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Caspase -1, -3, -8 and antioxidant enzyme genes are key molecular effectors following *Vibrio parahaemolyticus* and *Aeromonas veronii* infection in fish leukocytes

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ABSTRACT

Caspases are a family of proteases involved in many important biological processes including apoptosis and inflammation. In order to get insights into the caspase gene family and antioxidant enzymes in *Totoaba macdonaldi* during bacterial infection, an *in vitro* assay was performed involving three different types of caspases (Casp-1, Casp-3 and Casp-8) and antioxidant enzymes (catalase, gluthathione peroxidase 1 and 4) after *Vibrio parahaemolyticus* and *Aeromonas veronii* infection, using head-kidney and spleen leukocytes from the teleost fish totoaba at 12 and 24 h post-exposure. Characterization of caspases by bioinformatics analyses showed that *TmCas-1*, *TmCas-3* and *TmCas-8* shared overall sequence identities of 82-61%, 85–97% and 77-63%, respectively, with other teleost fish. Caspase-1, -3 and -8 proteins revealed a conserved penta-peptide sequence at the catalytic site and three amino acid residues involved in the catalysis (H, G and C), as well as two conserved domains. The expression levels of the three caspases were detected in a wide range of fish tissues; however, they varied among tissues and caspases, which were highly up-regulated in immune organs, such as head-kidney, liver and/or spleen. The pathogen-induced gene expression pattern revealed two interesting facts; first, that the expression of all the caspase genes and antioxidant enzyme genes evaluated in this study were strongly induced following *V. parahaemolyticus* infection; second, these up-regulations reached a maximum level at 24 h post-infection in head-kidney whereas in spleen leukocytes, it was observed at 6-h post-infection. In conclusion, based on these observations, the acute toxic effects of *V. parahaemolyticus* are associated to cell death and release of free radicals. This information provides a better understanding of the effects and nature of early immune response against common bacterial infections in totoaba leukocytes.

1. Introduction

Vibrio parahaemolyticus, a Gram-negative bacterium, causes vibriosis of fish, shellfish, and other aquatic animals, and it is the agent to one of the most severe infectious diseases causing histopathological changes and death, and thus great economic losses (Broberg et al., 2011; Peng et al., 2016). Strategies using immunotherapy may be the most effective method for preventing vibriosis and other bacterial infectious diseases (Cardenas et al., 2016). Nonetheless, the identification of infection mechanisms is critical to develop an improved aquaculture. In this regard, immune responses evoked by pathogens, such as *Vibrio* spp., have been examined in yellow croaker, orange-spotted grouper, zebrafish, Pacific red snapper and totoaba that have illustrated broad changes in immune signaling mechanisms during infection (Huang et al., 2011; Pang et al., 2016; Zhang et al., 2016; Cardenas et al., 2016; Reyes et al., 2016). In the last one, our research group studied leukocyte susceptibility and immune response against *V. parahaemolyticus* in *Totoaba macdonaldi*. Generally, the results have shown a significant decrease in cellular immune responses during the infection, principally in phagocytic ability and respiratory burst. The survival or viability of stimulated leukocytes was significantly reduced causing necrosis and apoptosis, indicating a robust killing response by *V. parahaemolyticus*. Associated with this response, it is important to know the different mechanisms that act against a broad range of pathogens. One of these mechanisms is the caspases-mediated pro-inflammatory and pro-apoptotic pathways where multifunctional caspases are involved. Apoptosis is critically important for the survival of multicellular organisms as it destroys damaged or infected cells that may interfere with normal

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Table 1
Sequences of the primers used in this study.

Gen	Gen abbreviation	Accession No.	Primer sequence (5´-3´)
Caspase 1	Casp1	KY689034	CCA TCG ACA ACA TTT ACA AAC AC TTG CAT CAT CAC AAA CAA TTA CTG
Caspase 3	Casp3	KY689035	GAT GAA AGT GTT TGC AAA GTT G CAT CCG TAC CAA AGA ACA CTC
Caspase 8	Casp8	KY689036	AAA GAT AGA GGA GGG ACT GAG CAG TCA TCA TTC AAA AAA TTC CTT CTG
Catalase	CAT	KY689029	CTCTGCTGGTCCAAGATGTG CACTTCAAAGTAGCCAAACG
Glutation peroxidase 1	GPx1	KY689031	GCCAAGGGACTCGTTATTC GCATCCTTTCCATTCACATTC
Glutation peroxidase 4	GPx4	KY689032	CAGAGCGCCAAGTCAATATAC CTGAGTGTAGTTTACCCTGGTC
Elongation factor-1α	EF-1α	KX524957	CATTGTCAAACTCATTCCACAG CACGGTCTGCCTCATGTC

function (Sun et al., 2015). The central molecules of signaling pathway leading to apoptosis are the caspases (Nicholson, 1999). Cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases (caspases) are a family of cysteine proteases that take part in vital roles in apoptotic cell death, necrosis, and inflammation (Grütter, 2000; Boatright and Salvesen, 2003). Caspases can be categorized into three main divisions based on different motifs near the C-terminus: Caspases 1, 4 and 5 are pro-inflammatory and help to control microbial infections (Ren et al., 2017). Caspases 2, 8 and 9 are upstream initiators required to activate apoptosis. Caspases 3, 6 and 7 are downstream "executioners" that cleave most substrates in cells undergoing apoptosis (Takle and Andersen, 2007). The host responds against both intracellular and extracellular pathogens by inducing different caspase-mediated pyroptosis (or pro-inflammatory pathway) (Miao et al., 2011), extrinsic and intrinsic apoptosis pathways (Ashida et al., 2011). Thus, caspase plays an indispensable role against pathogens, so it is necessary to understand the changes in caspase expression patterns during pathogenic infection and/or derived toxic compounds. To our knowledge, up to date no reports about the role of caspases in totoaba have been available. Our research group is interested in knowing the biology and immune response of this important endemic marine fish to the Gulf of California. Currently, this species is being evaluated as a potential aquaculture candidate because of its fast growth and good adaptation to culture conditions (True and Castro, 1997). However, the totoaba *in vivo* study is limited because it has been classified as critically endangered since 1975, which is why the *in vitro* experiment (using leukocytes) is an important alternative. Therefore, this study aims to characterize the full-length sequences of Casp-1, Casp-3 and Casp-8 from *T. macdonaldi*. Thus, we have conducted an *in vitro* study using totoaba head-kidney and spleen leukocytes to assess gross cytotoxic effect and oxidative stress caused by pathogenic bacteria such as *V. parahaemolyticus* and *Aeromonas veronii*. Information about its immune system under infection conditions is necessary, and disease management is essential to the successful production of this marine fish species.

2. Materials and methods

2.1. Vibrio parahaemolyticus and Aeromonas veronii

The *V. parahaemolyticus* strain used in this study was provided by Centro de Investigaciones Biologicas del Noroeste (CIBNOR, Mexico) from its bacterial collection. Firstly, *V. parahaemolyticus* was cultured in TSB (tryptic soy broth, BD #211,825) supplemented with 2.5% NaCl and incubated at 28 °C for 24 h. Then, its culture was centrifuged at 8000 *g* at 4 °C for 20 min. The supernatant was removed and the bacterial pellet suspended in sterile 0.9% PBS to 1×10^8 cell ml^{-1} (Reyes et al., 2016).

The *A. veronii* biotype *veronii* strain A186 used in this study was obtained from the Microbial Culture Collection of the Hospital of the University of Lund (Sweden) and was kindly provided by Prof. T. Wadström. *Aeromonas veronii* was identified according to Vázquez-Juárez et al. (2003) and re-activated according to Reyes et al. (2010). Finally, the bacterial suspensions were diluted with phosphate-buffered saline (PBS, pH 7.4) to a final concentration of 1×10^8 cell ml^{-1} for the challenge experiment.

2.2. Experimental fish

Healthy juvenile totoabas (two-months-old) without history of bacterial or parasitic infections were provided by the marine hatchery of Earth Ocean Farms Pichilingue Unit in La Paz, Baja California Sur, México where head-kidney and spleen leukocytes were sampled and transported immediately to CIBNOR lab. Temperature was maintained at 23.0 ± 1.0 °C, salinity averaged 35 ± 0.5‰ and photoperiod was maintained on a 12:12 light:dark schedule. Oxygen concentration was kept higher than 6 mg l^{-1} (Mata-Sotres et al., 2015).

2.3. Isolation of head-kidney and spleen leukocytes

For the in *vitro study*, head kidney and spleen samples of nine healthy totoabas (50 ± 5 *g* mean body weight) were used to separate leukocytes under sterile conditions, following Lee et al. (2014). Briefly, tissues were removed and passed through 100-μM cell strainers (BD Falcon, Franklin Lakes, NJ, USA) in sRPMI [RPMI-1640 culture medium (Gibco, Waltham, MA, USA) with 0.35% sodium chloride, 100 IU ml-1 penicillin (Sigma, St. Louis, MO, USA), 100 mg ml-1 streptomycin (Sigma, St. Louis, MO, USA), 10 IU ml-1 heparin (Sigma, St. Louis, MO, USA), and 5% fetal bovine serum (Gibco, Waltham, MA, USA)]. The volume of the cell suspension was adjusted to 2 ml. All samples were carefully layered over 51% Percoll gradient (Sigma, St. Louis, MO, USA) and centrifuged at 500 *g* at 23 °C or 45 min without break. The leucocyte layer was collected, and washed with sRPMI medium by centrifuging at 800 *g* at 23 °C for 10 min. Head kidney leukocytes were observed and counted with a TC20 Coulter Particle Counter (BioRad, Hercules, CA, USA) and adjusted to 10^6 cells ml^{-1} of sRPMI. A drop of leukocyte suspension was stained with trypan blue (Sigma, Cat. T-8154, St. Louis, MO, USA) to calculate viability, and leukocyte suspension with a viability of more than 95% was used for in vitro experiment (Reyes-Becerril et al., 2016).

2.4. Stimulation test

One milliliter of head-kidney and spleen leukocytes was dispensed into 24-well flat-bottomed microtitre plates (Nunc, Thermo Scientific, Waltham, MA, USA) containing 1×10^6 cells ml^{-1} per well. Leukocytes were stimulated with 20 μl of *V. parahaemolyticus* or *A. veronii* (1×10^8