20 *Mycobacterium spp.* in Fischen mit meist unbekannter Pathogenität diagnostiziert. *M. marinum* ist eine Spezies mit bekannter hoher Pathogenität bei Fischen und wird häufig mit Zoonose Fällen in Verbindung gebracht. 47 Fälle mit nachgewiesener oder vermuteter Fischtuberkulose aus zwei Zoologischen Gärten, die während der letzten 10 Jahre am Zentrum für Fisch- und Wildtiermedizin untersucht wurden, wurden in die Studie integriert. PCR und Sequenzierung wurden durchgeführt und die Daten mit klinischen und pathologischen Befunden verglichen. Die klinischen Symptome waren bei allen *Mycobacterium spp.* Fällen vergleichbar, mit Ausnahme der Fälle verursacht durch *M. marinum*, welche keine Hautläsionen zeigten. Die histologischen Veränderungen durch *M. marinum* waren akuter und stärker ausgeprägt als die durch andere *Mycobacterium spp.* verursachten Läsionen. Im Gegensatz zu den bisherigen Studien war *M. marinum* der vorwiegende Keim bei den Süsswasserfischen. Bemerkenswert war die Entdeckung von *M. holsaticum* in einem Meereswassersystem, dies ist somit die erste Publikation dieser *Mycobacterium* Spezies in Fischen.

Schlüsselwörter: Fischtuberkulose; *Mycobacterium marinum*; Pathologie; PCR; Sequenzierung

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Introduction

Piscine mycobacteriosis (fish tuberculosis) is a worldwide disease that affects an array of different fish species and has been associated with multiple *Mycobacterium spp.*[8,16]. The course of this disease is known to be chronic progressive with varying clinical signs. Most lesions are unspecific, as weight loss, non-healing skin ulcerations, scoliosis, loss of colour, and exophthalmia.[3] The mortality rate is usually low, while the development of multiple granulomas in the internal organs and in the skin is a common histological finding in routine fish necropsies.[7,22] The histopathology of these granulomas often presents as a chronic inflammatory response with epitheloid macrophages, pigmented macrophages, an increased amount of fibroblasts and a necrotic centre where these bacteria are typically located.[23] The bacteria located within these lesions can be identified using special stains, like Ziehl Neelsen. Mycobacteria are acid-fast aerobic organisms which react to the carbol fuchsine of the Ziehl Neelsen stain.[5] Advanced technical molecular methods have now made it possible to differentiate *Mycobacterium* down to the individual species level. As a result of these molecular advancements, it was possible to identify the three leading pathogenic agents responsible for fish tuberculosis.[8,20] From these three known pathogenic agents, *M. marinum* is supposed to be the most pathogenic one, and is known to occur both in marine and freshwater fish.[8,23,27,32] Additionally, *M. marinum* has the potential to infect other vertebrates, including humans.[3,14,17,24]

The transmission of *M. marinum* is still not completely understood, but infection is thought to be spread by ingestion of infective material, such as bacteria loaded skin cells from an infected host, or through dermal injury if the density of bacteria in the environment is high enough.[9,12] The prevalence of *Mycobacterium spp.* in European aquaria is reported to range between 41,7% and 46,8%[31,40], while in wild populations little is known about its prevalence and impact.[10] Once the infection is established, many factors make it challenging to eradicate these bacteria from the aquatic systems. Three main factors are responsible for the difficulties of eradication 1) longevity of the bacteria, up to two years in the environment, 2) difficulty to detect in standard screening tests,[2,26] and 3) disinfection methods are not practical in a running aquatic system.[23] All these factors contribute to an almost impossible eradication of some mycobacteria species.[23] To avoid a potential introduction of the bacteria, imported fish should undergo a quarantine period, however, due to the long incubation period of the infection, it is difficult to establish an adequate quarantine time.

Currently, there is no accepted treatment protocol for mycobacteria infections in fish. Antibiotic resistance testing on fish is rarely performed. If a resistance test was done, susceptibility of mycobacteria towards different antibiotics seems to be dependent on the infecting *Mycobacterium* species and strain.[7] It has been shown in experimentally infected yellowtails (*Seriola quinqueradiata*) that were orally treated with different antibiotics (rifampicin, streptomycin or erythromycin) that the treatment failed to eradicate the mycobacteria.[15]

In the presented study, we selected fish that were formerly diagnosed or suspected to be infected by mycobacteria from two zoological gardens in Switzerland (Zoo Basel and Tierpark Bern), with the goal to identify involved species of mycobacteria. Specifically, we addressed the following questions: (i) which mycobacteria species are involved in piscine mycobacteriosis and (ii) are there clinical or pathological differences between cases induced by *M. marinum* versus cases induced by other mycobacteria species? To investigate these questions, we performed a retrospective study using histological material from cases sent to the Centre for Fish and Wildlife Health (CFWH), University of Bern, Switzerland during the last ten years (2007-2016).

Materials and Methods

Sample selection

Archived material from 2007 to 2016 from previous fish cases were selected based on histological findings that confirmed or suspected piscine mycobacteriosis, originating from the Zoo Basel or Tierpark Bern. Confirmed cases are defined as the presence of acid-fast bacteria in histology, while suspected cases were selected based on the presence of indicative pathology without acid-fast bacteria in histology. In total, 30 cases from the Zoo Basel and 17 cases from the Tierpark Bern were selected for further examination.

PCR and sequencing

To identify the different bacterial species, two 20 µm sections of paraffin embedded material were prepared. Each section was deparaffinized, lysed, and DNA was extracted using the DNeasy tissue Kit (QIAGEN, Hombrechtikon, Switzerland) according to the manufacturer's protocol. Samples were incubated with proteinase K at 56 °C overnight in a shaking incubator. The DNA yield was determined by spectrophotometry using the NanoDrop photometer (NanoDrop Technologies, Inc., Wilmington, USA). Conventional PCR was performed using HotStarTaq DNA Polymerase (QIAGEN, Hombrechtikon, Switzerland) according to the manufacturer's instructions. Amplification of a 200 to 300 bp part (depending on the *Mycobacterium* strain) of the 16S-23S

spacer was performed with primers Sp1 (5′-ACC TCC TTT CTA AGG AGC ACC-3′) (AAGGA corresponds to the beginning of the spacer sequence) and Sp2 (5′-GAT GCT CGC AAC CAC TAT CCA-3′) as described.[28] A positive control sample obtained from confirmed cases in a group of guppies (*M. marinum*, 218 bp, ACCTCCTTTCTAAGGAGCACCACGAGAAA CACTCCAATTGGTGGGGTGTAAGCCGTGAGG GGTTCTCGTCTGTAGTGGACGGAAGCCGGGTG CACAACAACAAGCAAGCCAGACACACTATTG GGTCCTGAGGCAACATCTCTGTTGGTTTCGG GATGTTGTCCCACCATCTTGGTGGTGGGGTGT GGTGTTTGAGAATTGGATAGTGGTTGCGAGCAT) and a negative control using water were included in the PCR procedure. To confirm the specificity of the PCR products, they were purified with WIZAR RD®SV Gel and PCR Clean-Up System (Promega AG, Dübendorf, Switzerland). The products were checked on a 1,5 % agarose gel for amplification and molecular weight. Samples with a weak band were reamplified before being sent to sequencing (Microsynth AG, Balgach, Switzerland). Sequencing results were determined by BLAST-n based on a search in the GenBank database (www.ncbi.nlm.nih.gov).

Classification of histological findings

Histological lesions were classified into three different groups: acute, chronic or chronic-active. Acute lesions were defined as the presence of macrophage accumulation without circumscribed granuloma formation, no fibrotic capsule, pigmented macrophages present or absent, multifocal central necrotic areas in the macrophage aggregations. Chronic lesions were classified based on the presence of a well circumscribed granuloma with central necrosis, surrounded by low numbers of macrophages and a rim of fibroblasts. Finally, chronic-active lesions were determined if both components (acute and chronic) were present in one organ. The histopathological changes were graded on a scale from 0-6, with 0 meaning no lesions and 6 severe. Additionally, the distribution was judged from focal to systemic.

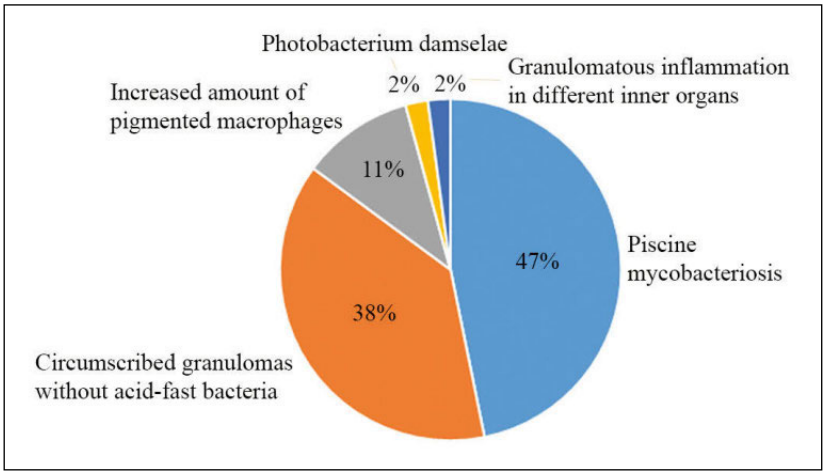

Figure 1: Overview of the initial diagnoses according to the histological findings, shown are 47 cases sent to the CFWH between 2007 and 2016

Classification of clinical findings

Only confirmed cases of piscine mycobacteriosis were included, which resulted in six reports from the Zoo Basel and five reports from the Tierpark Bern. The clinical signs were classified according to the following criteria: dyspnea, variation of coloration, skin ulcerations, apathy, anorexia, motoric incoordination, others. Additionally, the origin of the affected fish was documented and classified according to fresh- or seawater and cold or warm water.

Cause of disease

As in many cases several infectious agents were present, mycobacterial infections were classified as either (i) the most probable cause for clinical signs and mortality, or as (ii) possible cause for clinical signs and mortality, or as (iii) a secondary finding.

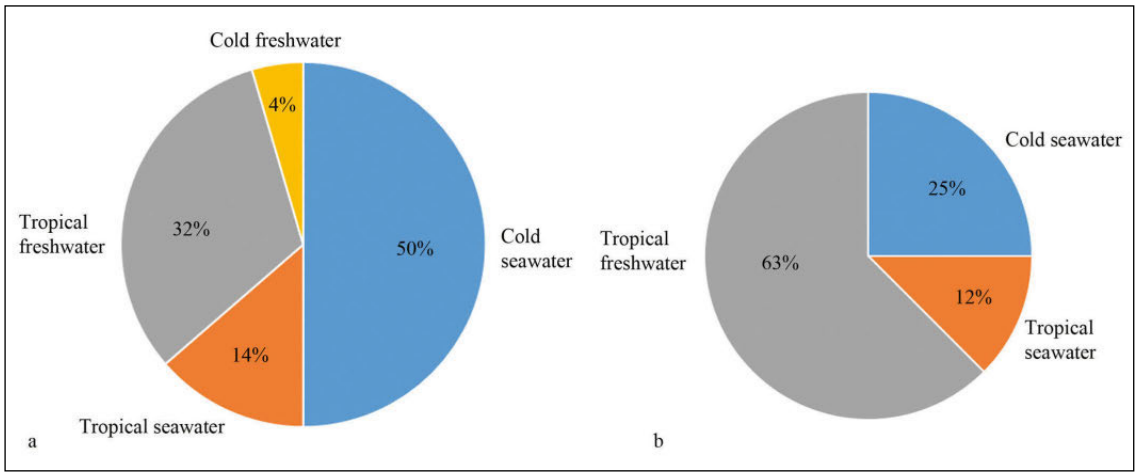

Figure 2: a. Water source of the submitted suspicious or confirmed mycobacteria cases from the Zoo Basel; b. water type of confirmed piscine mycobacteriosis cases from the Zoo Basel

Results

Origin of cases

Forty-seven cases indicative or suspicious for mycobacteria infections were included in this study (Figure 1). Twenty-two cases were diagnosed as piscine mycobacteriosis, confirmed by acid fast bacteria indicative for mycobacteria detectable in the histological slides (totalling 47% of all studied cases). In 18 cases, we detected multiple well circumscribed granulomas in different inner organs or the skin, without detectable acid fast bacteria. In one additional case, the granulomatous inflammation was diffuse without well demarcated granuloma formation. In five cases, an increased amount of pigmented macrophages was present. In one case, *Photobacterium damselae* was isolated on a bacteriology plate, however, it was undetermined if it was the responsible agent for the granulomatous inflammation. Thirty cases originated from the Zoo Basel with four different water sources (cold/warm freshwater and cold/warm seawater) used in 45 aquaria (Figure 2). The majority of all examined cases (64%) originated from seawater with half of these cases belonging to the cold seawater group. The majority of the remaining cases belonged to the warm freshwater group (Figure 2a).

The majority of confirmed piscine mycobacteriosis cases originated from the warm freshwater group (63%), while only 25% belonged to the cold seawater group (Figure 2b). The Tierpark Bern cases were only differentiated between fresh- and seawater groups because only warm water is used in the Tierpark Bern. Out of the 17 submitted cases, nine cases originated from the freshwater group (53%).

Identification of involved bacteria

Of the initial 47 cases, mycobacteria could be confirmed in 17 cases. Fourteen out of the 22 histologically positive cases were also positive by PCR and sequencing. In three out of the 25 suspicious submissions, the involvement of mycobacteria was confirmed by molecular techniques (Table 1). Additionally, positive results were further subdivided into *M. marinum* and other *Mycobacterium spp.* (Table 1), where it was determined that 86% of the *M. marinum* isolated cases belonged to fish originating from freshwater. Often multiple mycobacteria species were identified within a single case, which resulted in the total number of bacteria exceeding the total number of positive cases (Table 2).

Pathology

Lesions caused by *M. marinum* were dominated by multifocal infiltration of high numbers of macrophages and small necrotic areas (Figure 3, 4) which were interpreted as acute. Two cases, where *M. marinum* involvement was identified, showed chronic-active changes, while only one case presented with chronic lesions (Figure 3, 4).

On the other hand, cases that exclusively consisted of other mycobacteria species, the pathology consisted of more chronic alterations (30%) with the lesions showing a high proportion of fibrosis (Figure 4).

Cases associated with *M. marinum* were always classified as severe, while cases involving other *Mycobacterium spp.* were classified to be less severe (Figure 4b).

Clinical signs and cause of death

Within the eleven reports, we discovered that the majority of clinical signs consisted of apathy, variation of

Table 1: Correspondence of histological results and results obtained by PCR and sequencing; proportion of *M. marinum* in cases diagnosed as piscine mycobacteriosis based on histological findings and other diagnoses

Initial diagnosis (based on histology) (n)	PCR and sequencing positive for *Mycobacterium spp.* (n) (%)	*M. marinum* (n)	Other *M. spp.* (n)
Piscine mycobacteriosis (21)	13 (62)	6	7
Other Diagnoses (26)	4 (15)	1	3

n = number of cases

Table 2: Identified *Mycobacterium* spp. in cases originating from freshwater or seawater aquaria, the accession numbers of sequences are given in parenthesis.

Water Source (n)	*Mycobacteria sp.* (n) (Accession number)
Freshwater (19)	*M. marinum* (6) (CP000854.1, AM396475.1, AB548718.1, HG917972.2), *M. ulcerans* (3) (AP017635.1, CP000325.1), *M. chelonae* (3) (AM396443.1, EF362384.1), *M. liflandii* (2) (CP003899.1, AY500840.1), *M. stomatapiae* (1) (AM902938.1), *M. fortuitum* (1) (CP011269.1), *M. gallinarum* (1) (AF312318.1), *M. chubuense* (1) (CP003053.1), *M. elephantitis* (1) (HM229790.1)
Seawater (12)	*M. ulcerans* (2) (AP017635.1, CP000325.1), *M. marinum* (1) (HG917972.2), *M. chelonae* (1) (EF362384.1), *M.holsaticum* (1) (AJ310468.1), *M. gallinarum* (1) (AF312318.1), *M. angelicum* (1) (AM902930.2), *M. szulgai* (1) (KC315739.1), *M. nonchromogenicum* (1) (KT168287.1), *M. arabiense* (1) (KC010493.1), *M. cosmeticum* (1) (KP012257.1), *M. acapulcensis* (1) (AF191094.1)

n = number of cases

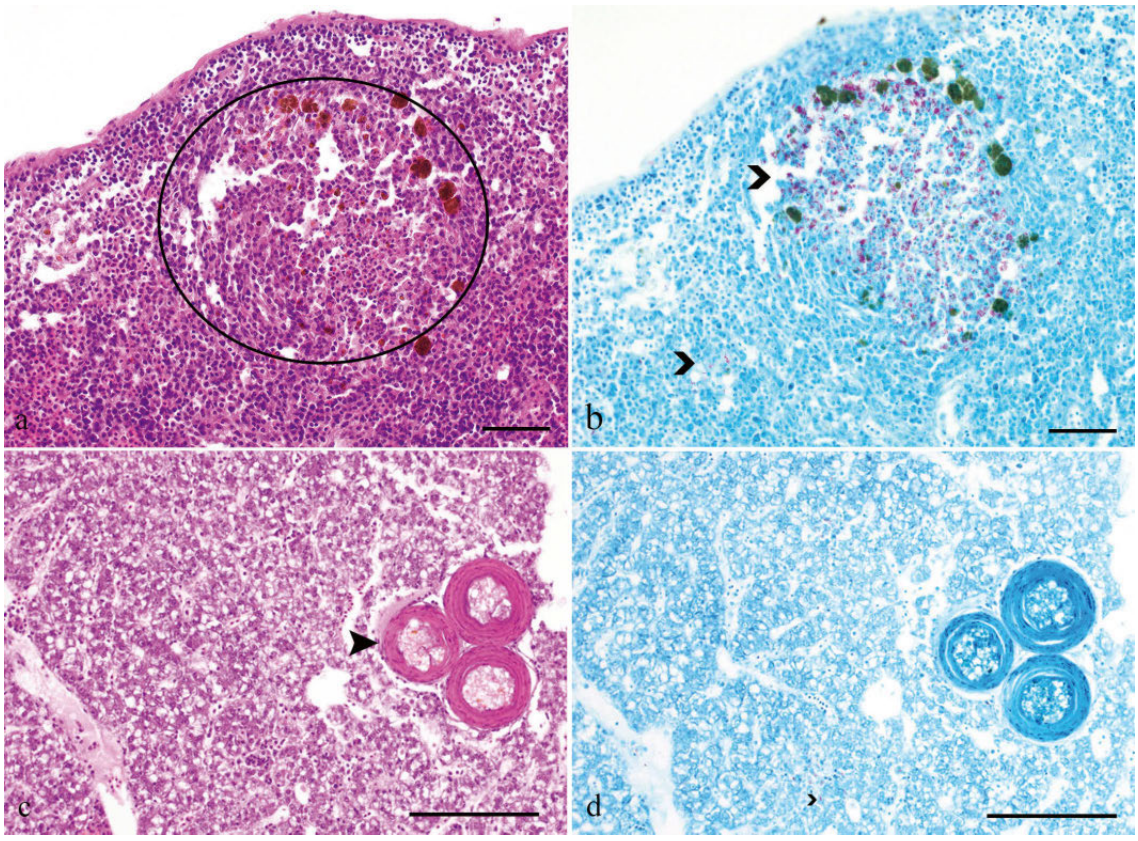

Figure 3: Histological picture of acute (a, b) and chronic cases (c, d); a. spleen of a lyretail coral fish (*Pseudanthias squamipinnis*) showing pathology interpreted as acute lesions, like infiltration with high numbers of macrophages and single cell necrosis in the centre of the infiltration (circle); b. acid fast bacteria are visible in high numbers intracellular in the macrophages and extracellular in the surrounding tissue (open arrowheads); c. liver of a lyretail coral fish (*Pseudanthias squamipinnis*) showing chronic lesions characterized by multiple well circumscribed granulomas with a central necrosis, a small rim of macrophages and peripheral thick rim of fibroblasts (closed arrowhead); d. acid fast bacteria are present only extracellular in the surrounding tissue (open arrowhead); bars = 50mm; HE stain (a, c), ZN stain (b, d)

coloration, skin ulcerations and anorexia. *M. marinum* cases never presented skin lesions, but all other clinical symptoms were similar to cases involving other *Mycobacterium spp.* (Table 3).

Three bacteria species (*M. marinum*, *M. ulcerans* and *M. chelonae*) dominated in seven out of the eight sequenced cases, where fish tuberculosis was diagnosed as cause of death (Table 4).

Table 3: Clinical signs described by the zookeepers before sending the cases to the CFWH for necropsy

Clinical signs *M. marinum* (n)	Clinical signs other *Mycobacterium spp.* (n)
variation of coloration (2), bleaching (2), apathy (4), anorexia (2)	variation of coloration (2), bleaching (2), skin lesion (6), apathy (2), anorexia (2), blindness (2), swollen abdomen (1)

n = number of observations

Table 4: Distribution of *M. marinu*m and other *Mycobacterium* spp. as probable cause of illness, as participating disease or as secondary finding

Causing agent of illness (n of confirmed cases)	*Mycobacteria spp.* (n of identified sequences)
Piscine mycobacteriosis as only probable cause of illness (n=8)	*M. marinum (4), M. ulcerans (3), M. chelonae (3), M. liflandii (2), M. holsaticum (1), M. stomatapiae (1), M. gallinarum (1)*
Piscine mycobacteriosis as participating disease (n=2)	*M. acapulcensis (1), M. cosmeticum (1)*
Secondary finding (n=7)	*M. marinum (3), M. ulcerans (2), M. chelonae (2), M. arabiense (1), M. angelicum (1), M. szulgai (1), M. chimae (1), M. nonchomogenicum (1)*

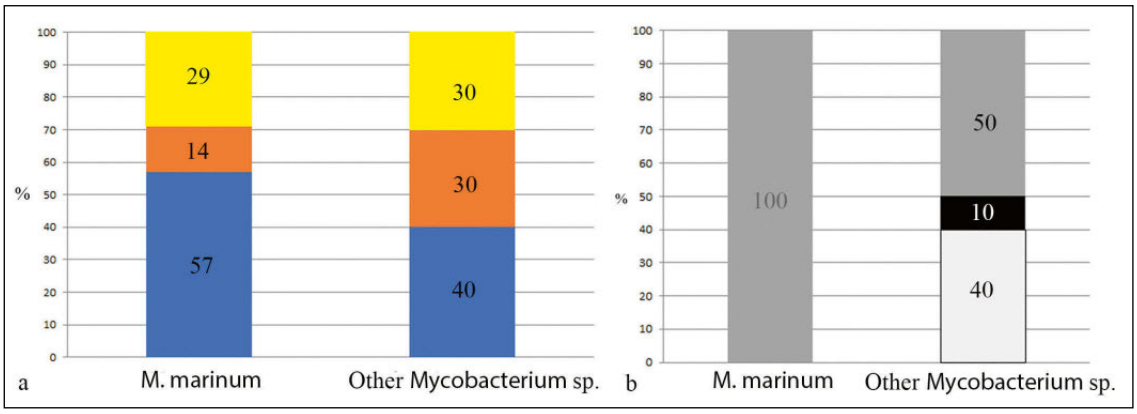

Figure 4: a. Pathology of fish infected with *M. marinum* or with other *Mycobacterium* spp., blue bars = acute lesions, orange bars = chronic lesions, yellow bars = chronic-active lesions; b. Severity of lesions in fish infected with *M. marinum* or with other *Mycobacterium* spp., grey bars = severe lesions, black bars = moderate lesions, white bars = mild lesions.

Discussion

For many years, the identification of mycobacteria was determined by gathering phenotypic data and biochemical test results of isolated species in culture medium.[3,6] However, in the last few decades, new methods, using advanced technology, have provided a tool that allows for rapid and accurate identification of mycobacterial species, thus replacing older, more laborious methods.[6,13,21,25] A particular method on the rise is multiplex polymerase chain reaction (PCR), which allows for the simultaneous amplification of more than one sequence of target deoxyribonucleic acid (DNA) in a single reaction, thus saving time and money.[11] One drawback of using PCR is that the sensitivity of detection has not been fully confirmed yet.[28,30,37] In previous studies that tested the efficiency of the PCR detection found a success rate between 67-77%,[25,30] while we had a slightly lower detection success rate of 64%.

As a first step in the diagnostic cascade, presence of clinical signs is often judged. However, clinical signs of fish tuberculosis are mostly unspecific and cannot lead to final diagnosis. Signs, such as apathy, variation of coloration and anorexia were present in our study and these signs have been well documented in other studies as well.[3,4,28] In addition to the aforementioned clinical signs, the presence of shallow irregular ulcerations have also been described as a typical sign for piscine mycobacteriosis.[3,4,8,28] The presence of skin lesions can be interpreted as a sign of a chronic prolonged course of the disease, which can also be a path for shedding infectious material via the wounds. Interestingly, we determined approximately 30% of our cases induced by *Mycobacterium spp.* beside *M. marinum* to show chronic lesions with a high proportion of skin ulcerations. However, skin ulcerations were absent in all fish affected by *M. marinum*. Additionally, *M. marinum* infections mostly showed a systemic distribution and were characterized by acute changes.

M. marinum is a slow-growing atypical *Mycobacterium* that is commonly found in freshwater and marine fish.[1] It is the most prominent, pathogenic *Mycobacterium sp.* found in fish tuberculosis cases.[4,8,23,34] The combination of severe and mostly acute lesions supports the high pathogenicity of this species. The majority of cases involving *M. marinum* infections are found in fish living in warm seawater.[28] However, in this study, 86% of *M. marinum* cases were found in freshwater aquaria.

M. marinum is closely related to another important *Mycobacterium* species, *M. ulcerans*.[36] In our study, five isolates were confirmed as *M. ulcerans*. This species is considered to have the highest risk to develop a zoonotic potential, and it was described as the causative agent for the Buruli ulcer in humans.[38,39] This disease has been recently categorized as a new emerging infectious disease in humans.[34] Clinical signs in humans are thought to be related to the toxin mycolactone.[33] In fish, no toxic effects of mycolactone have been reported so far.[8,20] In medaka and zebrafish experimentally infected with M. ulcerans, no mortality occurred and only a mild inflammatory response was seen in histopathology.[19] In accordance to our cases, *M. ulcerans* was always diagnosed as co-infection with *M. marinum*.

Furthermore, a mycobacteria strain was identified not reported before to occur in fish. *M. holsaticum* was described so far to occur only in mammals, mostly humans, causing pulmonary tuberculosis.[18,35] We identified *M. holsaticum* in a silver mooney fish (*Monodactylus argenteus*) from a seawater aquarium, presenting multiple skin ulcerations. In the same animal, *M. marinum* and *M. ulcerans* were demonstrated. Therefore, the role of *M. holsaticum* for the health of the fish remains unclear. The potential of fish as reservoirs for this *Mycobacterium* species has to be further investigated.

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Mycobactériose du poisson – Implication d'espèces bactériennes et reflets en pathologie

La mycobactériose du poisson est une maladie létale avec un potentiel zoonotique qui se trouve dans le monde entier chez les poissons d'eau douce et marins. Plus de 20 souches de *Mycobacterium spp.* sont à ce jour connues chez les poissons, mais la pathogénicité est actuellement inconnue pour la plupart d'entre elles. Cependant, *M. marinum* est signalé comme l'un des agents les plus pathogènes pour les poissons et il est impliqué dans des cas de zoonoses. Nous avons examiné 47 cas différents provenant de deux jardins zoologiques où la tuberculose du poisson a été identifiée ou suspectée au cours des dix dernières années. Nous avons recueilli des données de PCR et de séquençage qui ont ensuite été comparées aux données cliniques et à la pathologie précédemment collectées. Les signes cliniques causés par *Mycobacterium spp.* étaient similaires dans tous les cas, à l'exception des cas infectés par *M. marinum*, chez lesquels manquaient les lésions cutanées. Les lésions histologiques observées dans les infections par *M. marinum* tendaient à être plus aiguës et graves comparées aux lésions provoquées par d'autres espèces de *Mycobacterium spp.* La majorité des cas de *M. marinum* ont été documentés chez des poissons marins. Contrairement aux études précédentes, nous avons constaté que cette espèce était la principale bactérie présente chez les poissons d'eau douce. Fait intéressant, nous avons détecté *M. holsaticum* dans l'un des systèmes d'eau de mer examinés dans ce projet, ce qui est le premier cas confirmé de la présence de cette espèce de Mycobacterium chez un poisson.

Mots-clés: tuberculose du poisson; *Mycobacterium marinum*; pathologie; PCR; séquençage

Tubercolosi ittica – Coinvolgimento di specie batteriche e considerazione patologiche

La micobatteriosi ittica è una malattia letale che si riscontra sia nei pesci di acqua dolce che salata in tutto il mondo, ed è potenzialmente una zoonosi. Al giorno d'oggi più di 20 specie di *Mycobacterium spp.* sono conosciute, ma per molti di loro, la patogenicità è sconosciuta. Tuttavia, *M. marinum* è identificato come uno dei maggiori agenti patogeni ed è coinvolto in casi di zoonosi. In questo studio abbiamo analizzato 47 casi provenienti da due zoo, nei quali la tubercolosi ittica è stata identificata o è stata sospettata negli ultimi dieci anni. Abbiamo raccolto i dati della PCR e del sequenziamento, per poi paragonarli a dati clinici e patologici raccolti in precedenza. I sintomi clinici causati dal *Mycobacterium spp.* erano simili in tutti i casi esaminati eccetto per quelli infettati da *M. marinum*, nei quali lesioni cutanee erano assenti. La lesioni causate da *M. marinum*, osservate in istologia, risultano più acute e gravi rispetto a quelle causate da altri *Mycobacterium spp.* La maggior parte dei casi di *M. marinum* sono stati rilevati nei pesci d'acqua salata, contrariamente a studi precedenti, dove la presenza di questa specie di batterio prevaleva nei pesci d'acqua dolce. Per la prima volta in uno studio, è stata constatata la presenza di *M. holsaticum* in uno dei sistemi di acqua marina, facendone dunque il primo caso documentato di questo agente in un pesce.

Parole chiave: Tubercolosi ittica; *Mycobacterium marinum*; patologia; PCR; sequenziamento

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