

A novel agent (*Endozoicomonas elysicola*) responsible for epitheliocystis in cobia *Rachycentrum canadum* larvae

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ABSTRACT: Aquaculture of cobia has gained popularity in the last decade, and this species is now farmed in several countries in Latin America and Asia. Despite recent improvement in production techniques that allowed the expansion of the industry, little is known about the diseases that affect cobia during the larviculture stage. In this article we investigated the cause of mass mortalities occurring 13–20 d post-hatching in 3 cycles of cobia larviculture. Wet mounts from diseased larvae gills revealed the presence of cyst-like basophilic inclusions. DNA from the cysts was extracted and PCR amplified using the 16S rRNA gene universal primers for prokaryotes. The amplified products were sequenced and analyzed using BLAST, finding a similarity of 99% with *Endozoicomonas elysicola*, a Gram-negative bacterium. Confirmation of *E. elysicola* was conducted by designing a specific probe for *in situ* hybridization. Specific primers were also designed for diagnostic purposes. This is the first report of epitheliocystis in cobia larvae and also the first report of *E. elysicola* as an epitheliocystis-causing agent.

KEY WORDS: Epitheliocystis · *Endozoicomonas elysicola* · Cobia · Gill inflammation

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INTRODUCTION

Epitheliocystis is a common disease of fish characterized by the presence of cyst-like basophilic inclusions in the skin and gills (Nowak & LaPatra 2006). Although in wild fish epitheliocystis is usually a benign disease, in aquaculture-reared fish it can produce high mortalities (Crespo et al. 1990, Venizelos & Benetti 1996), especially in infections during early life stages. Epitheliocystis has been detected as early as 7 d post-hatching (dph) in sharpnose sea bream *Diplodus puntazzo* larvae (Katharios et al. 2008).

Cobia *Rachycentrum canadum* is a demersal finfish with almost worldwide distribution in tropical

marine waters. The quality of the flesh, fertility, fast growth and relative ease for reproduction and larviculture has made it attractive for the industry. Cobia aquaculture has expanded from 3 t in 1995 to over 40 000 t in 2010 (FAO 2012), and at present it is cultivated in at least 15 countries in Asia, America and Oceania (Benetti et al. 2010). In Colombia, cobia culture began in 2007 and by 2011 total production reached 110 t. The Colombian Aquaculture Research Center (CENIACUA) adapted and developed procedures for maturation and reproduction in captivity and over the past 3 yr it produced and reared larvae to supply the Colombian industry. Although survival in several larviculture cycles has been as high as

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30%, at least 3 severe episodes of mortalities in larvae have occurred, with final survival in the range of 2 to 10%. Wet-mount analysis and histopathology of gills from diseased fish showed characteristic lesions of epitheliocystis. In this article we present the first description of epitheliocystis causing severe mortalities in cobia larvae.

MATERIALS AND METHODS

Animals

Cobia larvae were produced and reared at CENIACUA's laboratory in Punta Canoas Bolivar, Colombia. Mature breeders were held in captivity at CEINER (Center for Research, Education and Recreation) and fertilized eggs were shipped to CENIACUA. Larviculture was performed in circular larval rearing tanks (6–7 t) under flow-through production according to published protocols (Benetti et al. 2008).

Background

During cobia larviculture at CENIACUA, 3 independent cycles presented with sudden events of mortality starting at 13 to 22 dph. The first signs were detected in Outbreak A at 13 dph, in Outbreak B at 22 dph and in Outbreak C at 15 dph. Mortalities started 12 h after the initial symptoms appeared, reaching almost 100% by 7 d. After the first outbreak, screening was initiated on 9 dph animals by wet mount and histopathology of gill tissue in order to detect early pathological signs of the disease.

Histology

Moribund larvae from the 3 outbreaks were anesthetized using clove oil and necropsied. Whole larvae were fixed in neutral 10% buffered formalin for 24–48 h, embedded in paraffin, sectioned and stained with hematoxylin and eosin as described by Faulk et al. (2007). Selected sections were also analyzed by Gram-Twort staining (Ollett 1951). Each larva was examined, and the severity of the disease was determined with a grading system based on the number of cysts per primary lamellae as counted under 100× magnification: slight, ≤10 cysts; moderate, 11 to 20 cysts; and severe, ≥21 cysts.

Molecular biology

The bacterial aggregates were separated from the gills of three individuals and collected using an eyelash under an optical microscope at 100× magnification (Fig. 1). Aggregates were transferred to sterile microcentrifuge tubes, and the DNA was extracted using QIAamp DNAm mini kits (Qiagen). PCR amplification of bacterial 16S rRNA gene was performed with universal primers (63F and 1387R) (Marchesi et al. 1998) using conditions previously described (Kabelitz et al. 2009). Negative controls were carried out without template DNA. PCR products were separated in 2% agarose gel to verify product quality, and purified using the QIAquick PCR Purification Kit (Qiagen). Sequencing in both directions was done directly on the purified amplified product. Electropherograms were analyzed using Geneious software (Biomatters). BLAST searches were then performed to compare the sequences with existing databases such as GenBank and the Ribosomal Database Project (RDP) (Cole et al. 2009)

PCR and *in situ* hybridization

Sequence results were used to design PCR-specific primers for identification of the bacteria. Primers ECF (5'-AAC TGG GCA GCT AGA GTG CGG-3') and ECR (5'-GTC ACC GGC AGT CTC CCC AGA-3'), which amplify a 528 bp fragment, were designed using Geneious software. Their specificity was BLAST tested against the sequences in RDP. No matches longer than 3 consecutive nucleotides were

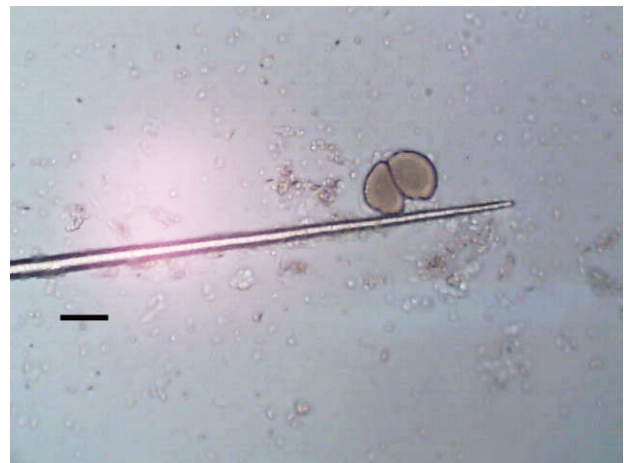


Fig. 1. Two cysts collected from the gills of diseased cobia *Rachycentrum canadum* larvae using an eyelash. Scale bar = 100 μ m (100×)

identified with any of the sequences with the exception of *Endozoicomonas elysicola*.

DNA samples from diseased and healthy larvae, and DNA extracted from *Vibrio* sp., *Pseudomonas* sp. and *Aeromonas* sp. isolates, were PCR amplified in an Axygen thermocycler. The PCR reaction mixture contained 10 mM PCR buffer (Tris-HCl, 50 mM KCl, pH 8.3), 1 mM of each dNTP, 10 pmol of each primer, 2.5 U Go-Taq Hot start polymerase (Promega), 1.5 mM MgCl₂, 100 to 300 ng DNA template and sterile H₂O in a 20 µl volume. Cycling conditions were initial denaturing step for 4 min at 95°C; 30 cycles of denaturing for 30 s at 95°C, annealing for 30 s at 62°C and extension for 30 s at 72°C; and final extension at 72°C for 4 min.

For *in situ* hybridization, a digoxigenin (DIG)-labeled probe was obtained by PCR amplification using the primers and cycling conditions described above and the DIG-11dUTP labeling system (Roche Applied Science). *In situ* hybridization was performed according to Alonso et al. (2004). Overnight hybridization with the probe was carried out in a humid chamber at 42°C and the colorimetric detection of hybridization signal was carried out with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate. Sections were counter-stained with 0.5% Bismarck Brown Y. Staining controls were performed in tissue sections without the DNA probe. Negative controls were run in tissue sections of healthy animals without the presence of histological lesions in gills.

RESULTS

Clinical signs of the outbreak

During the 3 outbreaks in 2010 and 2011, the first clinical signs were the presence of lethargic animals and decreased feed consumption. Gill edema and congestion, manifested as 'red head', was characteristic of symptomatic animals. Flared operculum and air gasping at the water surface occurred at late stages. Cumulative mortality reached 100% in one of the episodes. In wet mount analysis, numerous spherical dark brown cysts between 50 and 70 µm in diameter were detected in the gills of symptomatic larvae. In severely affected animals nearly all gill filaments had numerous cysts (Fig. 2).

Histopathology and *in situ* hybridization

Histopathological examination of larvae from the 3 outbreaks showed the presence of a large number of



Fig. 2. Wet mount of gills from diseased *Rachycentrum canadum* larvae. The cysts appear as rounded dark bodies. Scale bar = 100 µm (100×)

dense basophilic bodies, located within the hypertrophied epithelial cells of gills (Fig. 3). Cysts were located predominately at the base of the filaments with fewer cysts detected near the distal area. Furthermore, inclusions were not evenly distributed among the filaments; some filaments had up to 6 cysts while others showed no pathology. Gill rakers were also affected, mainly with one cyst per raker. Cysts stained clearly Gram-negative using the Gram-Twort staining.

Lesion development progressed as follows. (1) Small cyst-like bodies (1–2) found in less than 30% of the gill primary lamellae. (2) Increase in the number and size of the bodies reaching 5–6 per filament and compromising more than 50% of the gill filaments. (3) The number of bodies can reach up to 20 cysts per filament. Although only slight pathological changes were found near the cysts, some of the larvae with severe infestation showed mild, multifocal hyperplasia with occasional focal lamellar fusion and clubbing.

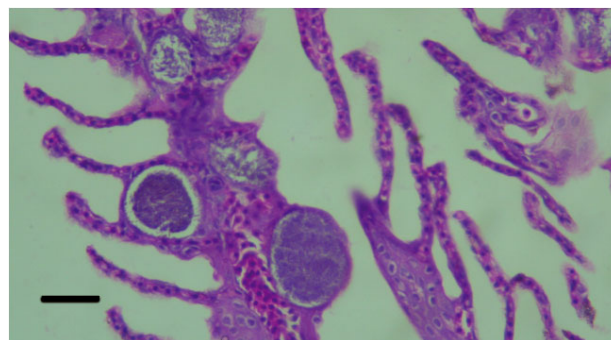


Fig. 3. Hematoxylin and eosin stained sections from formalin-fixed and paraffin-embedded gill tissues from symptomatic cobia *Rachycentrum canadum* larvae. The cysts appear in epithelial cells as regular rounded, basophilic structures. Scale bar = 100 µm (100×)

***In situ* hybridization**

There was a positive reaction to the DIG-labeled probe (presence of a dark blue/black precipitate) within the cyst-like bodies in gills (Fig. 4A). Occasionally, free bacteria were also found in the vicinity of the cysts. No positive signals were found in other organs, or in the tissues of healthy larvae (Fig. 4B).

Molecular identification

BLAST search of the sequenced 16S rRNA using the GenBank database revealed a 99.04% similarity with *Endozoicomonas elysicola* strain MKT110 GenBank accession number NR_041264. BLAST search within the RDP had a similarity absolute score (S_{ab} score) of 0.963 with *Endozoicomonas elysicola* MKT110, genus *Endozoicomonas*, family *Hahellaceae*, order *Oceanospirillales*, class *Gammaproteobacteria*. Other matches with an S_{ab} score above 0.92 were obtained with 4 environmental samples

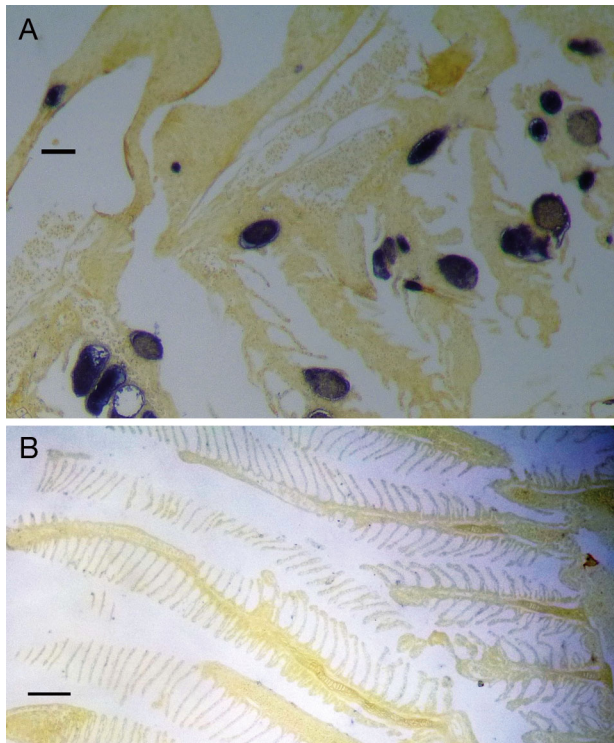


Fig. 4. A PCR digoxigenin-labeled probe was prepared using primers specific for *Endozoicomonas elysicola* and colorimetric *in situ* hybridization was performed in sections of gill tissues from (A) moribund *Rachycentrum canadum* larvae from the first outbreak and (B) healthy larvae. Most of the cysts show a strong positive response with the probe (A), while there is no signal in the tissues of the healthy larvae (B). Scale bars = 100 μ m (100 \times)

isolated from coral reefs (Sunagawa et al. 2010) and classified within the genus *Endozoicomonas*.

Nucleotide sequence accession numbers

The obtained 16S rRNA gene sequence was deposited at GenBank/EMBL/DDBJ under the accession number KC312958.

PCR amplification

The DNA samples previously described were amplified by PCR using both the bacterial and *Endozoicomonas* primer pairs. Except for the DNA from the healthy larvae, and the negative control, all samples amplified with the universal bacterial primers showing the expected ~1300 bp fragment. In contrast, only DNA samples extracted from diseased larvae and from the cyst-like structures shown in Fig. 1 were positive in the reaction with the *Endozoicomonas*-specific primers (data not shown).

DISCUSSION

At present, cobia culture is practiced in more than 15 countries from Asia, Oceania and America. The expansion of cobia culture has been supported by the development and optimization of protocols and techniques for broodstock management, reproduction in captivity and larviculture. However, in spite of these advances, final survival during larviculture is still low, ranging from 5 to 38% (Benetti et al. 2010). Both cannibalism and diseases are important factors in survival of larvae and they also threaten production. Cannibalism and disease incidence can sometimes be intimately related. Lethargic diseased animals are prime candidates for cannibalism, and cannibalism is an extremely effective means of disseminating causal agents of disease (Astrofsky et al. 2000, Baxa-Antonio et al. 1992).

Several diseases have been reported in cobia. Protozoan infestations caused by *Amyloodinium ocellatum*, *Epistylis*, *Nitzschia*, *Pseudorhabdosynochus epinepheli*, *Benedenia* and *Trichodinia* have been detected in Taiwan and the USA (Liao et al. 2004, Benetti et al. 2008), and even a viral disease, viral nervous necrosis, with confirmed vertical transmission has been reported (Le & Svennevig 2005). However, the knowledge of diseases affecting cobia during larviculture stages is still limited.

In recent years, epitheliocystis disease outbreaks have increased due to the introduction of new species and to the expansion of aquaculture. At present, it is considered a serious disease with a major potential impact on aquaculture production systems (Rigos & Pantelis 2010). Epitheliocystis has been described as early as 7 dph in cultured sharpnose sea bream larvae (Katharios et al. 2008), with mortalities reaching 100% at 25 dph, and Venizelos & Benetti (1996) described epitheliocystis disease at the metamorphosis stage (28 to 42 dph) of the Pacific yellowtail *Seriola mazatlana*. In the outbreaks in cobia larvae, reported here, the earliest lesion development appeared 12 dph, and larvae mortality was up to 100% 5–7 d after the initial symptoms appeared. In the few published reports on epitheliocystis in larvae (Venizelos & Benetti 1996, Katharios et al. 2008), mortality was very high regardless of the presence of proliferative response in gill cells, suggesting that the infection could compromise respiration and osmoregulation in the gill interlamellar space. Katharios et al. (2008) also suggested that the presence of inclusions near the mouth opening could block the entrance of the food, leading to starvation at a very sensitive developmental stage, or could affect the development or the function of sensory cells implicated in larval food detection. In the cobias examined in this study, inclusion bodies were present in the developed gill but very few were localized near the mouth opening. This suggests that inclusions in the mouth openings were not the main cause of the mortalities; instead we suggest that alteration of both gas exchange and osmoregulatory capacity could be the cause of rapid death in the infected animals.

Epitheliocystis has been widely attributed to infection by a relatively diverse group of intracellular Gram-negative bacteria within the phylum *Chlamydiae*, as confirmed by molecular characterization (Meijer et al. 2006, Draghi et al. 2004, 2007, Mitchell et al. 2013, Kumar et al. 2013) and immunohistochemistry using monoclonal antibodies against chlamydial lipopolysaccharide antigens (Nowak & La Patra 2006). However, in many reported cases, lesions failed to react with the antibodies, further illustrating the diversity of the chlamydial agents causing epitheliocystis, or indicating that other bacterial species could be involved in disease pathogenesis (Nowak & La Patra 2006). More recently, Toenshoff et al. (2012) have shown that a newly identified *Betaproteobacteria*, tentatively called '*Candidatus Branchiomonas cysticola*', caused epitheliocystis in Atlantic salmon *Salmo salar*, and they proposed that

bacteria from different evolutionary branches may cause epitheliocystis. A similar multifactorial hypothesis for epitheliocystis was also proposed by Steinum et al. (2010), who identified a microsporidian, *Desmozoon lepeophtherii*, present at high loads in Atlantic salmon with proliferative gill inflammation (PGI), suggesting that '*Candidatus Piscichlamydia salmonis*' and the microsporidian could be contributing causes of epitheliocystis.

In the present study, sequencing of 16S rRNA genes from the lesions of moribund larvae, and BLAST analysis against the RDB and NCBI databases, showed a 99% similarity with *Endozoicomonas elysicola* strain MKT110 GenBank accession number NR_041264. This organism was first isolated from the sea slug *Elysia ornata* collected in Japan in 2007 (Kurahashi & Yokota 2007). Analysis of 16S rRNA sequences indicated that the isolate constituted a new lineage in the gammaproteobacteria class, belonging to the order Oceanospiridales, family Hahellaceae (Garrity et al. 2005). The Hahellaceae family contains *Endozoicomonas* and 4 more separate genera: *Hahella* (Jeong et al. 2005), *Halospina* (Sorokin et al. 2006), *Kistimonas* (Choi et al. 2010) and *Zooshikella* (Yi et al. 2003). Although literature reports on *E. elysicola* are scarce, it has been identified in salmon suffering from PGI (Steinum et al. 2009). In that study, *Endozoicomonas* was isolated from 29% of the animals with no lesions and from 57% of animals with PGI grade III, indicating that it may have a role in the development of PGI. Furthermore, an uncultured gammaproteobacterium clone GAMMA 7, almost identical in 16S rRNA sequence to *E. elysicola*, was isolated from gills of Atlantic salmon in Tasmania affected by amoebic gill disease (Bowman & Nowak, 2004).

Although, as pointed out by one of the reviewers, the primers used for the amplification of 16S rRNA genes from the cysts are not optimal for the detection of some of the members of the phylum *Chlamydiae*, in our samples amplification was strong and BLAST analysis from the sequence of the amplicons showed only the presence of *Endozoicomonas* sp. Moreover, *in situ* hybridisation experiments unambiguously confirmed the presence of *Endozoicomonas elysicola* inside the cyst-like bodies. The fact that all of the cysts were labelled with the probe confirms that *Endozoicomonas* was indeed responsible for the lesions observed and for the mortality that followed.

In conclusion, we have described an outbreak of epitheliocystis in cobia larvae that was caused by *Endozoicomonas elysicola*, thus adding a new member to the list of the causal agents of epitheliocystis.

Recent advances in parallel sequencing and bioinformatics have made it possible to identify marine microorganisms that are associated with disease outbreaks. We believe that the identification and characterization of the causal agents are fundamental steps in understanding the etiology of epitheliocystis outbreaks in marine fishes, in addition to their control.

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