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Granulomas Caused by *Mycobacterium* sp. in farmed turbot *Scopthalmus maximus* (Linnaeus, 1758)

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Abstract

Turbot, *Scophthlalmus maximus*, is a pleuronectiform fish that occurs in the northeast Atlantic along the European coast and in the Mediterranean Sea, and is produced in fish farms since the last quarter of the twentieth century. During a survey conducted in a turbot fish farm, nodular formations were occasionally observed in several organs, especially in the kidney and in the spleen. Microscopic observations showed that these nodules contained acid-fast bacilli. Molecular identification of the isolated bacteria revealed the *Mycobacterium* genus. Although no abnormal mortalities were evident morbidity was observed. The normal development and welfare of infected fish decrease and the condition factor, the haematocrit and haemoglobin concentration in blood decreases significantly with the increase of nodule abundance.

Keywords: Turbot, Aquaculture, bacterial infection, *Mycobacterium.*

Introduction

The turbot *Scophthlalmus maximus* (Linnaeus, 1758) is a pleuronectiform fish that occurs in the northeast Atlantic along the European coast and in the Mediterranean Sea and is produced in fish farms since the last quarter of the twentieth century. This fish species is very much appreciated due to its refined flesh and thus reaches high market prices. Spain is the major European producer, accounting for more than 80% of European production (Eurostat, 2009). In Portugal, turbot production is experiencing an expansion due to the development of some farms and the recent opening of a large scale operator. According to literature, the most deleterious infections for turbot culture in the Iberian Peninsula are caused by bacteria and parasites. Concerning bacteria *Aeromonas salmonicida*, *Vibrio* spp., *Photobacterium damselae* subsp. *piscicida* (as *Vibrio damsela*), *Edwardsiella tarda*, *Tenacibaculum maritimum* like species, *Streptococcus parauberis*, *Mycobacterium marinum* and *M. chelonae* have been reported in turbot (Fouz *et al*., 1991; 1992; Alsina & Blanch, 1993; Angulo *et al*., 1994; Nougayrede *et al*., 1994; Toranzo *et al*., 1994; Domenech *et al*., 1996; Cerdà-Cuèller *et al*., 1997; Santos *et al*., 2002; Montes *et al*., 2003; Villamil *et al*., 2003; Padrós *et al*., 2006; Piñeiro-Vidal *et al*., 2007; Najimi *et al*., 2008).

Members of the genus *Mycobacterium* are Gram-positive, acid-fast, non-motile, rod-shaped bacteria that cause a progressive chronic disease. Some species are zoonotic pathogens that can cause lesions in skin, mainly in the hands and especially in immune debilitated individuals (Aubry *et al*., 2002; Ghittino *et al*., 2003; Decostere *et al*., 2004; Streit *et al*., 2006; Gauthier & Rhodes, 2009; Jacobs *et al*., 2009; Wu *et al*., 2012; Seneviratne & Herieka, 2013). This condition is often reported as fish tank hobby diseases or fish tank, swimming pool or aquarium granuloma. In recent years, an increased number of *Mycobacterium* species has been isolated from fishes but *M. marinum*, *M. chelonae* and M. *fortuitum* are the most commonly identified species infecting fish (Gauthier & Rhodes, 2009). Although this infection may remain asymptomatic for long periods, the most common symptoms are exophthalmia and nodular lesions in kidney and spleen, occasionally in liver, heart, and mesentery and sporadically in branchial tissue and gonads (Woo *et al*., 2002; Gauthier & Rhodes, 2009; Noga, 2010; Novotny *et al*., 2010; Roberts, 2012). This disease has been reported in numerous cultured and wild fish species namely European seabass (*Dicentratrchus labrax*), gilthead seabream (*Sparus aurata*), snakehead (*Channa striata*), channel catfish (*Ictalurus punctatus*), striped bass (*Morone saxatalis*), turbot (*Scophthalmus maximus*), Atlantic salmon

(*Salmo salar*), European tench (*Tinca tinca*) and Atlantic guitarfish (*Perca fluviatilis*) (Knibb *et al.* 1993; Santos *et al*. 2002; Brocklebank *et al*. 2003; Aranaz *et al*. 2008; Bozzetta *et al*. 2010; Anderson *et al*., 2012). Nevertheless, mycobacteriosis is not listed by the European Union (Annex IV, Part 2, of Council Directive 2006/88/EC), and no consequences of the disease exist in Portuguese aquaculture.

In this work, we report on the infection levels and the pathology caused by *Mycobacterium* sp. in turbot during a 10-month period.

Material and Methods

Fish examined

From September 2009 to June 2010, turbot from an intensive fish farm were surveyed in order to evaluate their health status. Fish (n=102) were measured in cm to the nearest decimal point (length to peduncle, L_p = 25.2 ± 1.5), weighed in g to the nearest decimal point (W= 579.7 ± 121.2) and the condition factor ($K=100WL_p^{-3}$) was calculated. All fish were examined, dissected and observed for the presence of any abnormality / lesion according to routine methods (see Noga, 2010). The abundance of detected nodules in kidney was scored as zero (absence of nodules), low (a few nodules dispersed in organ), high (nodules occupying 1/3 to 2/3 of the organ volume) and very high (nodules occupying more than 2/3 of the organ volume).

Haematological analysis

Blood was collected from the caudal vein using heparinized syringes and the haematocrit (Ht) was determined by the microhematocrit standard method, haemoglobin concentration (Hb) was determined by the cyanomethemoglobin method and red blood cells (RBC) were counted on diluted blood samples (1:100 dilution in Dacie`s fluid) in a Neubauer chamber (Blaxhall & Daisley, 1973).

Histology

Samples of kidney, spleen, liver, digestive tract and gonads were fixed in 10% phosphate-buffered formalin, routinely processed, stained with Haemotoxylin and Eosin (H&E) or Ziehl-Neelsen (ZN) and examined under a light microscope (LM).

Mycobacterium isolation and identification

Granulomatous tissues were aseptically homogenized and treated with the BBL Myco Prep® decontamination Kit (Becton Dickinson and Company, USA), according to the manufacturer's instructions and 200 µL of the supernatant were inoculated on the Lowenstein-Jensen (L-J) medium and incubated at 25ºC according to the methodology used by Santos *et al*. (2002). DNA was extracted from bacterial isolates using the E.Z.N.A.™ Bacterial DNA Kit (Omega Bio-Tek Inc., USA). DNA from−thirty-four isolates was subjected to PCR with primers T39 and T13 as described by Talaat *et al*. (1997); obtained fragments were treated with restriction enzymes *Ban*I and *Apa*I, in order to identify *Mycobacterium* species through unique restriction patterns, as described by the same authors. After restriction, the fragments were separated by electrophoresis on a 1% agarose gel and visualized with SYBR Safe DNA gel stain. Four isolates were selected with the aim of confirming the identification of these mycobacteria to species level through 16s RNA gene sequencing. DNA of these isolates (extracted as stated above) was subjected to PCR with primers 27F (domain Bacteria) and 1492R (universal), and the 16S RNA genes amplified were purified with Diffinity RapidTip® (Pre-sanger sequencing; Diffinity Genomics Inc.; USA) and sent to Stab Vida (Lisbon, Portugal) for sequencing. The sequences obtained were aligned with other GenBank sequences.

Statistical analysis

The SPSS 18.0 software package was used for data analyses. Mean and standard deviation were calculated for K, Ht, Hb and RBC considering classes of abundance of kidney nodules (Class 0 - kidney without nodules, Class 1 - kidney with low numbers of nodules, Class 2 - kidney with high numbers of nodules and Class 3 kidney with very high numbers of nodules). The relationship between abundance of nodules in kidney and K, Ht, Hb and RBC was analysed using Spearman's rank correlation test. The correlations were considered to be significant at $p < 0.05$.

Results

Fish examination

Most of the fish did not present external pathological changes except for nodular formations in the fin bases (Fig. 1) and unilateral or bilateral exophthalmia (Fig. 2) observed in 9.8% and 2%, respectively, of the examined fish. Internally, the most significant macroscopic pathological change was the presence of miliary white to greyish nodules in kidney, spleen and occasionally in liver in 71.6%, 65.7% and 17.6%, respectively, of the inspected fish (Fig. 3). This symptomatology was rarely observed in the digestive tract (4.9%) and in the gonads (1%). Nodule intensity varied greatly from a small number dispersed in the organ (46%) to a very high number occupying almost all the organ (5%). In almost all examined fish, kidney and spleen nodule abundance was similar (only 7 fish presented low numbers of nodules in kidney and no nodules in spleen and 1 presented low numbers of nodules in spleen and no nodules in kidney).

Fig. 1: Haemorrhage and white to yellowish nodular formations at the base of fins.

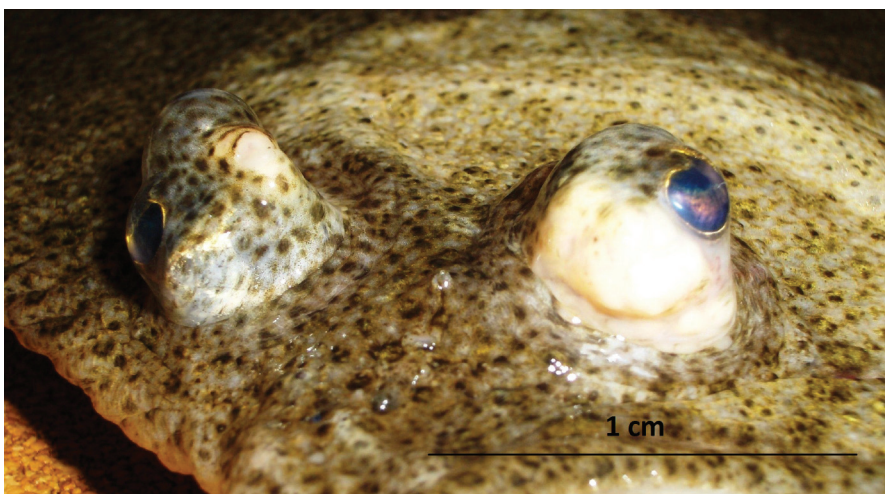

Fig. 2: Bilateral exophthalmia caused by large whitish nodules.

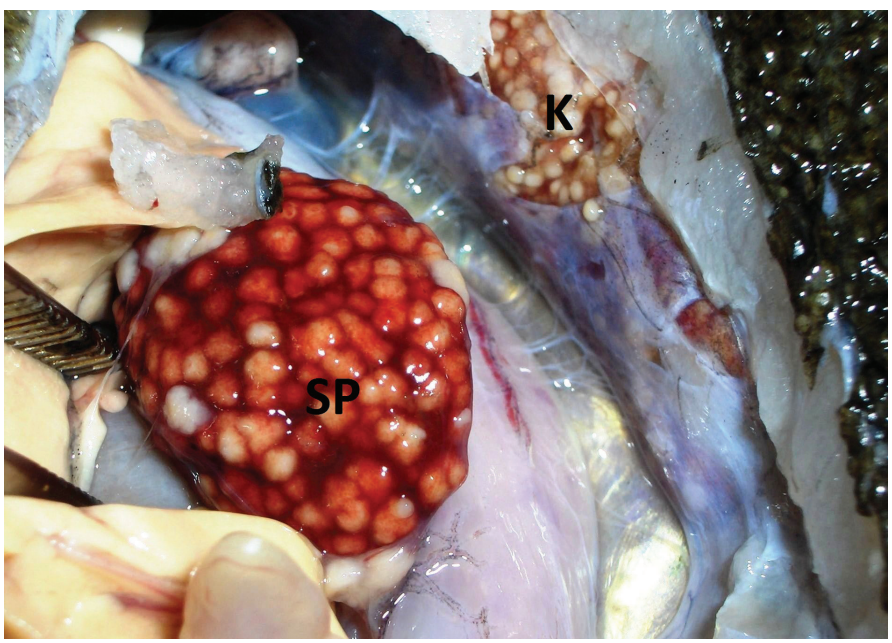

Fig. 3: Miliary white to greyish nodules in kidney (K) and spleen (SP).

Condition Factor and Haematological analysis

The mean values of the condition factor, haematocrit, haemoglobin concentration in blood and RBC from each kidney nodule abundance classes are presented in Table 1. Excluding RBC, significant negative correlations were detected between these parameters and the abundance of nodules in kidney.

Histopathology

Histological examination revealed granulomatous lesions characterized by a central region constituted by epitheloid cells surrounded by mononuclear inflammatory cells, especially lymphocytes (Fig. 4). However, most of the granulomatous lesions presented a central necrotic region surrounded by an inflammatory tissue capsule rich in mononuclear cells (Fig. 5). Structurally, the most affected organs were strongly injured. In some Ziehl-Neelsen staining sections it was possible to observe acid–fast bacilli in the core of the granulomas (Fig. 6).

Mycobacterium isolation and identification

Bacterial growth was obtained from all symptomatic and some asymptomatic fish. Pure cultures of rough, cream coloured colonies, developed after 7-10 days of incubation at 25ºC. After exposure to light, all colonies turned yellow (photochromogenic). A 924 bp DNA fragment was amplified from all thirty-four isolates (Fig. 7). Restriction Enzyme Analysis (REA) of this fragment revealed that, from isolate ACP1.03.10, two fragments were obtained with each enzyme (approximate sizes 562 bp and 363 bp with enzyme *Ban*I, and 812 bp and 112 bp with enzyme *Apa*I; Fig. 8). From the other isolates, the same two fragments were obtained with *Apa*I treatment, but with *Ban*I the 924 bp fragment remained undigested (Fig. 9). Four isolates (ACP1.03.10, ACP25.03.10, ACP10.10.09 and ACP1.10.09), representative of the two patterns obtained with PCR and REA, were chosen for 16S RNA gene sequencing. Alignment of the sequences with other sequences in GenBank confirmed that our isolates belong to the genus *Mycobacterium*, but didn't support identification to species level.

Table 1. Relationship between abundance of nodules in kidney (Cl 0 – absence of nodules, Cl 1 - low number of nodules, Cl 2 – high number of nodules, Cl 3 - very high number of nodules) and condition factor (F), haematocrit (Ht), blood haemoglobin concentration (Hb) and red blood cell counts (RBC) (mean ± standard deviation). N – number of fish examined. Spearman correlation coefficient (S – significant, NS - not significant).

Abundance Class of nodules	N	F	Ht (%)	Hb (g/100 mL)	RBC (x10^6/mm^3)
Cl 0	29	3.695 ± 0.478	21.1 ± 6.0	4.22 ± 1.59	1.924 ± 1.124
Cl 1	47	3.652 ± 0.497	20.0 ± 5.7	4.00 ± 1.50	2.196 ± 1.190
Cl 2	21	3.467 ± 0.330	16.4 ± 5.9	3.27 ± 1.95	1.445 ± 0.538
Cl 3	5	3.317 ± 0.348	19.2 ± 3.5	3.23 ± 0.98	3.332 ± 1.761
Spearman correlation		S	S	S	NS
p		0.015	0.010	0.006	0.785
(r)		(- 0.241)	(- 0.253)	(- 0.269)	(- 0.027)

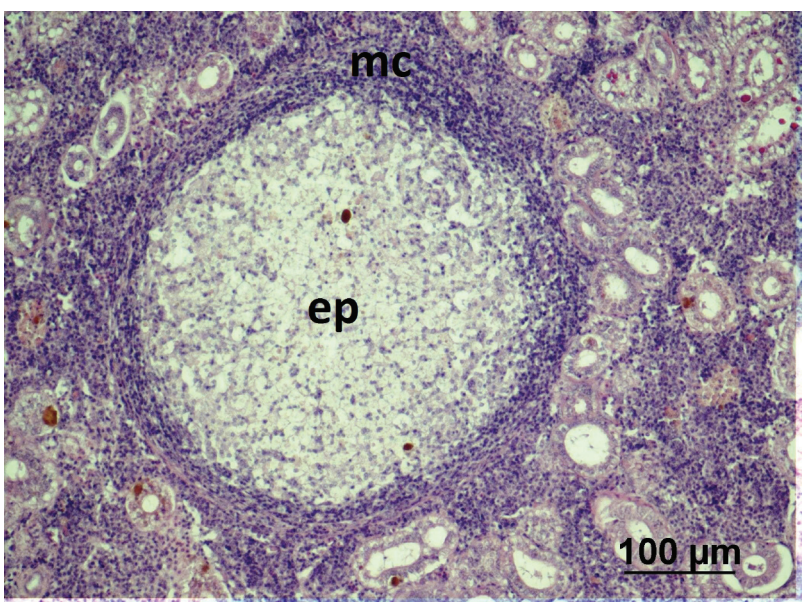

Fig. 4: Early kidney granulomatous lesions with epitheloid cells (ep) and mononuclear cells (mc) (H&E).

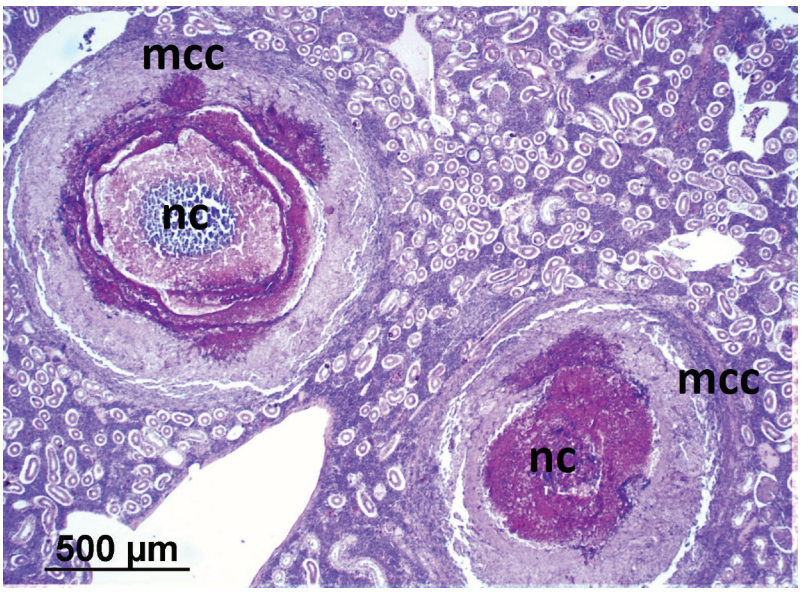

Fig. 5: Kidney granulomatous formations with central necrotic material (nc) and mononuclear cells capsule (mcc) (H&E).

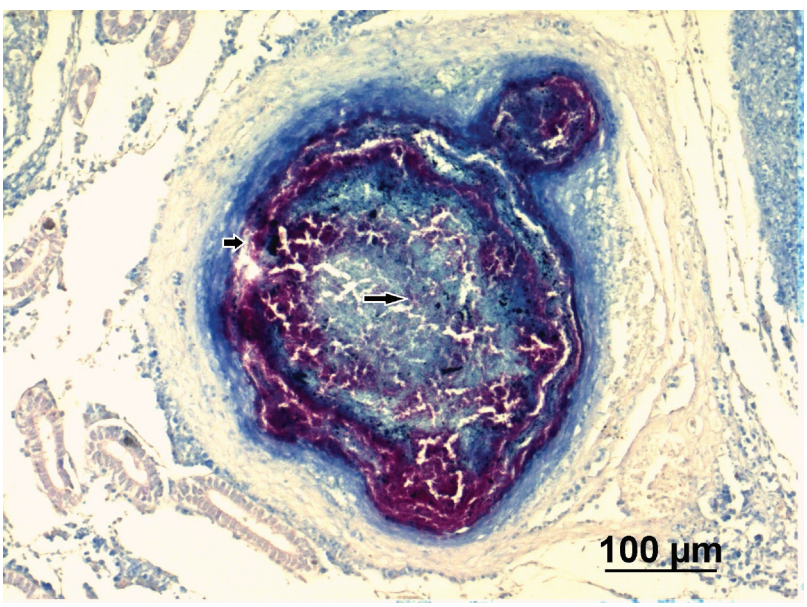

Fig. 6: Granuloma with acid fast bacteria (arrow) (ZN).

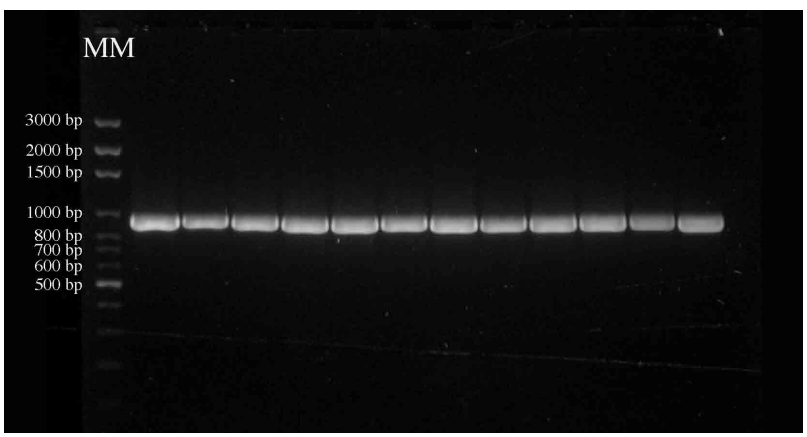

Fig. 7: Fragment of 924 bp amplified from the 16S RNA gene with gene-specific primers T39 and T13 designed by Talaat *et al*. (1997) (MM – molecular weight marker).

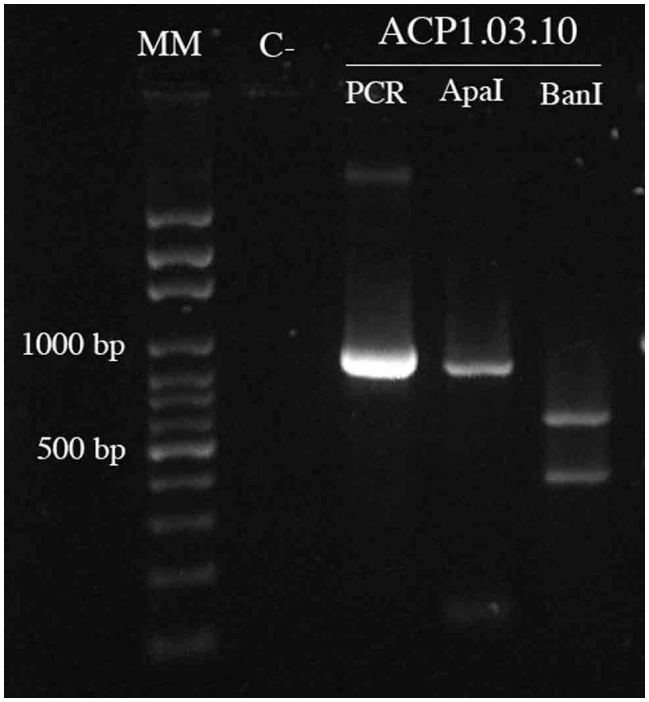

Fig. 8: Restriction enzyme analysis of *Mycobacterium* isolate ACP1.03.10 (PCR, 924 bp undigested PCR product; *Apa*I, fragments obtained after restriction with enzyme *Apa*I; *Ban*I, fragments obtained after restriction with enzyme *Ban*I; MM – molecular weight marker).

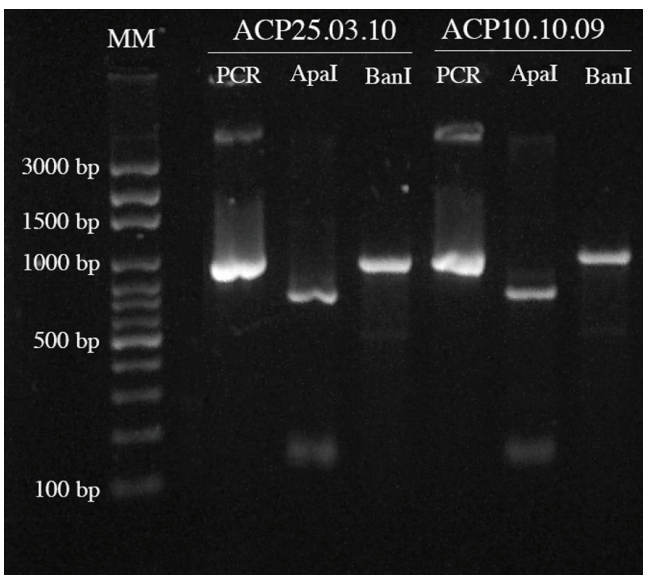

Fig. 9: Restriction enzyme analyses of all *Mycobacterium* isolates included in this study, except isolate ACP1.03.10 (legend as in Fig. 8).

Discussion

Growth at 25ºC in 7-10 days on the Lowenstein-Jensen (L-J) medium and the photochromogenic character of the colonies indicated that the isolates belong to the genus *Mycobacterium*, presumably *M. marinum*. To confirm this assumption, the protocol developed by Talaat *et al*. (1997) was employed and the expected 924 bp fragment was amplified from the 16S rRNA gene. These authors developed an assay based on PCR and REA for a rapid diagnostic test to identify *M. marinum*, *M. chelonae* and *M. fortuitum*, the most commonly reported *Mycobacterium* species infecting fish. According to this assay, restriction of the 924 bp fragment with *Ban*I yields two fragments (562 bp and 363 bp) in *M. chelonae* and *M. fortuitum*, but the fragment remains undigested in *M. marinum*, while treatment with *Apa*I yields two fragments (812 bp and 112 bp) from *M. chelonae* and three fragments (677 bp, 132 bp and 115 bp) both from *M. marinum* and *M. fortuitum*. In our study, only the restriction pattern of isolate ACP1.03.10 permitted its identification as *M. chelonae* according to Talaat *et al*. (1997). The pat-

tern obtained with all other isolates was inconclusive, as the 924 bp fragment amplified from the 16S RNA gene remained intact when treated with enzyme *Ban*I, which is consistent with *M. marinum*, but its restriction with enzyme *Apa*I resulted in 2 fragments, with sizes consistent with those reported by the same authors for *M. chelonae*, but not consistent with *M. marinum*. Thus, PCR amplification and REA, according to the assay reported by Talaat *et al*. (1997), confirmed that all our isolates belong to the genus *Mycobacterium*, but only in one case (isolate ACP1.03.10) allowed assignment to a particular species (*M. chelonae*). The confirmation that our isolates are members of the genus *Mycobacterium* also resulted from sequencing the 16S RNA gene, but sequencing didn't assign any of our bacteria to any particular species. In fact, all the sequences obtained were found to be close – although not identical – to many strains belonging to different species of this genus. This result is not surprising, as high homology in 16S RNA gene, as well as in other genes, of *Mycobacterium* isolated from fish has been reported by a number of authors (see Jacobs *et al*., 2009). Although our sequence results didn't allow unequivocal species determination, a difference was obtained in the most probable hits with GenBank sequences, suggesting that isolate ACP1.03.10 could be closer to *M. chelonae* and the other isolates closer to *M. marinum*. This being the case, it would be in agreement with the findings of Santos *et al*. (2002), who also detected these two species, identified by rate of growth, colonial morphology and biochemical methods.

Although no unusual mortalities were observed, it was noticed that fish did not grow as fast as usual. Apart from the occurrence of granulomas, *Amyloodinium ocellatum* trophonts were observed at very low levels (see Saraiva *et al*., 2011) in examined fish. Nevertheless, the low growth observed seems to be caused by these nodular lesions since the condition factor decreased with the increase of nodules in internal organs. The statistical analysis shows that beyond the condition factor, the haematocrit and the haemoglobin concentration in blood presented a negative correlation with the abundance of nodules in the most infected organ, the kidney. The replacement level of functional haematopoietic tissue in the infected fish is probably the cause of these observations. These conditions are reported in renal and splenic diseases of different aetiology (see Roberts, 2012) and according to Noga (2006) myelophthisic anaemia due to fibrosis of haematopoietic tissue may occur in chronic granulomatous diseases such as mycobacteriosis. As a conclusion, it can be stated that although *Mycobacterium* infection does not cause significant mortality, it affects the growth and welfare of fish. This disease can be transmitted from fish to fish, but also vertically; the pathogen can survive in the environment for years and can be insidious and difficult to eradicate (see Noga, 2010), being a threat for many aquaculture industries.

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