ISOLATION AND CHARACTERIZATION OF PATHOLOGY IN CASE OF MASSIVE MORTALITY BY *Photobacterium damselae* subsp. *piscicida* IN *Rachycentron canadum*

ISOLAMENTO E CARACTERIZAÇÃO DA PATOLOGIA EM CASO DE MORTALIDADE MACIÇA POR Photobacterium damselae subsp. piscicida *EM* Rachycentron canadum

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ABSTRACT: This study aimed to investigate outbreak with high mortality in cultured juvenile cobia occurred in Southeast Brazil in 2011. Fish displayed retarded growth rates, lethargy, fin ulceration, skin depigmentation, corneal opacity, and physical deformities. Internally, livers were increased in volume and pale in different degrees. Firm whitish nodules were disseminated in the liver, kidney and spleen. A moderate number of parasites identified as *Neobenedenia melleni* were recovered from the body surface. Microscopically, severe hepatic steatosis and extensive granulomatous lesion were identified in all fish sampled. Microbiological analysis of moribund fish revealed the presence in pure culture of a Gram-negative bacterium identified as *Photobacterium damselae* subsp. *piscicida* using biochemical and molecular characteristics. Analysis of the partial 16S rRNA sequences confirmed the results demonstrating high identity (98%). The isolates were sensitive to chloramphenicol and enrofloxacin and resistant to ciprofloxacin, florfenicol, doxycycline hydrochloride, norfloxacin, oxytetracycline, and tetracycline. Chronic pasteurellosis was considered as the main problem in the farm, while hepatic steatosis and parasitic infestation may have contributed to the development of the process.

KEYWORDS: Cages. Pseudotuberculosis. Granulomas. Antibiotic resistance. *Neobenedenia melleni.*

INTRODUCTION

Cobia *Rachycentron canadum* is a carnivore fish distributed worldwide in tropical and sub-tropical seas, except the eastern Pacific (COLLETTE et al. 2015). Cobias have great potential for commercial aquaculture in Brazil due to their fast growth rate, good market demand, feed conversion rates, and their easy adaptation to captivity (BENETTI et al. 2007). However, commercial production is still low (CAVALLI et al. 2011).

The rapid expansion and intensification of cobia production led to an increase of disease outbreaks (CHU et al. 2013). Infectious disorders caused by virus, parasites and bacteria occur in all stages of production. However, bacterial diseases are the main problem in cultured cobias. Vibriosis, pasteurellosis, mycobacteriosis, furunculosis, and streptococcosis are the most common diseases (LIAO et al. 2004).

Pasteurellosis is recognized as one of the most threatening problems in cage-cultured cobia, also known as photobacteriosis or pseudotuberculosis, is caused by the bacterium *Photobacterium damselae* subsp. *piscicida* (ROMALDE 2002). The etiological agent is a halophilic, Gram-negative, non-motile, and bipolar coccobacillus. The first description of the disease occurred in white perch, and striped bass, in 1963, in Chesapeake Bay, USA (SNIESZKO et al. 1964). Photobacteriosis is characterized as an acute septicaemia, while the chronic form of the disease is unusual (HAWKE 2012).

Parasitic infections rank second only after bacterial diseases in cobia aquaculture (MCLEAN et al. 2008). Cobia harbor all classes of parasites (crustacean, myxosporidia, flukes, worms, and protozoan) that infest the gastrointestinal tract, gills and skin (CHU et al. 2013). The impact of parasites on fish health is correlated with the level of infestation. Minor infections can cause reductions in

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growth and provide portals for the entry of other pathogenic agents (MCLEAN et al. 2008). Monogenean *Neobenedenia sp.* parasitizes the skin, fins and eyes and it has been related to high mortality in cobias (LOPEZ et al. 2002; OGAWA et al. 2006).

The present work describes a case of massive mortality in juvenile farmed cobias, in Brazil. Diagnostic investigation demonstrated multiple causes involved such as chronic pasteurellosis by *P. damselae* subsp. *piscicida*, ectoparasitic infestation by *N. melleni* and severe hepatic steatosis. The diagnostic procedure, histopathological findings and sensitivity pattern to antimicrobials from the bacterial isolate were described.

MATERIAL AND METHODS

Fish and farm conditions

In the winter of 2011, a disease outbreak occurred in cobias from sea shore cages located on the northern coast of Sao Paulo State, Brazil (23° 48' 54" S 45° 22' 14" O). This fish farm presented twenty see cages (400 m^3/ 10 x 10 x 4 m). Fish exhibited high mortality rates, retarded growth and lethargy. Water temperature during the first half of the year fluctuated between 19ºC and 29ºC. Sixty-one moribund juveniles cobias, mean mass ± SD (229.6 ± 84.4 g), mean size ± SD (27.5 ± 2.3 cm), were sampled for microbiological and histopathological analysis. The fish farm started with approximately 20 000 fries in November 2010 and after the outbreak there were only 500 fish (97.5% of mortality). The owners applied a treatment with florfenicol for 10 days; however, they did not observed positive effects of the treatment. The study was carried out according to the Brazilian animal welfare standards and ISO - International Organization for Standardization (2006) and was approved by the Ethics Committee (protocol nº 016000/12).

Isolation and characterization of the bacteria

Fish that showed clinical signs of disease (n=15) were euthanized with overdose of benzocaine solution (150 mg/L). Samples of brain, head kidney, spleen, and liver were collected and plated onto brain heart infusion broth (BHIB, Difco™, USA) with 2% NaCl. Samples were kept on ice and send to the laboratory facilities. Broth aliquots were plated on brain heart infusion agar (BHIA, Difco™, USA) with 5% sheep blood and 2% NaCl. Identification of presumptive colonies was performed as described by Thyssen et al. (1998) and based in the standard procedures described in the Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). The strain ATCC 51736 was used as a positive control.

Putative colonies were inoculated on the surface of two different plates (3 per fish): thiosulphate citrate bile salt sucrose agar (BBL™, USA) supplemented with 1.5% NaCl (TCBS-1.5) and 5% sheep blood agar supplemented with 3% NaCl. Both plates were incubated at 30°C for 24h. Pure presumptive colonies were used for further identification by using a commercial miniaturized biochemical tests kit API 20E (bioMérieux, France) according to the manufacturer instructions. Pure stock cultures were maintained at 80°C in 15% glycerol (v/v) trypticase soy broth (TSB) supplemented with 2% NaCl.

16S rRNA polymerase chain reaction (PCR), sequencing and phylogenetic analysis

Total RNA from pure all cultures were extracted with the commercial kit DNeasy (Qiagen, Germany) and the 16S rRNA gene of the isolate was amplified by PCR and two random samples were sequenced. Sequences were aligned and compared with available sequences in the NCBI GenBank (http://www.ncbi.nlm.nih.gov) using BLAST. Procedures were performed as previously described by Mian et al. (2009).

P. damselae sp. *piscicida* was confirmed by polymerase chain reaction (PCR), using the forward primer (5'-AGGGGATCCGATTATTACTG-3') and reverse primer (5'-TCCCATTGAGAAGATTTGAT-3') (RAJAN et al., 2003).

Antibiotic susceptibility

Antibiotic susceptibility was determined using the Kirby-Bauer (K-B) diffusion method according to the Clinical & Laboratory Standards Institute (CLSI) standards for antimicrobial susceptibility testing. Bacterial colonies from BHI plates were suspended in sterile phosphate buffered solution (PBS) and turbidity adjusted to 0.5 McFarland standard. The mixture was inoculated onto Mueller-Hinton Agar (BD Difco™) supplemented with 2% NaCl, ten minutes later antibiotic discs were added (chloramphenicol, florfenicol, ciprofloxacin, doxycycline hydrochloride, enrofloxacin, norfloxacin, oxytetracycline, and tetracycline). These antibiotics are the most common used in aquaculture worldwide. After 24h of incubation at 30°C, the diameters of the inhibition zones were measured using Vernier calipers. Based on this, the

susceptibility of the isolate to each antibiotic was determined according to CLSI (2012).

Histopathology and Parasitological analysis

Fragments from skin, eyes, gills, heart, liver, head kidney, spleen, stomach, pyloric caeca, and intestine (n=46) were processed according to Survana et al. (2013) and stained by hematoxylin-eosine and Ziehl-Neelsen method (ZN) (MANRIQUE et al., 2012). Examination was performed by light microscopy (Olympus BX 51), and histopathological photos were taken with a camera DP72 Olympus (Software cellSens v. 1.5). Ectoparasites were found over the body surface and they were collected with the aid of tweezers and magnifying glass. Fixation and preservation were performed in 5% buffered formalin and staining with Carmim and Gomori's trichrome solution according to the methods of Kerber et al. (2011).

RESULTS

Bacteria isolation, characterization and analysis of the 16S rRNA gene

Only one type of colonies was isolated from the tissues of the all diseased cobias. The isolate (PDP) showed to be a Gram-negative, nonhemolytic, rod-shaped, nonmotile bacterium, positive for oxidase and catalase. PDP did not grow in TCBS-1.5 (HAWKE, 2012). It exhibited an API 20E profile: 2005004. Comparison with 16S rRNA sequences in GenBank (http://www.ncbi.nlm.nih.gov) showed that the isolate was most similar to members of *P. damselae* subsp. *piscicida* (98% identity, Figure 4 / 71).

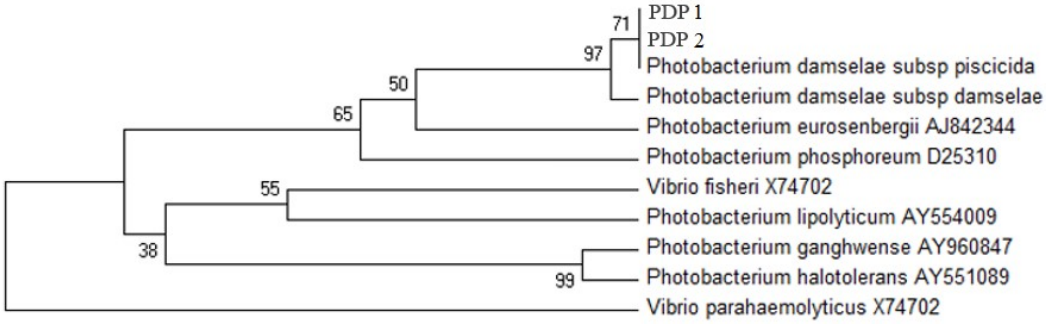

Figure 4. Phylogenetic tree based on 16S rDNA gene sequences of two samples (PDP1 and PDP2 / "Amostra").

Antibiotic susceptibility patterns

The strain was susceptible to two antibiotics: chloramphenicol and enrofloxacin, and resistant to ciprofloxacin, florfenicol, doxycycline hydrochloride, norfloxacin, oxytetracycline and tetracycline (Table 1).

Table 1. Susceptibility test for PDP to seven different antibiotics.

Antibacterial	Concentration	Susceptibility
Chloramphenicol	30 µg	S
Florfenicol	30 µg	R
Ciprofloxacin	5 µg	R
Doxycycline hydrochloride	30 µg	R
Enrofloxacin	5 µg	S
Norfloxacin	10 µg	R
Oxytetracycline	30 µg	R
Tetracycline	30 µg	R

S, Strain is sensitive to antibiotic; R, Strain is resistant to antibiotic

Histopathology analysis

Diseased fish presented ulcers around the dorsal fins with bone exposure, depigmentation, loss of muscle mass, and erosion of the tegument. Almost all fish were affected. Internal examination evidenced accumulation of a clear liquid in the coelomic cavity (100% of cases). Livers were enlarged, pale-yellowish, soft (100%) and some of

them presented whitish nodules of 0.5 to 2.0 mm diameter in a diffuse or multifocal pattern (39.13%). Some fish displayed the nodules in other organs like kidney or spleen (Figure 1).

Figure 1. Gross appearance of whitish nodules in kidney of cobias (arrows).

Monogeneans, identified as *Neobenedenia melleni* MacCallum, 1927 (Monogenea: Capisalidae) were seen in around the dorsal region of the head of all fish (Figures 2A and 2B). The monogenoids were identified according to their morphological characteristics (WHITTINGTON; HORTON, 1996).

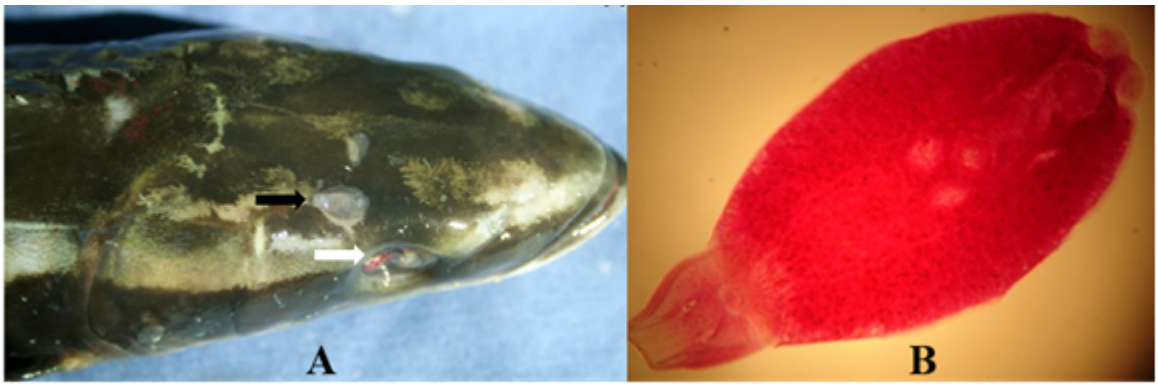

Figure 2. Neobenedenia melleni, identified as parasites in cobias, Rachycentron canadum, from a commercial mariculture situated on the north coast of São Paulo State, 2011. A. Macroscopic view from a body surface parasite (black arrow), ocular hemorrhage (white arrow). B. Stereoscopic view of a specimen stained with Mayer's carmalum.

Histopathology revealed severe diffuse steatosis characterized by the presence of large well-defined vacuoles and nuclei displaced to the cytoplasm periphery (Figure 3A). In addition, liver tissue showed congestion of blood vessels, chronic granulomatous inflammation and coagulative necrotic centers containing bacterial accumulations limited by macrophages, few lymphocytes, and extensive fibroplasia (Figures 3B and 3C). Bile canaliculi also showed necrosis of epithelium and macrophage infiltration. Granulomatous lesions were negative for Ziehl-Neelsen staining. Kidneys presented hyperplasia of melanomacrophages centers with extensive necrosis compared to cobias of the same age (SHIMADA et al., 2014). Granulomatous lesions, similar to the ones found in liver, were seen around the tubuli (Figure 3D). In the intestine, mucosal detachment and mononuclear inflammatory infiltrate in the mucous and sub mucous membranes were verified. Around 50% of fish displayed granulomas in piloric cecum and epithelial necrosis with severe inflammatory

infiltration in the intestinal mucous and submucosa. Gills from all animals showed hyperplasia multifocal of secondary lamellae and in two fish.

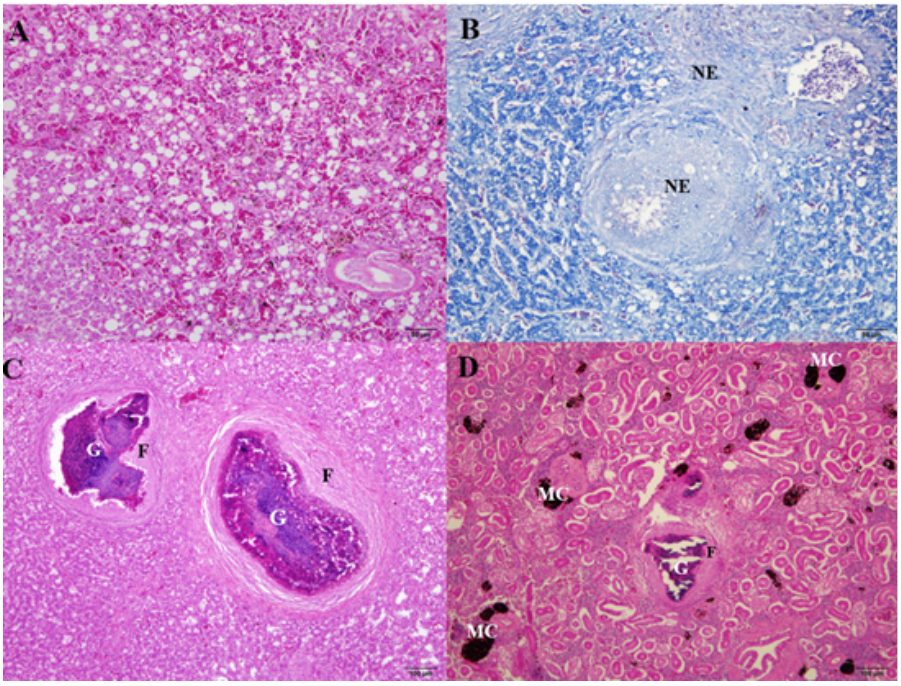

Figure 3. Histopathology micrographs of liver and head kidney collected from diseased Rachycentron canadum, Brazil, 2011. A. Hepatocytes with large intracytoplasmic lipid droplets (hepatic steatosis, black arrows) and moderate congestion of hepatic sinusoids (arrow heads) (H&E, 20X). B. Extensive necrosis in liver, negative for the presence of Mycobacterium sp. (Ziehl Nielsen, 20X). C. Hepatic granulomas with necrotic center and extensive fibroplasia. D. Granulomas in head kidney surrounded by fibrous tissue and abundant melanomacrophages centers.

DISCUSSION

Bacteriological, histological and molecular analysis indicated that *P. damselae* subsp. *piscicida* was the main causative agent of the mortality. Only one type of colony was isolated and it exhibited a unique API 20E profile, in agreement with earlier reports for the pathogen (TORANZO et al. 1991; MLADINEO et al. 2006). *Mycobacterium* spp., *Nocardia* sp. and *P. damselae* subsp. *damselae* were considered for the differential diagnosis due to similarities of anatomopathological lesions (ISHIKAWA et al. 2001). Mycobacteriosis and nocardiosis were ruled out by bacterial morphology, biochemical identification, negative result for Ziehl-Neelsen staining, and DNA sequencing.

P. damselae subsp. *piscicida* has a broad host range and presented worldwide distribution. This pathogen has been isolated from farmed cobias in Taiwan (LIU et al. 2003). High mortality rates have been reported in other species such as gilthead seabream (*Sparus aurata*) (TORANZO et al. 1991) and sole (*Solea senegalensis*) (ZORRILLA et al. 1999) in Spain, Atlantic bluefin tuna (*Thunnus thynnusi*) in Croatia (MLADINEO et al. 2006), *Macropodus opercularis* in Taiwan (LIU et al. 2011), sea bass and sea bream in Egypt (ESSAM et al. 2016).

The external signs observed were similar to those previously described in naturally infected species. Whitish granulomatous lesions on the kidney, liver and spleen have been described before in outbreaks of photobacteriosis in cobias (LIU et al. 2003) and other species (LIU et al. 2011). Nonetheless, a report in tuna fish described the kidney as the only organ with granulomatous lesions (MLADINEO et al. 2006). Tubercle-like structures can be induced in fish inoculated with the bacteria (NOYA et al. 1995).

Histopathologically, the necrotic lesions and presence of bacteria observed in the internal organs indicates a septicaemic process. The granulomas consisted of a necrotic center with agglomerations of bacteria delimited by epithelioid cells, dense infiltrate of macrophages, few lymphocytes and fibroplasia, similar with other reports (NOYA et al. 1995; MLADINEO et al. 2006).

The necrotic areas observed in the liver, kidney, spleen and pyloric cecum may be related to extracellular products such as phospholipases,

cytotoxic, hemolytic or apoptosis-inducing exotoxins secreted by *P. damselae* subsp. *piscicida* (ANDREONI; MAGNANI, 2014). These exotoxins induce intense apoptosis of neutrophils and macrophages and allows evasion of the phagocytosis (Do vale et al. 2005) and could explain cases of persistent infection. Other virulence factors is the production of superoxide dismutase and catalase, enzymes that inactivate reactive oxygen species (ROS) synthetized by macrophages during the respiratory burst (BARNES et al. 1999). These facts may explain the failure of the initial treatment and the development of chronic inflammation.

Antibiotics have been the first line of defense in fish aquaculture to control photobacteriosis outbreaks. However, recently, the pathogen acquired resistance to various antibiotics (ANDREONI; MAGNANI, 2014). Resistance to the bactericidal mechanisms is an important contributor to the virulence of fish pathogen (ESSAM et al., 2016). The differential presence of plasmids has contributed significantly to their divergence in virulence gene content of the two *P. damselae* subspecies (OSORIO et al., 2015). In the present study, *P. damselae* subsp. *piscicida* strains were resistant to ciprofloxacin, florfenicol, doxycycline hydrochloride, norfloxacin, oxytetracycline, and tetracycline. Probably that is why the use of florfenicol had no effects in fish mortality. This added to sick fish decrease food intake consequently the intke of antibiotics.

Antibiotic resistant strains of *P. damselae* subsp. *piscicida* were reported worldwide, in Japan (KIM; AOKI 1993), Taiwan (LIU et al. 2003), China (WANG et al. 2007), Italy (LAGANÀ et al. 2011), and Egypt (ESSAM et al. 2016). The extensive and incorrect use of chemotherapeutics is correlated with the increase of drug resistant strains (MAGARIÑOS et al. 1996). This fact illustrates the need for surveillance of antibiotic resistance in *P. damselae* subsp. *piscicida* strains for an effective treatment and prediction of occurrence of drug resistant strains in Brazil. Despite the susceptibility observed with chloramphenicol. It is not possible to threat the fish with this antibiotic due to it is banned in most countries for causing aplastic anemia (LU et al., 2009).

Development of a disease in fish is the result of unbalanced pathogen-host-environment interactions that lead to suppression of fish immune system (TORANZO et al. 2005). Outbreaks of photobacteriosis are related to water temperature variations (HAWKE 2012). The optimal temperature range for cobias is between 27º C and 29º C (SUN et al. 2006), while in our study, the temperature variation was within 19º C and 29º C. This strong variation could cause stress in the animals, being a predisposing factors for the outbreak.

Lesions that compromise the hepatic parenchyma may predispose to infections by opportunistic pathogens such as bacteria and parasites (SPISNI et al. 1998). The severe diffuse steatosis found in our study reflects nutritional and metabolic disturbances, caused by an unbalanced artificial diet containing excessive oleic acid, details of the development of this condition in the sampled farm were presented previously by Shimada et al. (2014). There is enough evidence to suport that in this case liver steatosis favored the infection by *P. damselae* subsp. *piscicida.*

Ectoparasites such as *Neobenedenia* sp. feed on the mucus and epithelium of fish, exposing the dermis to secondary infections by bacteria, fungi or virus (ROBINSON et al. 1992). Parasitic infections can also cause stress in the host, and are responsible for decreased immunity and greater susceptibility to other diseases (CHU et al. 2013). *Neobenedenia melleni* has been previously reported in farmed cobia (KERBER et al. 2011) and grouper (ROUMBEDAKIS et al., 2013) in Brazil, and should be considered as an emerging problem for marine fish farming in Brazil.

CONCLUSIONS

Multiples causes may have contributed to the mortalities, however *P. damselae* subsp. *piscicida* was considered as the principal cause. Other factors such as hepatic steatosis, parasitism and wide water temperature variation may have acted as predisposing factors.

In addition, there is concern about antibiotic resistance and surveillance programs are needed to secure the production of cobias in Brazil.

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RESUMO: Este estudo objetivou investigar um surto com alta mortalidade em cobia juvenis cultivadas na região Sudeste do Brasil em 2011. Os peixes apresentavam baixa taxa de crescimento, letargia, ulceração nas nadadeiras, despigmentação da pele, opacidade da córnea e deformidades físicas. Internamente o fígado apresentava aumentado e pálido em diferentes graus, com nódulos esbranquiçados e firmes disseminados no fígado, rins e baço. Na superfície corporal dos peixes foram observados moderado número de parasitas identificados como *Neobenedenia melleni*. Microscopicamente verificou-se esteatose hepática grave e extensa lesão granulomatosa em todos os peixes amostrados. A análise microbiológica dos peixes moribundos revelou a presença, em cultura pura de uma bactéria Gram-negativa identificada como *Photobacterium damselae* subsp. *piscicida* usando características bioquímicas e moleculares. A análise das sequências parciais de 16S rRNA confirmou os resultados demonstrando alta identidade (98%). Os isolados foram sensíveis a cloranfenicol e enrofloxacina e resistente a ciprofloxacina, florfenicol, cloridrato de doxiciclina, norfloxacina, oxitetraciclina e tetraciclina. A pasteurelose crônica foi considerada como o principal problema na maricultura, enquanto a esteatose hepática e a infestação parasitária podem ter contribuído para o desenvolvimento do processo.

PALAVRAS-CHAVE: Gaiolas. Pseudotuberculose. Granulomas. Resistência a antibióticos. *Neobenedenia melleni.*

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