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Discovery of immunoglobulin T in Nile tilapia (*Oreochromis niloticus*): A potential molecular marker to understand mucosal immunity in this species

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ABSTRACT

Immunoglobulin molecules play an important role in the immune defense system in all jawed vertebrates, by protecting the organism from a wide variety of pathogens. Nile tilapia (*Oreochromis niloticus*) is extensively cultivated worldwide, with a strong established market demand. It constitutes one of the model species for the study of fish immunology and its genome is currently fully sequenced. The presence of the immunoglobulin M gene in this species is well documented, as well as its major role in systemic immunity. To date, the IgT gene from *O. niloticus* has not been identified and, therefore, no information is available on the role of this immunoglobulin isotype in the immune response in tilapia. In the present work, novel secreted and membrane immunoglobulin T isotypes and a fragment of IgM were isolated from tilapia head kidney lymphocytes. Their transcriptional profiles were analyzed by quantitative PCR in larval development and in different tissues of healthy or lipopolysaccharide/*Edwardsiella tarda*-challenged tilapia adults. The presence of IgT and IgM were detected in early stages of larval development. Additionally, these genes exhibited differential expression profiles in basal conditions and after *E. tarda* infection in adult tilapia, in accord with the proposed effector functions of these immunoglobulins in the systemic and mucosal compartments. Our results suggest the potential involvement of this new Ig in mucosal immunity in tilapia.

1. Introduction

All Gnathostomes (vertebrates with jaws) possess an adaptive immune system (Raida et al., 2011; Sunyer, 2012). One of the fundamental features of the adaptive immune response is the production and secretion of antibodies or immunoglobulins (Igs) for defense against infectious agents. The study of immunoglobulin genes in teleost fishes has both applied and basic value: it can help improve the health of economically valuable fish species for aquaculture and gives a better understanding of the genetic basis and evolutionary divergence in the antibody response in each species (Hikima et al., 2011).

Immunoglobulins are produced mainly by plasma cells. They can be found in their secreted form as antibodies, a component of bodily fluids (including serum and mucosal secretions), or in their membrane-bound form on the surface of B cells where they act as receptors (BCR) (Salinas et al., 2011). Ig molecules are composed of two heavy chains (IgH) and two light chains (IgL), which remain linked by interchain disulfide bonds. The antigen binding sites are located in the variable region of the light and heavy chains, while the constant region of the heavy chains determines the effector functions of the different Ig isotypes (Abbas et al., 2012).

Mammals possess five functionally distinct Ig isotypes (IgM, IgD, IgG, IgA, and IgE), whereas three different Ig isotypes have been recognized in teleost fishes (IgM, IgD, and IgT/Z) (Sunyer, 2013; Mashoof and Criscitiello, 2016). Teleost IgM was first identified by Marchalonis in 1971, and is present in all vertebrate species (Fillatreau et al., 2013). The soluble (secreted) form of IgM of teleost fishes is generally found as a tetramer, unlike the mammalian pentameric secreted IgM (Parra et al., 2013). It constitutes the most abundant Ig class in fish serum, and is the isotype that plays the most important role in the adaptive immune response at the systemic level (Cuesta et al., 2004). In addition, IgM also has a role in mucosal immune responses. IgD has a wide distribution among vertebrates, and was initially discovered in 1997 by Wilson and colleagues in channel catfish (*Ictalurus punctatus*). However, its function is still not well defined (Parra et al., 2013). The most recently discovered Ig isotype is IgT (also called IgZ due to its

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identification in zebrafish), which likely exists in most teleost species and seems to be exclusive to this group of vertebrates (Mashoof et al., 2014; Mashoof and Criscitiello, 2016). It was first identified in 2005 in zebrafish (*Danio rerio*) (Danilova et al., 2005) and in rainbow trout (*Oncorhynchus mykiss*) (Hansen et al., 2005), and to date there are several reports of its identification in most model teleost species, with the notable exception of medaka (*Oryzias latipes*) and channel catfish (Mashoof and Criscitiello, 2016). Protein characterization and functional localization studies in rainbow trout show that IgT can play a crucial role in the specific immune response against infectious agents in the intestinal, epithelial and gill mucosal surfaces (Zhang et al., 2011; Xu et al., 2013, 2016).

Nile tilapia (*Oreochromis niloticus*) constitutes one of the most important species for aquaculture worldwide, with a strong established market demand. The production of this freshwater species has increased considerably in recent decades because they are very resistant, easy to reproduce and breed, and due to the quality of their meat (Wang et al., 2015). However, intensive production of tilapia, as with most cultivable species, faces serious challenges among which, the high incidence of pathogens in the aquatic environment is a major concern (Munang'andu et al., 2016). Therefore, the ability to measure the humoral immune response (systemic and mucosal) facilitates the evaluation and understanding of the immune response developed in tilapia under different environmental conditions and stimuli, as well as the protective effects induced by newly developed vaccines.

Nile tilapia is also one of the model species for the study of fish immunology (Fillatreau et al., 2013) and its genome is currently sequenced. Despite this, to date there are no reports of the third Ig isotype, IgT/Z. Taking this into account, in the present study we isolated for the first time the cDNAs encoding the heavy chain of the secreted and membrane-bound forms of IgT in *O. niloticus* and characterized their nucleotide and deduced amino acid sequences. Furthermore, we analyzed their expression profiles by real-time PCR in early larval developmental stages, in different tissues in healthy adult fish and after stimulation with lipopolysaccharide (LPS) and infection with *Edwardsiella tarda* in comparison to IgM. Our results suggest that IgT (comparing with IgM) is more specialized for mucosal immunity in Nile tilapia, as seen in other teleost species.

2. Materials and methods

2.1. Animals

Nile tilapias (*Oreochromis niloticus*) were provided by the CIGB Aquaculture Station, Havana, Cuba, and were kept in aerated freshwater under natural photoperiod. Water temperature was maintained between 28 °C and 30 °C. All animal experiments were previously approved by the Ethics Committee of the Center for Genetic Engineering and Biotechnology, Havana, Cuba. Only the healthy fish, as determined by the general appearance and the level of activity, were used for the studies.

2.2. Identification of immunoglobulin sequences in tilapia nucleotide databases

A search using known sequences of teleost fish immunoglobulin T/Z (GenBank Accession No.: *Epinephelus coioides* GU182366; *Ctenopharyngodon idella* DQ478943, DQ489733; *Cyprinus carpio* AB004105, AU301009; *Danio rerio* AY643750, AY643752, AY646263) was performed to identify possible IgT sequences in the databases of expressed sequence tags (EST) of Nile tilapia (taxid:8128) (http://www.ncbi.nlm.nih.gov/). As a result, homologous EST sequence (GenBank Accession No.: GR632518) and Hypothetical Protein (GenBank Accession No.: XM_003457108) hits were retrieved and translated into protein sequence for analysis using BlastX (https://blast.ncbi.nlm.nih.gov/Blast.cgi/) and Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Based on the sequence information found, specific pairs of oligonucleotides were designed and synthesized to allow the cloning of the identified transcripts (Supplemental Table 1).

For amplification of a fragment of tilapia IgM sequence, two sequences found in the databases (GenBank Accession No.: KJ676389 and KF305823) were analyzed using BlastX and Clustal Omega. Based on the sequence information found, specific oligonucleotides (shown in Supplemental Table 1) were designed and synthesized to allow amplification by fragments of the cDNA encoding the consensus region between both sequences.

2.3. Isolation of tilapia head kidney lymphocytes and total RNA extraction

Six adult tilapias with an average body weight of 200 g were previously acclimated for 1 week. Then, fish were sacrificed and head kidney samples were aseptically extracted. Head kidney lymphocytes were isolated using Ficoll-Paque Plus (GE Healthcare) according to the manufacturer's recommendations, with minor modifications. The organs were macerated in 2 mL sterile PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 $7H_2O$) using a 100 µm cell strainer, then placed on Ficoll-Paque and centrifuged at 2500 rpm for 25 min. The leucocyte band was collected, washed twice with PBS and kept at −70 °C until use. Total RNA was extracted with Tri-Reagent (Sigma) according to the manufacturer's recommendations. Concentration and purity was determined in a NanoPhotometer NP80 (IMPLEN) measuring optical density at 260 nm and the 260/280 and 230/280 ratios. The product was analyzed in denaturing gel electrophoresis.

2.4. cDNA synthesis for sequence analysis

The first complementary DNA (cDNA) strand was obtained from 5 µg of total RNA previously treated with DNase (RQ1 RNAase-Free Dnase, Promega). Reverse-transcription was performed using a Reverse Transcription System (Promega) according to the product manual. The reactions were performed in a final volume of 20 µL in the following conditions: 4 µL of $MgCl_2$ 25 mM, 2 µL of Reverso Transcriptase enzyme buffer, 2 µL of dNTP 10 mM mix, 0.5 µL of inhibitor RNasin ribonuclease, 15 U of AMV Reverso Transcriptase enzyme, 0.5 µg of Oligo $(dT)_{15}$ and nuclease free water. Reactions were incubated for 15 min at 42 °C and 5 min at 95 °C for inactivating reverse transcriptase. Afterwards, the reaction was stored at −20 °C until further use.

2.5. DNA amplification using the PCR

Amplification reactions were performed with Platinum® Pfx DNA Polymerase (Invitrogen) according to the product manual, in a total volume of 50 µL, using 3 µL of the product from the reverse transcription reaction and 50 pmol of specific oligonucleotides designed for the EST sequence, the Hypothetical Protein and for the IgM fragment (Supplemental Table 1). In each reaction was included a negative control with all reactants excluding the DNA. Reactions were carried out in a Minicycler TM intelligent heating block (MJ Research, Inc.). Reaction steps were: 2 min initial denaturation at 94 °C, 1 min denaturation at 94 °C, 30 s annealing at 50 °C (for EST and Hypothetical Protein) and 62 °C (for IgM), 1 min extension at 68 °C (35 cycles) and 5 min final extension at 68 °C. Then, electrophoresis was performed in a 1% (w/v) agarose gel. The run was performed at 120 V in TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8) supplemented with ethidium bromide to a concentration of 0.5 µg/mL.

The DNA amplified in each reaction by PCR was purified using a QIAquick Gel Extraction Kit (Qiagen), inserted into the pGEM-T Easy vector (Promega) and transformed into competent TOP 10 *Escherichia coli* cells. Plasmid DNA from at least 12 independent clones was purified at analytical scale using alkaline lysis mini-plasmid preparation. Positive clones identified by restriction analysis were subjected to DNA sequence analysis using Macrogen DNA Sequencing Services (http://