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C-type lectin 17A and macrophage-expressed receptor genes are magnified by fungal β-glucan after *Vibrio parahaemolyticus* infection in *Totoaba macdonaldi* cells

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ABSTRACT

C-type lectins are a principal carbohydrate recognition mechanism as glucans on cell surfaces. This study identified and investigated molecular characterization and immune roles of a novel c-type lectin 17A from *Totoaba macdonaldi* (*TmCLEC17A*), which were described in head-kidney leukocytes after immunostimulation with fungal β-glucan 197A and *Vibrio parahaemolyticus* infection. This nucleotide sequence from totoaba was acquired using NGS and bioinformatics tools. Its full-length cDNA sequence consisted of 1128 bp (including the stop codon) and an open reading frame (ORF) of 771 bp encoding a 256 amino acid protein, 5´-UTR of 48 bp and 3´-UTR of 309 bp. The *TmCLEC17A* protein revealed a C-terminal-C-type lectin (CTL, also named carbohydrate-recognition domain, CRD), a N-terminal trans-membrane domain and a coiled coil motif, showing the highest similarity (80%) and identity (96%) with *Larimichthys crocea*. Fungal β-glucan 197A plus *V. parahaemolyticus* enhanced transcriptions of *CLEC17A* and TLR2 significantly besides the macrophage receptors, such as macrophage mannose receptor 1 and macrophage colony-stimulating factor 1 receptor 2. In addition, natural resistance-associated macrophage protein 2 was significantly up-regulated in leukocytes challenged with live *V. parahaemolyticus*. Overall, these results indicated that *CLEC17A* might be implicated in *T. macdonaldi* innate immunity as a pattern recognition receptor; fungal β-glucan 197A could stimulate cellular immune mechanisms in head-kidney leukocytes; and it could be used as potential immunostimulant in fish aquaculture.

1. Introduction

During the last decade, functional foods and natural plant products have recently originated considerable interest as alternative agents for fish health. Natural fungus-derived molecules are being promissory pharmaceutical products, including glucans (Borchani et al., 2016). Glucans are structural molecules present in the cell walls of multicellular and unicellular fungi (Free, 2013). Fungal β-glucans are well-known immunostimulatory molecules that can help against many diseases in animals and human beings (Chen and Seviour, 2007). In a previous study, the immunostimulatory effect of β-glucans-derived from fungus was evaluated in fish leukocytes (Cárdenas-Reyna et al., 2017). Interestingly, *Aspergillus niger*-β-glucan strain 197 A could activate immune cells and promote phagocytic and antioxidant enzymatic activities. Indeed, *A. niger*-β-glucan inhibited the *Aeromonas hydrophila*-induced apoptosis in fish leukocytes, indicating a potent immunostimulant induced response by fungal derived-glucans (Cárdenas-Reyna et al., 2017).

The critical initial process for the induction of immune mechanisms is the detection of antigenic molecules, which results in the activation of down-stream pathways (Arancibia et al., 2007). This recognition has been accomplished by a diverse group of pattern recognition receptors (PRRs), which identify specific molecules found in pathogens, named pathogen-associated molecular patterns (PAMPs) (Li et al., 2017). Several families of PRRs have been identified such as Toll-like receptors (TLRs) and C-type lectin-like receptors (CTLRs), among others (Zhang and Chen, 2015). C-type lectins have a typical domain named C-type lectin domain (CTLD) or carbohydrate recognition domain (CRD), which has strong affinity to carbohydrates present in microorganisms (Zelensky and Gready, 2005). By binding to these structures, C-type lectins mediate diverse crucial cellular processes including leukocyte adhesion and fast stimulation of defense mechanisms against harmful microorganisms (Drickamer and Taylor, 1993). In fish, various types of lectins have been characterized and they can be central molecules to

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develop immunity against pathogens (Vasta et al., 2011; Johansson et al., 2016; Nakamoto et al., 2017). In Atlantic salmon (*Salmo salar*), three kinds of C-type lectin receptor (CLRA, CLRB, and CLRC) genes were expressed in peripheral blood and head kidney leukocytes, and the mRNA transcription of these genes was up-regulated after bacterial infection (Soanes et al., 2004). In other recent study, a C-type lectin domain family 4 homologue gene was strongly up-regulated in primary and secondary immune organs after *Yersinia ruckeri* infection in rainbow trout (*Oncorhynchus mykiss*) (Johansson et al., 2016). Recently, collectin, a subfamily member of the C-type lectins, was involved in host defense against Gram-positive (*Streptococcus agalactiae*) and Gram-negative (*Aeromonas hydrophila*) bacterial pathogens in Nile tilapia (*Oreochromis niloticus*) (Mu et al., 2018).

On the other hand, fungal-glucans possess many of the characteristics attributed to pathogen-associated molecular pattern molecules (PAMPs) and have been shown to interact with several receptors on the surface of macrophages (Underhill et al., 1999; Brown and Gordon, 2001). In mammals, dectin-1 a small type II receptor expressed on the cell surface of innate immune cells, including macrophages and dendritic cells, has been shown to mediate the response of macrophages to intact yeast and zymosan (Brown and Gordon, 2001). However, the exact mechanisms of receptor binding and internalization of glucan particles, as well as downstream signaling pathways leading to macrophage activation, have not been fully studied yet in fish.

Recently, the development of totoaba aquaculture has been started in Baja California, Mexico, allowing its successful management from broodstock and juvenile stages. Therefore, this species is gaining interest and efforts are being pursued in intensive aquaculture due to its rapid growth. Given the remarkable function of C-type lectin in innate immunity and that the relevance of bioactive compounds-derived from fungi has been increased in recent years, particular attention has been paid to the carbohydrate molecules and their interactions with the fish immune system. Taking all these previous considerations into account, this study characterized the first C-type lectin family 17 member A from *Totoaba macdonaldi*. The mRNA expression profile of *TmCLEC17 A* and its modulation by fungal β-glucan 197 A and *Vibrio parahaemolyticus* infection were characterized in head-kidney leukocytes.

2. Materials and methods

2.1. Fungal β-glucan

Fungal β-glucan isolated from *Aspergillus niger* (197 A) was characterized by Cárdenas-Reyna et al. (2017) and the spectra of anomeric proton resonances identified a (1/3)-β-D-glucan.

2.2. Vibrio parahaemolyticus

The *Vibrio parahaemolyticus* strain was obtained from the bacterial collection of Centro de Investigaciones Biologicas del Noroeste (Mexico). *V. parahaemolyticus* was cultured as described Reyes-Becerril et al. (2016); and the bacterial numbers were finally adjusted in PBS (NaCl 137 mM / L, KCl 2.7 mM / L, Na_2HPO_4 10 mM / L, KH_2PO_4 1.8 mM / L, pH 7.3) to 1×10^8 cell ml^{-1} immediately before the experiments.

2.3. Fish and ethical statement

The commercial farm Earth Ocean Farms S.R.L.C.V., BCS, Mexico, kindly donated healthy juvenile *Totoaba malcdonaldi* (n = 6) for this study. CIBNOR Bioethical Committee approved the experiments performed in this study. Each organism was anesthetized (Eugenol, 50 mg l^{-1}) before exsanguination and then decapitated to obtain tissue samples (head-kidney, liver, spleen, skin, muscle, thymus, and intestine). Tissue samples were sampled and immediately stored at 80 °C in TRIzol Reagent (Invitrogen) for RNA extraction, which was used to examine CLEC17 A expression level in these tissues.

2.4. Leukocytes preparation

Leukocytes were isolated from head-kidney of healthy totoaba (Lee et al., 2014). Samples from head-kidney were filtered (100-μm, BD Falcon, Franklin Lakes, NJ, USA) for cell collection using RPMI-1640 medium (Gibco, Waltham, MA, USA) adding 0.35% NaCl, antibiotics, and fetal bovine serum (Reyes-Becerril et al., 2016). Then, cell suspensions were dispensed over Percoll gradient (Histopaque 1077 (Sigma, Cat: 10771, St. Louis, MO, USA) 51%) and centrifuged (500 *g*, 23 °C, 45 min without brake). Leukocyte film was obtained and after washing with sRPMI medium (800 g at 23 °C for 10 min), head-kidney leukocytes viability was estimated up to 95% and adjusted to 1×10^6 cells ml^{-1} per well before bioassays.

2.5. In vitro study

The *in vitro* study was performed following the protocol described by Cárdenas-Reyna et al. (2017). β-glucan (200 mg ml^{-1}) were added to head-kidney leukocytes (1×10^6 cells ml^{-1}) and after 8 h of incubation, 20 μl containing *V. parahaemolyticus* (1×10^8 cells ml^{-1}) were dispensed. Leukocytes incubated with zymosan A (50 mg ml^{-1}) (Sigma, Z4250, St. Louis, MO, USA) were used as positive controls. Twenty-four hours later, the cell suspension was centrifuged (11 000 g, 10 °C, 1 min) and one ml of Trizol reagent (Invitrogen, USA) was added to the cell pellet for RNA extraction.

2.6. In silico analysis: C-Type lectin domain family 17 member A (CLEC17 A)

The CLEC17 A gene sequence was determined using next generation sequencing (NGS) and bioinformatics analysis of ensemble transcripts. Briefly, mRNA was purified from *Totoaba macdonaldi* head-kidney, and Truseq stranded mRNA sample preparation kit (Illumina) was used for cDNA synthesis. MiSeq reagent kit v.2 (MiSeq, Illumina) was used for NGS analysis; the novo reads and contigs were obtained with the Trinity program (Grabherr et al., 2011). The tBLASTn program served to identify homologous sequences to the mammalian CLEC17 A (Altschul et al., 1990). Finally, primers were designed based on the obtained sequence.

The identity and similarity of CLEC17 A protein was compared with homologous proteins in other species (http://www.ncbi.nlm.nih.gov/blast). Subsequently, the predicted amino acid sequence was summited (EXPASY, https://www.expasy.org/) to obtain the molecular weight and isoelectric point. Protein domains, motifs and characteristic regions of the protein were predicted (http://smart.embl-heidelberg.de/ and http://prosite.expasy.org/). The Phyre2.0 program (http://www.sbg.bio.ic.ac.uk/phyre2/) was also used to predict the secondary structure by comparing it with several protein structures. Finally, multiple alignments at the amino acid level (http://www.ebi.ac.uk/Tools/msa/clustalo/) and the evolutionary tree were obtained (http://www.megasoftware.net) using 10,000 bootstrap replications.

2.7. Expression analysis

Relative mRNA transcription from leukocytes was determined using

Table 1
Oligonucleotide primers used for real-time PCR.

Gen	Gen abbreviation	Accession No.	Primer sequence (5´-3´)
C-type lectin family 17 member A	CLEC17A	MH051307	GTCTCTCCCAATGGTGTGA AGTTCCATCCACCCACTTC
Macrophage mannose receptor 1	MMR	MH051308	ACAAGGCTGGAAAAAAATTGAC GTTCGTGCGATCGTAAAAAG
Natural resistance-associated macrophage protein 2	NRAMP	MH051310	GTTTTTTCCCAGCATCAGTC GTATTTCCAGATGGCGTAGTAGTG
Macrophage colony-stimulating factor 1 receptor 2	MCSFR	MH051309	TGTTCGTCACACCTTACAAAAC TGGGGTCAAGGGTAACATTC
Toll like receptor-2	TLR2	MH051311	GATGCTAGCTGGGTGGAA CGAAATTCTCAGAGAGGACG
Elongation factor-1α	EF-1α	KX524957	CATTGTCAAACTCATTCCACAG CACGGTCTGCCTCATGTC

quantitative PCR as well as the 2^{DDCT} method (Livak and Schmittgen, 2001). The RNA from leukocytes was obtained after stimulation with fungal β-glucan and post-infection with *Vibrio parahaemolyticus* (at 24 h) and then reverse-transcribed to cDNA, which was used to examine the mRNA expression levels of C-type lectin 17 A, Toll-like repeceptor 2 (TLR2) and macrophage markers (MMR, macrophage mannose receptor 1; MCSFR, macrophage colony-stimulating factor 1, receptor 2; NRAMP, natural resistance-associated macrophage protein 2). The mRNA transcription of immune-relevant genes was determined by quantitative PCR (ABI PRISM 7500 instrument) following the protocol reported in Reyes-Becerril et al. (2016). The expression of elongation factor 1-alpha (ef-1α) gene was used as endogenous reference. Table 1 shows the primers used in this study. Triplicates samples were used for each quantitative PCR analysis.

2.8. Statistical analysis

The experiments and analysis were determined in triplicate. Means and standard deviations was calculated. A one-way analysis of variance (ANOVA) and Tukey's test were used to analyze the results of β-glucan on immunological parameters (SPSS v.23.0 software, Richmond, VA, USA). Data from gene expression analysis were calculated and showed as previously described (Reyes-Becerril et al., 2016). Statistical differences were significant at $P < 0.05$ level.

3. Results

3.1. Analysis and characterization of ORF sequence of C-type lectin family 17 member A

The cDNA of *Totoaba macdonaldi* CLEC17A (*TmCLEC17A*) (GenBank: MH051307) consisted of 1128 bp (including the stop codon) with an ORF of 771 bp encoding a protein of 256 amino acid sequence, a 5´-UTR of 48 bp and a 3´-UTR of 309 bp (Fig. 1). The calculated molecular weight of *TmCLEC17A* was 29.53 kDa with an estimated isoelectric point of 5.10.

3.2. Multiple sequence alignments

The TmCLEC17A protein revealed a C-terminal C-type lectin (CTL) or carbohydrate-recognition domain (CRD), a N-terminal transmembrane domain and a coiled coil motif (Fig. 2); however, these structural features were not conserved in all the species (Table 2). Two sugar binding motifs were identified in the C-terminal CTL domain, 221 × P × 223 (X for E, Q, D and N) and 241WND243. In this domain, the Ca2+ binding sites 1 (G196, E199, N224 and D230) and 2 (E221, N223, N242 and D243) were also found (Fig. 2); two pairs of conserved disulfide bonds (C164- C254 and C231- C246) were identified (Fig. 1). The predicted secondary structure elements of TmCLEC17 A protein (100% confidence and 47% coverage) consisted of four α helix, one transmembrane helix and seven β sheets. The tertiary predicted 3D structural model is shown in Fig. 2. The TmCLEC17 A showed the highest similarity (80%) and identity (96%) with *Larimichthys crocea* (Table 3).

3.3. Phylogenetic analysis

The phylogenetic analysis of TmCLEC17A protein showed different clusters for each species class (Fig. 3). Moreover, the TmCLEC17A amino acid sequence was found in the same cluster than *Larimichthys crocea*, revealing a close relationship with this species, which agrees with the highest similarity and identity observed in the multiple sequence alignment analysis (Table 3).

3.4. The expression pattern analysis of C-type lectin 17A (CLEC17A) in different tissues from totoaba

Real-time PCR was used to determine tissue distribution of the *CLEC17A* gene in seven totoaba tissues (head-kidney, liver, spleen, skin, muscle, thymus and intestine). The mRNA expression profiles of the *CLEC17A* gene differed substantially. The expression of *CLEC17A* was the highest in the hematopoietic head-kidney, liver and spleen tissues followed by skin, muscle, and thymus, and the lowest in the intestine (Fig. 4, $P < 0.05$).

3.5. Fungal β-glucan up-regulated the expression profiles of C-type lectin and TLR2 after V. parahaemolyticus infection

To investigate stimulus effects on *CLEC17A* and TLR2 expression *in vitro*, totoaba leukocytes were stimulated with fungal β-glucan and challenged with live *V. parahaemolyticus* after 24 h post-stimulation-infection (Fig. 5). In this study, we could observe a high up-regulation of *CLEC17A* and TLR2 genes in those leukocytes stimulated with fungal β-glucan plus *V. parahaemolyticus* compared with the other control groups.

3.6. Fungal β-glucan highly up-regulate macrophage receptors

Several *in vitro* reports have demonstrated that β-glucans are potent immunostimulatory molecules that increase functional activity of macrophages. This study confirmed that fungal β-glucan plus *V. parahaemolyticus* induced a high up-regulation in MMR and MCSFR genes compared with the zymosan group plus *V. parahaemolyticus* or *V. parahaemolyticus* alone (Fig. 6ab).

Fig. 1. Nucleotide and deduced amino acid sequence of C-type lectin family 17 member A for *Totoaba macdonaldi*. The star codon (ATG) is boxed; the stop codon (TAA) is marked with an asterisk; the polyadenylation signal (AATAAA) is in uppercase and bold; and the cystein residues involved in disulfide bonds are in bold (C^{164}, C^{254}, C^{231} and C^{246}). Transmembrane domain is highlighted in light grey (residues 34–56); coiled coil motif is highlighted in dark gray (residues 70–127); and C-type lectin (CTL) or cabohydrate-recognition domain (CRD) is highlighted in black (residues 134–255).

Interestingly, NRAMP (Natural resistance-associated macrophage protein 1) gene expression was highly up-regulated in leukocytes infected by *V. parahaemolyticus* followed by fungal β-glucan plus *V. parahaemolyticus* (Fig. 6c).

4. Discussion

C-type lectins (CTLs) are an important type of pattern recognition receptors (PRRs) with crucial functions in the immune system (Rauta et al., 2014; Zhu et al., 2013), which have been described as Ca^{2+}-dependent sugar-recognition proteins and functioned as PRR to distinguish between safe and danger molecules by recognizing and binding the carbohydrates on the surface of microorganisms (Weis et al., 1998; Zelensky and Gready, 2005). The CTL receptor superfamily is classified into 17 groups considering different properties, such as structural, functional, and phylogeny (Monteiro and Lepenies, 2017). This study characterized for the first time the *CLEC17A* from *T. macdonaldi* based on bioinformatics analyses. We characterized the sequence of *CLEC17A* obtained from *T. macdonaldi*, which is a glycan-binding receptor probably involved in the carbohydrate mediated interaction among cells in germinal centers (Graham et al., 2009). The *in silico* analysis of the sequence revealed an ORF of 771 bp, which codified a protein of 256 amino acid. The *TmCLEC17A* protein showed a transmembrane domain, a coiled coil motif and the CTL domain; these structural features were even found conserved in the bird species: *Nipponia nippon*, *Phaethon lepturus* and *Mesitornis unicolor*. However, the CTL receptors can be also soluble proteins (Geijtenbeek and Gringhuis, 2009). Interestingly, the four cysteins (C164- C254 and C231- C246) associated in the formation of the internal disulfide bonds in the C-terminal CTL domain were conserved in twelve species analyzed in this study (Zhang et al., 2018; Wei et al., 2010). Particularly, the main role of CTL domain is the recognition of antigens with carbohydrate residues located on the surface of pathogens (Johansson et al., 2016). On the other hand, the sugar binding motifs 221 × P × 223 and 241WND243 were conserved in all the species (except for *Crassostrea gigas* and *Mizuhopecten yessoensis* in the WND motif). The XPX is important to the Ca2+/carbohydrate-binding, specifically the EPN motif found in almost all sequences is considered as a mannose-binding characteristic of mannose-binding proteins (Ao et al., 2015; Wei et al., 2010; Johansson et al., 2016). The Ca^{2+} binding sites were mostly conserved among the species, including *Homo sapiens* (Graham et al., 2009). In addition, *TmCLEC17A* protein showed 100% confidence and 47% coverage with the *H. sapiens* C-type mannose receptor 2 templates, which agrees with features found in the sequence (Johansson et al., 2016).

This research examined the mRNA transcription of *CLEC17A* gene observing a constitutive expression in all the studied tissues. Its expressions were the highest in head-kidney and liver, as well as in spleen and skin. Interestingly, a large number of fish CTLs from orange-spotted grouper *Epinephelus coioides* (Ji et al., 2014), tongue sole *C. semilaevis* (Zhou and Sun, 2015) and black rockfish *Sebastes schlegelii* (Liu et al., 2016) were prominently expressed in the liver. In healthy grass carp (*Ctenopharyngodon idella*), the mRNA expression of mannose receptor type 1, a C-type lectin, were constitutively expressed in several tissues with variable levels; however, the highest gene expression was found in spleen and head-kidney, indicating that mannose receptor type 1 might play a crucial role in innate immune responses (Wang et al., 2014). Therefore, the information of C-type lectin mRNA distribution in different tissues and their response to immunostimulants (*i.e.* glucans) or pathogens are useful to understand their particular functions in immunity.

Several studies have highlighted that β-glucans can activate immune cells, stimulating the expression of C-type lectin and pro-inflammatory mediators (Panagos et al., 2006; Zheng et al., 2015; Jaehrig

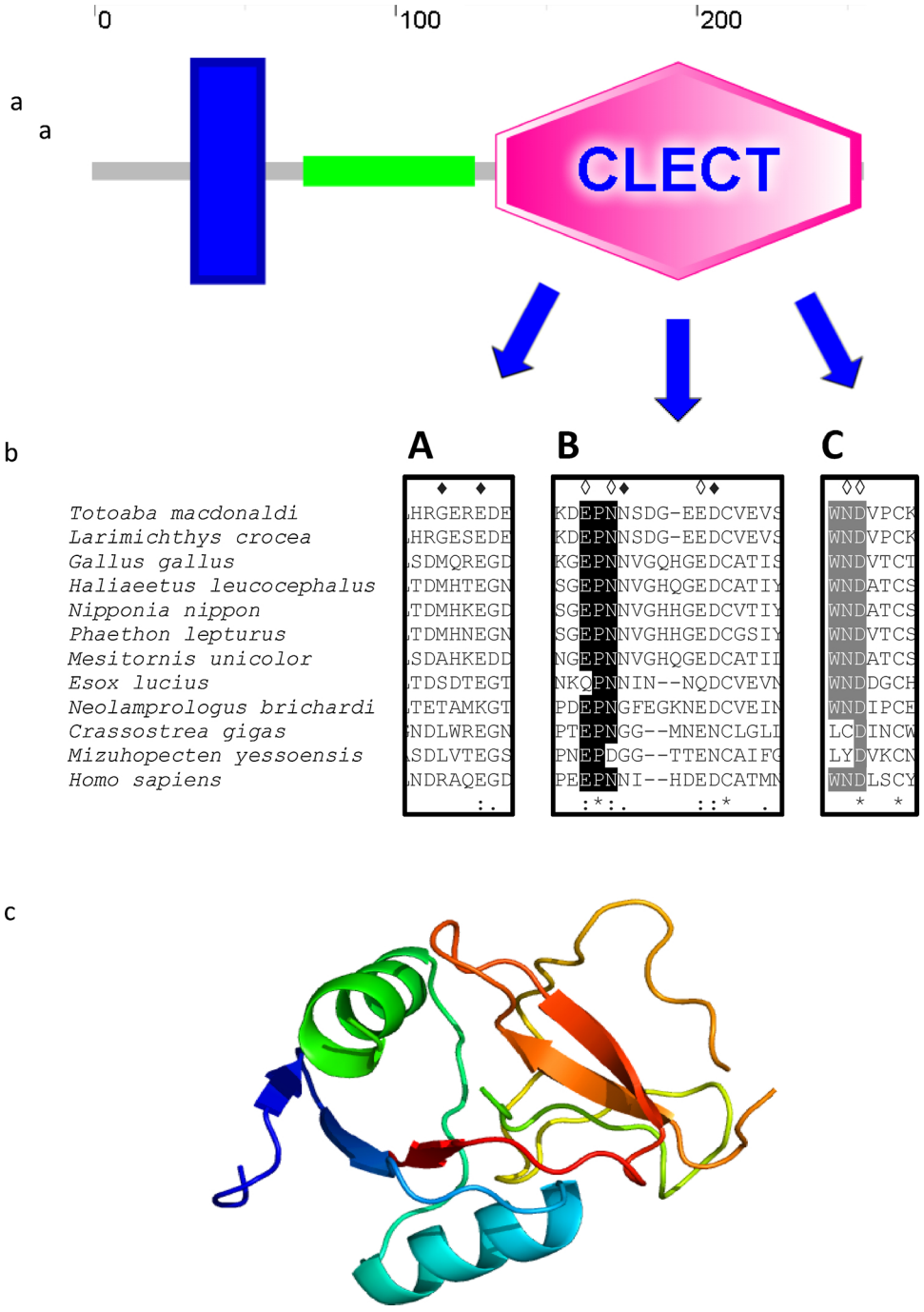

Fig. 2. Diagram showing the domain organization, conserve motifs and predicted 3D structure of *Totoaba macdonaldi* in C-type lectin family 17 member A: (a) The transmembrane domain, coiled coil motif and C-type lectin (CTL) or carbohydrate-recognition domain are shown in blue, green and pink, respectively; (b) The EPN and WND motifs are highlighted in black and dark gray, respectively. The Ca^{2+} binding sites 1 are marked with a solid diamond and the binding sites 2 are marked with a hollow diamond; and (c) Predicted 3D structural model of *Totoaba macdonaldi* C-type lectin family 17 member A with a confidence and coverage of 100 and 47%, respectively. The models were created with Phyre2 using a Homo sapiens C-type mannose receptor 2 template (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

et al., 2007; Sonck et al., 2010). Therefore, this study assessed the *in vitro* immunomodulatory effects of fungal derived-β-glucan and zymosan and its protection against a bacterial infection. Recently, our research group evaluated the effect of natural glucans obtain in the laboratory against purified form like zymosan (composed of insoluble β-glucan and mannan) due that this glucan (obtained from baker's yeast cell walls) has been utilized in numerous experiments owing to its ability to activate macrophages and improve protection against infection with a variety of pathogens (Pietretti et al., 2013). This study has demonstrated for the first time that stimulated leukocytes with glucan-derived fungus and infected with *V. parahaemolyticus* increase the mRNA transcription of *CLEC17A* gene in head-kidney leukocytes from totoaba compared with zymosan or *V. parahaemolyticus* treatments. In this regard, the CLRs are usually involved with carbohydrate recognition through specific motifs existing in the CTL domain (Drickamer and Fadden, 2002). Since fungi have (1→3)-β-D-glucans in their cell walls (Fontaine et al., 1996; Lipke and Ovalle, 1998; Cárdenas-Reyna et al., 2017), the immune system has specific receptors to identify those carbohydrates and attack fungal pathogens. Overall, β-glucans induce cellular immune responses by cell surface receptors, such as Dectin 1 and TLR2/6 dimer. Dectin-1 is a C-type lectin receptor expressed on monocytes, macrophages, neutrophils, dendritic cells, and Langerhans cells that recognizes fungal wall–derived β-glucans (Herre et al., 2004; Viriyakosol et al., 2005). Dectin-1 promotes the phagocytosis of live

Table 2
Structural features of *Totoaba macdonaldi* and its counterparts of other species with high identity in C-type lectin family 17 member A (96-24%). Domain and motif organization were predicted using SMART program.

Species	Amino acids residues	Length of		
		Transmembrane domain	Coiled coil motif	CLECT[a] domain
Totoaba macdonaldi	256	23	58	122
Larimichthys crocea	205	–	60	120
Gallus gallus	320	23	–	122
Haliaeetus leucocephalus	304	23	–	130
Nipponia nippon	303	23	37	120
Phaethon lepturus	297	23	31	130
Mesitornis unicolor	333	23	34	124
Esox lucius	204	–	31	124
Neolamprologus brichardi	213	23	–	122
Crassostrea gigas	246	–	–	128
Homo sapiens	264	21	–	122
Mizuhopecten yessoensis	247	–	–	121

(–) Indicates the absence of the domain and motif, respectively.
[a] C-type lectin (CTL) or cabohydrate-recognition domain (CRD).

Table 3
Query cover and identity of *Totoaba macdonaldi* C-type lectin family 17 member A amino acid sequences with other species.

Species	*Totoaba macdonaldi*		
	Accession	Query cover (%)	Identity (%)
Larimichthys crocea	KKF31470.1	80	96
Gallus gallus	XP_003643419.1	68	37
Haliaeetus leucocephalus	XP_010567194	68	32
Nipponia nippon	XP_009465922.1	68	32
Phaethon lepturus	XP_010282980.1	67	30
Mesitornis unicolor	XP_010188290.1	66	32
Esox lucius	XP_019900479.1	59	42
Neolamprologus brichardi	XP_006787634.1	53	42
Crassostrea gigas	XP_019926768.1	50	24
Homo sapiens	EAW84437	47	39
Mizuhopecten yessoensis	XP_021361020.1	39	24

yeast and fungal-derived zymosan particles, as well as promoting zymosan or fungal pathogen-induced proinflammatory response by macrophages and at least in some cases cooperating with TLR2 to mediate

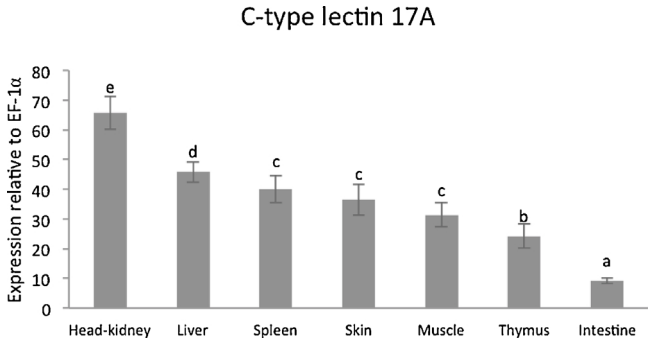

Fig. 4. Tissue expression pattern of *Totoaba macdonaldi* C-type lectin 17-A gene detected by quantitative PCR. The transcript levels in head-kidney, liver, spleen, skin, muscle, thymus and intestine were sampled from healthy totoaba (n = 6). The expression is normalized to EF-1α. Data are the means of three independent experiments and presented as means ± SD.

this response (Gantner et al., 2003; Viriyakosol et al., 2005). In this work, TLR2 gene expression was strongly up-regulated in leukocytes after fungus β-glucan 197A stimulation after infection with *V. parahaemolyticus*. Recently, Angulo et al. (2018) evaluated the effect of stimulated goat-leukocytes with *D. hansenii* β-glucan and observed a highly up-regulated of MyD88 gene expression, suggesting that these β-glucans could also activate TLR2/MyD88/NFκB pathway. Remarkably, Dectin-1 in cooperation with TLR2 activated human macrophage pro-inflammatory responses to promote phagocytosis activity (Gantner et al., 2003; Viriyakosol et al., 2005). Macrophages have a specific affinity for carbohydrate-based delivery systems *via* C-type lectin receptors (CLRs); thus, this study evaluated three important genes: MMR (macrophage mannose receptor 1), MCSFR (macrophage colony-stimulating factor 1, receptor 2) and NRAMP (natural resistance-associated macrophage protein 2), that have been characterized as phagocytic and/or signaling receptors in the context of various infection models (Yadav and Schorey, 2006). Interestingly, MMR and MCSFR gene expression were up-regulated after stimulation with the fungal β-glucan plus infection with the bacterium. One of the most intensively studied pattern recognition receptors (PRRs) is the MR, which has been implicated in pathogen recognition through binding terminal mannose, fucose, and N-acetylglucosamine residues (Lennartz et al., 1987; Wileman et al., 1986). The MMR is ubiquitously expressed on macrophages and has a strong affinity for mannose oligosaccharides playing a main role in anti-inflammatory responses (Taylor et al., 2005). Other similar C-type lectin receptors have critical participation in immune modulation during microbial infections in fish (Wang et al., 2014). On

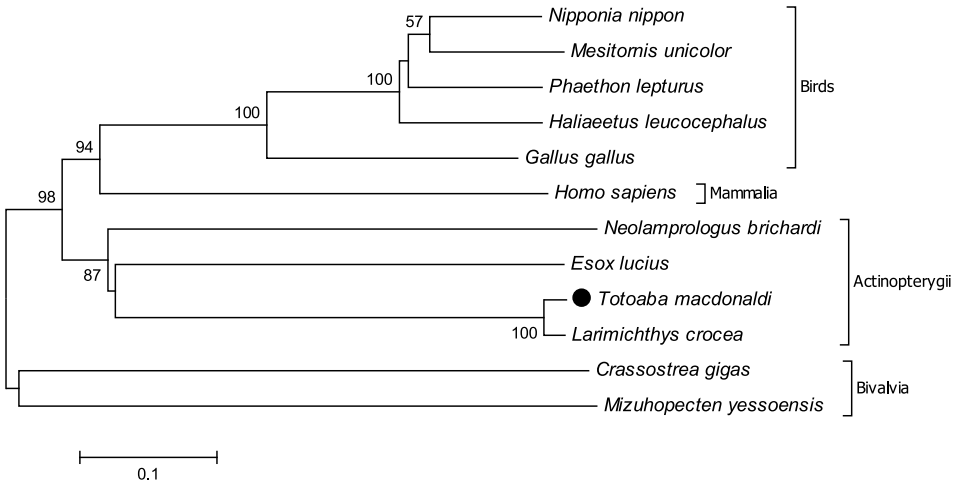

Fig. 3. Phylogenetic analysis of *Totoaba macdonaldi* C-type lectin family 17 member A. The tree is based on an alignment corresponding to the full length of the amino acid sequences using MEGA 6.0 with a cut less than 50 and 10,000 bootstrapping. Genetic distances were calculated based on protein differences (p-distance). Black circle shows C-type lectin family, reported in this study. The tree is grouped by species class.

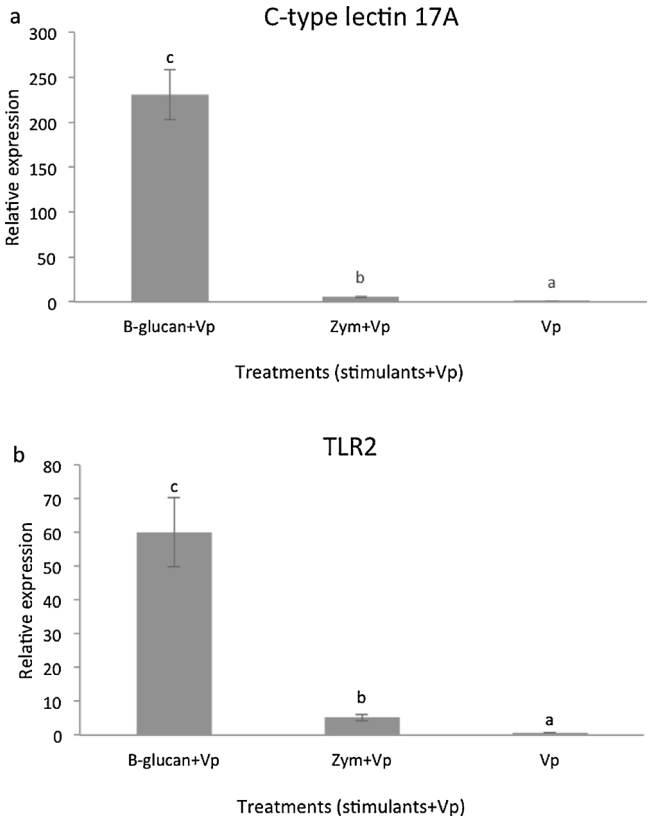

Fig. 5. Gene expression of C-type lectin 17-A (a) and TLR2 (b) in head-kidney leukocytes stimulated with β-glucan and zymosan (as positive control) plus *V. parahaemolyticus* (1×10^8 cell ml^{-1}) at 24 h post-stimulation measured by quantitative real-time PCR. Data are presented as mean ± S.D, and EF-1α was used as a reference gene; significant differences ($p < 0.05$) were calculated. Different letter in the block denotes significant difference between treatments.

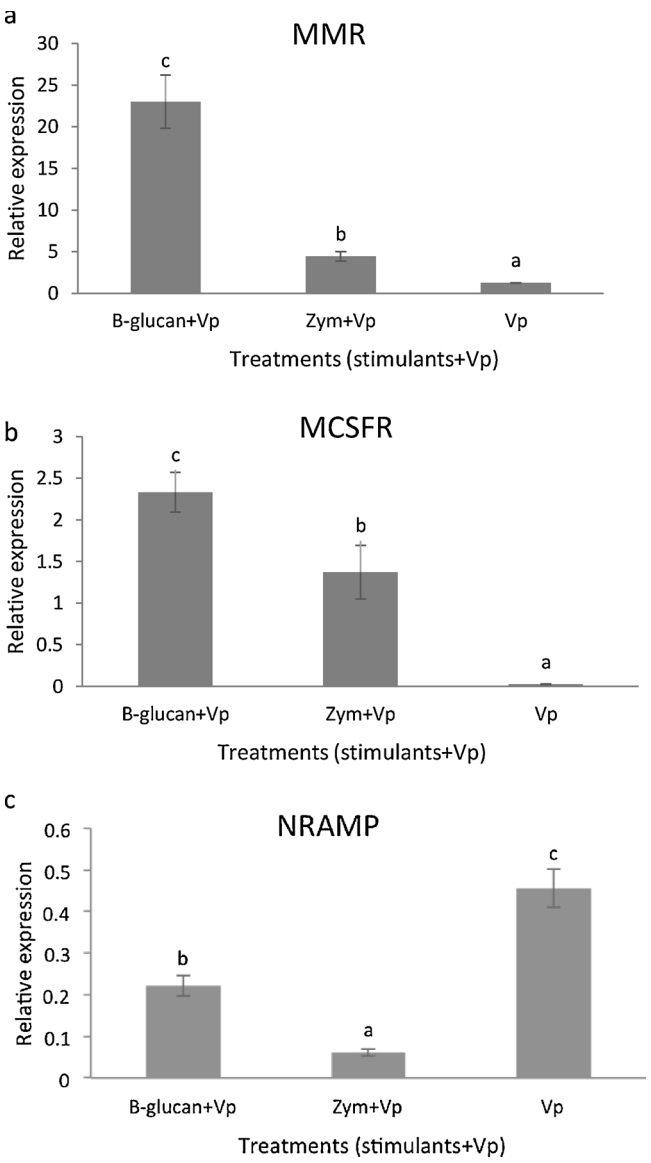

Fig. 6. Gene expression of (a) MMR (macrophage mannose receptor 1), (b) MCSFR (macrophage colony-stimulating factor 1, receptor 2) and (c) NRAMP (natural resistance-associated macrophage protein 2) in head-kidney leukocytes stimulated with β-glucan and zymosan (as positive control) plus *V. parahaemolyticus* (1×10^8 cell ml^{-1}) at 24 h post-stimulation measured by quantitative real-time PCR. Data are presented as mean ± S.D, and EF-1α was used as a reference gene; significant differences ($p < 0.05$) were calculated. Different letter in the block denotes significant difference between treatments.

the other hand, the MCSFR is considered a specific biomarker due to its transcription is essentially limited to the monocytes and macrophages (Hume et al., 1995; Lichanska et al., 1999). In teleost, zebrafish macrophages also express the MCSFR gene (Herbomel et al., 2001). Moreover, MCSFR expression is involved in the modulation of clonal expansion and maduration of macrophages (Pixley and Stanley, 2004). On the contrary, the NRAMP (natural resistance-associated macrophage protein 2) gene expression was highly up-regulated in leukocytes exposed only to the live *V. parahaemolyticus*. In mammals, NRAMP 1 regulates macrophage activation and antimicrobial activity (Blackwell and Searle, 1999-2000), and it is associated with infectious and disease resistance/susceptibility (Bellamy et al., 1998; Abel et al., 1998). The use of NRAMP as a marker for disease resistance in fish might has a promising potential for breeding and selection programs in aquaculture; it could also be useful for the identification of appropriate of immunomodulatory molecules and to analyze the functional characterization of macrophages during infectious diseases in fish.

To conclude, this is the first *C-type lectin 17A* (*CLEC17A*) protein characterized in totoaba fish. The totoaba *CLEC17A* protein conserve structural architectures with its mammalian counterparts, suggesting comparable functions. Moreover, this is the first study where fungal β-glucan isolated from *Aspergillus niger* has been evaluated follow the infection with live *V. parahaemolyticus* in head-kidney leukocytes. The results have shown that, C-type lectin 17A, TLR2 and macrophage markers (MMR and MCSFR) were strongly up-regulated after fungal β-glucan, suggesting that the fungal β-glucan 197A is an effective and powerful immunostimulant. Additionally, analysis of down-stream gene signaling pathways (Syk, TRAF6, MyD88 and NFκB) and pro-inflammatory cytokine (IL-1β and TNF-α) expression induced by β-glucan following *V. parahaemolyticus* infection merits further research. The next step will be to focus on dietary administration in an *in vivo* study for aquaculture implementation.

Conflict of interest

The authors declare that they have no conflicts of interest.

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