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Original Article

Ultrastructure of intracytoplasmic *Rickettsia*-like infection of the gills of the teleost *Archosargus probatocephalus* (Sparidae) in northeastern Brazil

Ultraestrutura de *Rickettsia* intracitoplasmática infectando as brânquias do teleósteo *Archosargus probatocephalus* (Sparidae) no Nordeste do Brasil

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Abstract

A histopathological survey was conducted to investigate the presence of microparasites in fish *Archosargus probatocephalus,* in a river near Maceió, Brazil. Light microscope observations of fragments of gill showed the presence of small cysts containing numerous myxospores that were morphologically identified as *Henneguya*. Transmission electron microscopy observations further revealed several gill cells containing groups of prokaryotic cells within large cytoplasmic vacuoles. Each infected host cell displayed a single vacuole containing a variable number of *Rickettsia*-like cells (up to 11), some of which presented the dumbbell shape characteristic of binary fission. The *Rickettsia*-like cells were pleomorphic, without a nucleus and with chromatin dispersed in the cytoplasm. They had a thin electron-dense wall of Gram-negative type. The morphology of these prokaryotic was similar to those of the order Rickettsiales and was described as a *Rickettsia*-like organism. Histopathological evaluation showed that several vacuole membranes had a lysed appearance. Some had ruptured, thus allowing direct contact between the *Rickettsia*-like organism and the cytoplasm of the host cell. The rupturing of the branchial epithelium may have contributed towards reduction of the surface area of the gills, but it is not possible to say that this was the cause of the host's death.

Keywords: Fish, Sparidae, cytoplasmic vacuoles, *Rickettsia*-like cell.

Resumo

Um levantamento histopatológico foi realizado para pesquisar a presença de microparasitas, no peixe *Archosargus probatocephalus* em um rio próximo a Maceió, Brasil. Observações ao microscópio óptico de fragmentos de brânquias mostraram a presença de pequenos cistos contendo numerosos mixósporos, identificados morfologicamente como *Henneguya*. Ocasionalmente, na microscopia eletrônica de transmissão, foram observados vários corpos citoplasmáticos de inclusão, grupo aparentemente de células procarióticas que vivem dentro de um grande vacúolo citoplasmático de algumas células branquiais. As células hospedeiras infectadas tinham um único vacúolo contendo um número variável de células do tipo *Rickettsia*, até 11, algumas das quais em forma do haltere, característica da fissão binária. Essas células eram pleomórficas sem núcleo, tendo a cromatina dispersa no citoplasma e possuíam uma parede densa de elétrons finos do tipo Gram-negativo. A morfologia dessas células procarióticas foi semelhante àquelas da ordem Rickettsiales e foram descritas como organismos tipo Rickettsiae. A histopatologia mostra várias membranas de vacúolos circundantes com aspetos lisados, enquanto outras apresentam rupturas que mostram contato direto do organismos tipo Rickettsiae com o citoplasma da célula

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hospedeira. A ruptura do epitélio branquial pode ter contribuído para a redução da superfície das brânquias, mas não é possível afirmar que foi a causa da morte do hospedeiro.

Palavras-chave: Peixe, Sparidae, vacúolo citoplasmático, célula tipo *Rickettsia*.

Introduction

Occurrences of cell-dependent prokaryotic microorganisms such as Bacteria, Rickettsiae, Mycoplasma and Chlamydia in eukaryotes has been described worldwide. These are commonly associated with differing pathogenicity in many geographical regions (Athanassopoulou & Karagouni, 2004; Azevedo, 1993; Azevedo et al., 2005; Birkbecka & Ringøb, 2005; Bower et al., 1994; Cusack et al., 2002; Fryer et al., 1990; Fryer & Lannan, 1994; Guo et al., 2004; Molloy et al., 2001; Turnbull, 1993; Villalba et al., 1999; Yuksel et al., 2006). Rickettsial and bacterial diseases that affect a wide range of freshwater, brackish water and marine fish (Bower et al., 1994; Mauel et al., 2003; Soares et al., 2013; Weinert et al., 2009), including ornamental species (Mauel et al., 2007) have been described. Elevated economic losses due to occurrences of rickettsial infections in cultivated fish have also frequently been reported (Branson & Diaz-Munoz, 1991; Chern & Chao, 1994; Corbeil et al., 2005; Cusack et al., 2002; Fryer et al., 1990; Mauel et al., 2003).

Subsequently, several experiments have been conducted to evaluate the effects of different chemical biocide products on the development of rickettsial species (Mauel et al., 2003; Miquel et al., 2003; Palmer et al., 1996; Smith et al., 1996).

Over recent years, rickettsial diseases have been observed in various locations and in a variety of species, infecting different organs, how: the clam gills from Island Lizard (Great Barrier Reef) (Goggin & Lester, 1990); the renal tissue of Chilean salmon (*Oncorhynchus kisulch*) (Fryer et al., 1992); the gills of the oyster (*Crassostrea gigas*) collected in Spain and the digestive gland of the shrimp (*Pandalus platyceros*) from Canada (Bower et al., 1994) and the kidney, liver and spleen of juvenile *Atractoscion nobilis*, from the USA (Chen et al., 2000). The rickettsiosis were found to be especially important in salmon farms, where high mortality was observed (Branson & Diaz-Munoz, 1991; Chern & Chao, 1994; Corbeil et al., 2005; Cusack et al., 2002; Fryer et al., 1990; Guo et al., 2004).

These prokaryotic microorganisms cause chronic systemic infections associated with internal and external lesions. *Rickettsia*-infected fish show lethargic and anorexic behavior, an enlarged abdominal cavity and lesions in various tissues and organs such as the liver, swim bladder and epithelial and muscle tissues, among others. They may also cause epithelial hyperplasia, which results in fusion of the gill lamellae (Branson & Diaz-Munoz, 1991; Chern & Chao, 1994).

Human infections caused by ingestion or handling of *Rickettsia*-infected fish have frequently been reported (Lu et al., 2019; Novotny et al., 2004; Shotts, 1987).

Prokaryotic parasites have most frequently been described infecting the cytoplasm (Azevedo, 1993; Cusack et al., 2002; Guo et al., 2004) and nuclei of eukaryotic hosts (Azevedo, 1989), or living in association with other prokaryotic cells (Azevedo & Villalba, 1991). They may be pathogenic and lethal (Austin & Austin, 2007; Chen et al., 2000; Mauel et al., 2003; Toranzo et al., 2005), and are considered to be obligate parasite (Austin & Austin, 2007).

The fish species *Archosargus probatocephalus* (Walbaum, 1792), commonly known as the "sargo" or sheepshead seabream, belongs to the family Sparidae of the order Perciformes, and has wide distribution, ranging from Nova Scotia (Canada) to Brazil (Adams et al., 2018). It is considered to be a species with high potential for aquaculture because of its tolerance to wide temperature ranges, its euryhaline and omnivorous nature and the excellent quality of its meat (Rojas-Castañeda et al., 2017).

Several studies have suggested that rickettsial species and *Rickettsia*-like organisms (RLOs) are pathogenic and capable of causing the death of their hosts (Almendras & Fuentealba, 1997; Yuksel et al., 2006; Rozas & Enríquez, 2014).

The aim of this paper was to describe the ultrastructure of intracytoplasmic prokaryotic cells that cause damage to the organization of the gill cells of *Archosargus probatocephalus*. These cells were morphologically identified as belonging to the group of RLOs and were found in a survey on microparasites of the gill tissues of a commercially important teleost in Brazil. This formed the first description of these cells in Brazil.

Methodology

Four adult specimens of the sheepshead seabream *Archosargus probatocephalus* (Brazilian common name "sargo or sargo-de-dente"), were not breeding, with lengths between 15 and 25 cm, were collected from the estuary of the Ipioquinha River (9° 29' S / 35° 34' W), a river partially preserved near the city of Maceió (Alagoas), Brazil, in June and August 2019. They were transported to the aquaculture laboratory of the Agrarian Sciences Center of the Federal University of Alagoas, where biometrics and dissection were performed. Collections were carried out under license number 56475-1, of November 15, 2016, renewed on December 8, 2019 MMA of Brazil.

Light microscopy (LM) and transmission electron microscopy (TEM)

Small fragments of tissues from different organs were checked under a light microscope, to detect any microparasites that might have been present. Small cysts were observed in the gills of a single specimen. The infected material was photographed under this microscope and was then processed for ultrastructural observations.

For TEM, infected fragments of gill tissue were fixed in 4-5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2-7.4) for 10-12 h. They were then washed overnight in the same buffer and postfixed with 2% osmium tetroxide in the same buffer for 3 h. All these steps were done at 4 °C. This was followed by dehydration through an ascending ethanol and propylene oxide series and embedding in Epon to form blocks. Semithin sections (~1 µm thick) were stained with methylene blue-Azur II. Ultrathin sections were double-contrasted with uranyl acetate and lead citrate, and were observed using a TEM (JEOL 100CXII; JEOL Optical, Tokyo, Japan), operated at 60 kV.

Results

A single specimen displayed infection by myxosporean species contained within cysts, which measured up to 125 µm and appeared located in the peripheral region of the gill filaments (Figure 1A). The cysts were surrounded by several layers of epithelial cells, some of which displayed cytoplasmic vacuoles containing small dense bodies that could not be identified in semithin sections (Figure 1A). TEM observations revealed that the ultrastructural aspects of these small bodies were congruent with the morphological features of prokaryotic organisms, namely RLOs (Figure 1B-F).

The RLOs were found in variable numbers per section (most frequently 5-7) (Figure 1B, F). Serial ultrathin sections showed variable numbers of RLOs (up to 11) located within several voluminous vacuoles of the infected host cells (Figure 1C-E). These vacuoles were more frequently located at the periphery of the epithelial cells in which the cysts were located (Figure 1B, C). Most of them displayed a degraded membrane and presented various aspects of rupture and RLO release (Figure 1C - F). The cytoplasm of the host cell surrounding the vacuole membrane also showed an appearance of severe lysis (Figure 1C-E).

The RLOs were pleomorphic, and their DNA appeared randomly distributed in the internal granular cytoplasm, forming a reticular nucleoid without a nuclear envelope (Figure 1D-F). The nuclear envelope and the nucleoplasm of the host cells displayed typical organization (Figure 1 C-E).

In these observations, it was not possible to detect morphological aspects that would suggest that the RLOs had influenced mortality.

Discussion

Intracellular prokaryotes have frequently been described in different aquatic species and in several geographical locations, as: *Ruditapes decussatus* (Mollusca, Bivalvia), collected in Portugal (Azevedo, 1989); *Venerupis rhomboides*, from Spain (Villalba et al., 1999); juvenile *Atractoscion nobilis*, collected in the California (USA) (Chen et al., 2000); *Mytella guyanensis* from, Bahia (Brazil) (Ceuta & Boehs, 2012); *Crassostrea gasar*, cultivated in northeastern Brazil (Silva et al., 2015) and *Oncorhynchus tshawytscha*, from farms in New Zealand (Brosnahan et al., 2019).

The ultrastructural characteristics of the prokaryotic cells observed in this study, namely their pleomorphic shape and lack of mitochondria and nuclear envelope, suggested that they belonged to the order Rickettsiales, genus *Rickettsia*. They are described here as *Rickettsia*-like organisms (RLOs). The morphological characteristics that identify these RLOs have already been described in numerous reports (Azevedo & Villalba, 1991; Cusack et al., 2002; Guo et al., 2004). The ultrastructure of the outer membrane of the cell wall and cytoplasmic membrane further

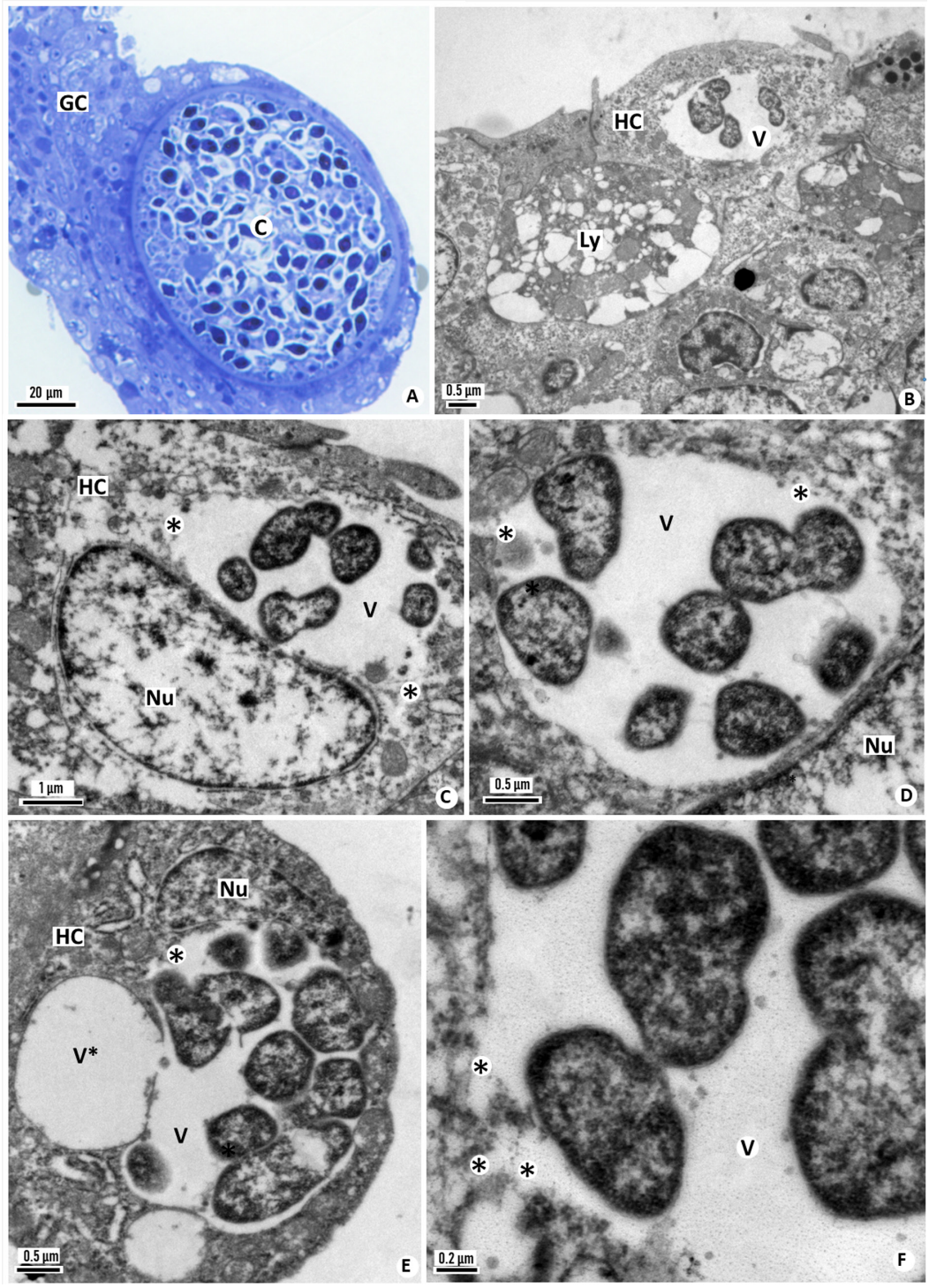

Figure 1 Light and transmission electron microscopy aspects of gill epithelial cells of the teleost *Archosargus probatocephalus*. Some of these cells presented generally a single large cytoplasmic vacuole containing *Rickettsia*-like organisms (RLOs). A - Semithin section of a cyst (C) containing myxospores surrounded by different gill cells (GC), infected by RLOs within vacuoles that were hardly observed in LM; B - Ultrathin section showing the periphery of the gill, where an infected host cell (HC) with a vacuole (V) containing some *Rickettsia*-like cells are present. Near the vacuole (V), some cellular material seems lysed (Ly); C - Ultrastructural appearance of an infected host cell (HC) located near to the periphery of the gill, in which the nucleus (Nu) is in contact with the vacuole (V) containing the RLOs. The HC shows high-grade of lysis (*); D - Ultrastructural appearance of the cytoplasm of a host cell containing a vacuole (V) with several pleomorphic RLOs. The cytoplasm of the host cell and the membrane of the vacuole show advanced stages of degradation (*). Partial portion of the nucleus (Nu) showing the closed contact with the vacuole content; E - Ultrastructural details of a host cell (HC) showing two type of vacuoles. The biggest containing several RLOs (V), while the smallest one (V*) appeared empty of RLOs. The vacuole membrane and the nucleus (*) show degraded aspects. F - Ultrastructural details showing some RLOs within the vacuole (V) that seems well preserved, while the membrane and the surrounding cytoplasm of the host cell appears highly lysed (*).

revealed them to be Gram-negative. This information was not given in most previous descriptions (Fryer & Lannan, 1994), with the exception of Fryer & Hedrick (2003).

This was the first description of an intracellular prokaryotic RLO from this Brazilian host fish (*Archosargus probatocephalus*). This seems to be a situation that has frequently been encountered, with occasional reports of numerous eukaryotic cells in aquatic species in different taxonomic groups. It also seems to occur more frequently as an intracytoplasmic infection (Azevedo, 1993; Azevedo et al., 2005), than as an intranuclear infection (Azevedo, 1989). In another report, a giant rickettsial organism associated with bacteria in the molluscan gills of *Crassostrea gigas* caused host mortality (Azevedo & Villalba, 1991).

Intracellular prokaryotic microorganisms have never before been reported from the sheepshead seabream. This fish species is distributed along large proportions of the North and South American Atlantic coasts (Rojas-Castañeda et al., 2017). Thus, the present study constitutes the first description of an intracytoplasmic prokaryote in this fish species, and specifically from a geographical region where it is commercially valuable for human consumption.

The lack of data in the literature with regard to vacuole-forming intracytoplasmic infections in fish hosts hampers recognition of the degree of tissue degradation that may result from these types of infections, along with detailed assessment of the histopathological effects of RLOs. Considering that the present study resulted from a chance finding during the course of a myxozoan survey conducted on *Archosargus probatocephalus* (work in progress), we are unable to say whether the RLOs described here can increase host mortality. Nevertheless, a previous study showed that infection by RLOs may occur through weakened host immunity, as a result of abiotic changes to the aquatic environment (Soares et al., 2013).

It is well known that various species of invertebrates, and especially arthropods and bivalves, act as transmission vectors for RLOs. However, no information is available regarding the possibility that infected fish might be active transmitters of rickettsiosis.

Our results do not allow any suggestions regarding the mode of infection or development of these prokaryotic parasites within the host. The way in which the RLOs were organized, with evidence of vacuole membrane rupture and degradation of host cytoplasm, suggests that they possibly had pathogenic activity.

Little is known about the mechanisms through which RLOs penetrate into host cells (Austin & Austin, 2007). Some studies have suggested that food vacuoles (phagosomes) may be a possible means through which RLOs enter into host cells (Silverman et al., 1992). Others have described action mediated by phospholipase, i.e. "induced phagocytosis" (Silverman et al., 1992). Occurrences of dissemination to neighboring cells, were reported by Weddle & Agaisse (2018), who observed that RLOs used the cytoskeleton of the host cell to move from infected cells and project to adjacent cells.

Experimental results from artificial infestation of fish with *Rickettsia* have suggested that cell penetration occurs through the skin and gills (Smith et al., 1999, 2004). Some authors have suggested that occurrences of rickettsiosis may result from biotic factors such as pathogens and abiotic causes such as pollution (Austin & Austin, 2007). Considering that, in the present study, RLOs were found contained within cytoplasmic vacuoles located on the periphery of gill epithelial cells, our observations support the idea that RLO penetration occurs mainly through this organ. In addition, the high number of RLOs contained in the same vacuole possibly suggests that penetration of host cells is accompanied by occurrences of cell division, from a single penetrating RLO.

Further detailed observations are needed in order to better understand the evolution of RLOs, which will help in the future to elucidate the mechanisms of host infection, life cycle, transmission and pathogenicity.

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