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Cloning And Analysis Of The Nile Tilapia Toll-Like Receptor Type 5 Mrna Sequence

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Abstract: Toll-like receptors (TLRs), which detect infections in vertebrates, are the most thoroughly understood innate immune receptors. However, bony fish's TLRs shows distinct features and substantial diversity, which are likely originated from the variation in evolutionary history of fish and the distinct environments that they life in. there are a limited data about the structure of the fish immune system. Our work aimed to identify and clone and sequence the Nile tilapia TLR5 for the first time as a model for freshwater fish species. The full-length sequence of Nile tilapia (Oreochromis niloticus) TLR-5 receptor has been identified, where it consisted of 2661 nucleotides. The consensus cDNA sequence showed 81% identity with the sequence from Takifugu rubripes, 77% identity with the sequence form Japanese flounder, and 75% identity with the sequence from Japanese medaka, which confirmed that the new sequence is considered probably homologous to fish TLR-5. The predicted protein structure encoded by the Nile tilapia TLR-5 mRNA composed of 887 amino acids, beginning with signal peptide as ATG, like other functional fish TLR-5 sequences. Analysis of the deduced amino acid sequence indicated that Nile tilapia TLR-5 has typical structural features and contains the main components of proteins in the TLR family. Our results reveal a complete and functional Nile tilapia TLR-5 that is orthologous to other vertebrate receptors.

Keywords: Nile tilapia - single nucleotide polymorphisms - fish - Toll-like receptor - TLR-5 - gene expression.

1. INTRODUCTION

In developing countries that focus on agriculture economy and consider as a major component, livestock, poultry and aquaculture consider main source for creation jobs and improve the economic condition; this sector suffer in middle east region and specially in Egypt with a lot of threats either in poultry industry (several infectious disease as viral pathogens: Newcastle virus, Infectious Bronchitis, Avian Influenza, and so on) and in

livestock sector (food and mouth disease, bovine diarrhoea and so on; bacterial pathogen like salmonella, E-coli..) and in aquaculture farms [1-13]. To overcome the risks of such threats, should make better understanding to the different species immune system structure. The immune system is extremely important in vertebrate life, and much work has been directed toward studying immune system structure in human and murine models; however, limited data are available regarding the immune system in other species, especially fish species [14-16]. Salmon is considered the major universal fish model, although the salmon immune system may function differently from the immune systems of other fish models. Our work focused on Nile tilapia as a model of freshwater bony fish, as Nile tilapia is one of the major fish subjected to large-scale cultivation worldwide, and little is known about the immune system of Nile tilapia from structural and functional points of view. Therefore, we studied Toll-like receptors (TLRs) in Nile tilapia, and here we show our results concerning Nile tilapia TLR-5. Pathogen-associated molecular patterns (PAMPs) are classes of pathogens associated with molecules recognised by cells of the innate immune system. It is possible to refer to these molecules as small molecular motifs that are retained within a microbe class. In both plants and animals, PAMPs are recognised by TLRs and other pattern recognition receptors (PRRs) [17, 18]. By recognising conserved non-self-molecules, these receptors stimulate innate immune responses that protect the host from infection. An endotoxin located on the bacterial cell membrane, bacterial lipopolysaccharide (LPS), is known to be the prototypical PAMP. TLR receptors are particularly involved in the recognition of one or more patterns; TLR-4 explicitly recognises LPS, recognises bacterial flagellin, and TLR-3 recognises nucleic acid variants generally associated with viruses, such as double-stranded RNA (dsRNA) or unmethylated CpG motifs [14, 16, 19-21]. TLR-3 and TLR-5 are recognised bacterial flagellin and nucleic acid variants commonly associated with viruses, such as double-stranded RNA (dsRNA) or unmethylated CpG motifs; TLR-2 [22-26] or TLR-15 and TLR-21 [27-30] are recognised for lipoteichoic acid from gram-positive bacteria and peptidoglycan, respectively. Although the expression "PAMP" is pretty new, the theory that receptors from multicellular organisms must sense molecules derived from microbes has been debated for several decades, and references to an "endotoxin receptor" are found in most of the early literature. The basic components of the system for vertebrate pathogen identification are TLRs. Even after their standardized general structure, remarkable heterogeneity can be found throughout species in the domain composition of individual TLRs. Typical type-I transmembrane proteins are TLRs and have 3 primary domains: a tandem repeat leucine-rich repeat (LRR) motif that recognises PAMPs, a transmembrane region and a signal-transmitting intracellular Toll/IL-1 receptor (TIR) domain. For the understanding of selective pressures on TLRs, understanding of interspecific distinctions is of particular importance. TLRs are inherent immune system membrane-bound sensors that recognise invariant and distinctive molecular features of invading microbes and are also essential for activating vertebrate adaptive immunity. Genetic variation in TLR genes has been specifically linked to human and livestock differential pathogen outcomes. Nevertheless, it is possible to gain unique insights into the consequence of TLR polymorphisms on the evolutionary ecology of infectious diseases by investigating additional classes of vertebrates that have not yet been extensively studied. TLRs are PRRs that detect and play a role in PAMPs in activating innate and adaptive immune response [24, 25, 31]. Via the detection of LPS, lipopeptides, flagellins, dsRNA and CpG DNA motifs, these receptors play a critical role in host immune responses [27]. The TLR system forms part of an ancient, evolutionarily preserved machinery with homologues found in insects, nematodes, plants, fish, mammals and birds [32]. In non-mammalian vertebrates, including birds and fish, a number of TLR genes have been identified [24]. Among different species, the number of TLR genes varies. Thirteen TLRs have been identified in mammals (TLR-1 through TLR-

13), and these receptors recognise and respond to a wide range of exogenous and endogenous ligands functionally. Of the 13 mammalian TLRs, only the murine genome was identified by TLR-11, TLR-12, and TLR-13. TLR-1 through TLR-5 and TLR-7 through TLR-9 orthologues have been reported in teleost fish, although different studies have suggested that TLR-6 and TLR-10 do not exist in teleost fish [33, 34]. Fish-specific TLRs have been registered, including TLR-18, TLR-19, TLR-20, TLR-21, TLR-22 and TLR-23, in addition to orthologues of TLRs in mammalian species [28, 33, 35, 36]. However, most of these fish TLRs and their components of the signalling cascade exhibit a strong structural resemblance to the TLR system of mammals. Many TLRs are currently only characterised by a limited number of model organisms, including salmon. To our experience, there is almost little data available on Nile tilapia TLRs, which is one of the most extensively farmed fish and is of major economic significance world - wide. Here we're discussing the Nile tilapia TLRs. In human and veterinary medicine, study dedicated to explaining the vast diversity of molecules involved in pathogen identification is of crucial importance. Equally important for understanding the evolutionary biology of host-parasite relationships is such research. Much effort was made to distinguish the components of the immune system in human and mouse models, but much less is actually understood in other organisms regarding the architecture of the immune system [37]. Knowledge relating to any of the many living species can offer new insights into the principles of immune function of vertebrates. Investigations of the fish clade can be especially useful for explaining general patterns of immune system evolution in terrestrial vertebrates. A well-diversified taxon with an origin distinct from that of mammals but with similar physiology is created by fish. Unlike mammals, however, our knowledge of the molecular structure of the immune system of fish is limited [21, 36].In order to investigate the universal validity of the results obtained in humans and mice, new models are needed for these reasons; we have therefore chosen Nile tilapia as a representative freshwater fish [33, 38-41].

2. MATERIALS AND METHODS

2.1 Samples

Kidney, brain, spleen, intestine, muscle, liver, gill, heart and skin samples were obtained from Oreochromis niloticus. Nile tilapia were bought as live mature fish from a standard farm and held under observation for 5 days to make sure they were free from any clinical infections. Then at -80°C, the samples have been collected and processed.

2.2 Primer design

The complete TLR-5 mRNA sequences have been retrieved from GenBank for rainbow trout (Oncorhynchus mykiss), Takifugu rubripes (Fugu rubripes), Larimichthys crocea (large yellow croaker), Paralichthys olivaceus (Japanese flounder), and Epinephelus coioides (orange-spotted grouper). Using the ClustalW software, the sequences were aligned, in which we designed degenerative primers that matched the alignment of the sequence to clone short sequences. Then to obtain the full-length sequence from both the 3 'and 5' directions, we used the RACE method with the SMARTer RACE cDNA Amplification Package (Clontech, CA, USA) according to the manufacturer's instructions. In our laboratory, all primers were designed to (unpublished data).

2.3 Molecular cloning of Nile tilapia TLR-5

Complete RNA was obtained by TRIzol (Invitrogen, USA) from fish kidney, brain, spleen, skin, intestine, muscle, liver, gill, heart and skin samples as per the manufacturer's protocol. RNA samples were used according to the manufacturer's instructions to synthesise the cDNA library in a 10 µl reaction mixture using a BioRT cDNA First Strand Synthesis Package

(Hangzhou Boiler, China). Using common primers, PCR amplification was performed to amplify the target gene using Ex Taq polymerase (Takara Bio, Dalian, China) as per the manufacturer's protocol and as used previous established protocol [17, 18, 42]. In order to obtain the full-length sequence from the 3 'and 5' ends, we used the SMARTer RACE cDNA Amplification Package (Clontech, USA) as instructed by the manufacturer and previously established protocol [17, 18].

2.4 Sequence analysis

Nucleotide BLAST has been used to scan for GenBank with the TLR-5 mRNA Tilapia fish genome, and the translated amino acids were scanned for the BLAST protein to decide if any other cloned gene was associated with the new sequence. The TLR-5 Nile tilapia sequence was contrasted with the known TLR-5 mRNA sequences of various species retrieved from GenBank and matched with ClustalW in the programme MEGA-5 [43]. From the amino acid alignments, a phylogenetic tree was built using the neighbour-joining process, with the options of pairwise deletion, All phylogenetic analyses were performed by MEGA-5 software [30, 43]. The extracellular, transmembrane, and cytoplasmic domains of these sequestrating proteins. The extracellular, transmembrane, and cytoplasmic domains of these protein sequences were predicted with the analysis tools provided at the following websites: http://smart.embl-heidelberg.de and http://split.pmfst.hr.

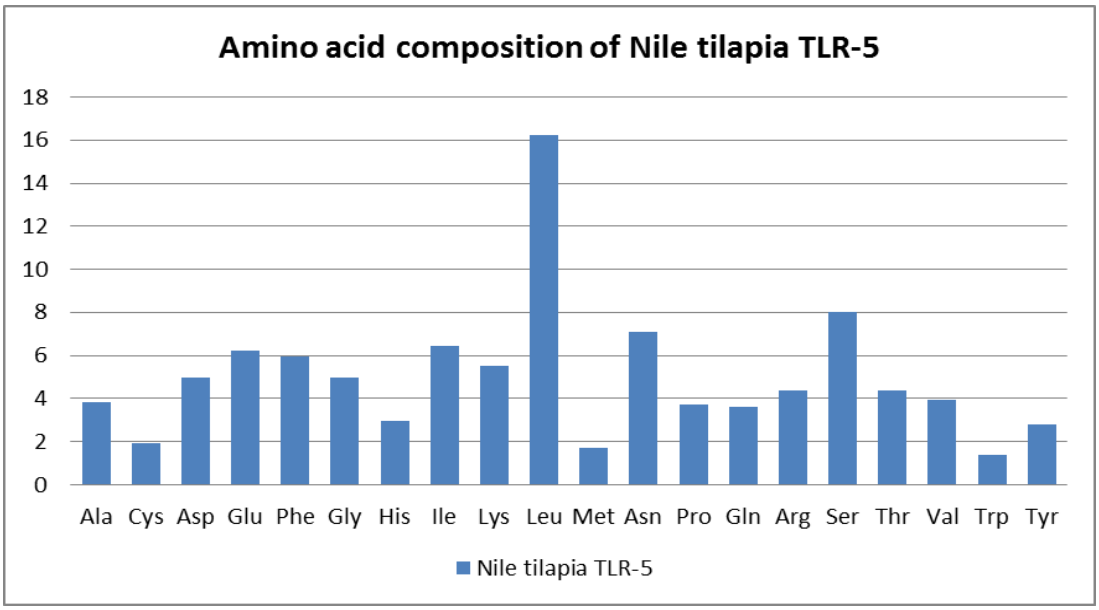
3. RESULTS AND DISCUSSION

3.1 Identity results for Nile tilapia TLR-5

The Nile tilapia TLR-5 full mRNA sequence was registered in the NCBI GenBank database under accession no. Such as JQ809461. The sequence composed of 2661 nucleotides, and 81 percent identity with Takifugu rubripes, 77 percent identity with Japanese flounder, and 75 percent identity with Japanese medaka were shown by the consensus cDNA sequence, which indicated that the new sequence is likely homologous to TLR-5 fish. The predicted protein encoded by the TLR-5 mRNA sequence of Nile tilapia consists of 887 amino acids, starting with ATG, which is identical to the TLR-5 sequence of other fishes. The composition of the amino acids of the encoded polypeptide is defined in the chart in Fig 1.

Figure 1; chart explain the amino acid composition and percentage of the encoded poly peptide in the Nile tilapia TLR-5 mRNA

3.2 Transmembrane and domain structure results

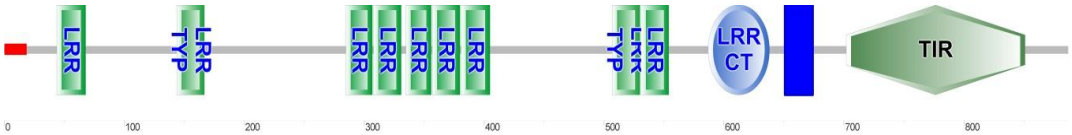


The SMART web tool was used to calculate the Nile tilapia TLR-5 domain structure; Nile tilapia TLR-5 began with a signal peptide (20 amino acids from 1-20), followed in the extracellular region by 9 LRR domains (residues 44-554) and one C-terminal LRR domain (LRR-CT, residues 587-638) and in the cytoplasmic region by a TIR domain (residues 702-851), as shown in Figure 2. A number of available amino acids is included in the encoded amino acids, with 20 distinct amino acids in total. Leucine was the most popular encoded amino acid and tryptophan was the least common amino acid. As shown in Figs 2 and 3, we developed a chart to display the quantities and ratios of the encoded amino acids in Nile tilapia TLR-5.

Figure 2: Nile tilapia TLR-5 repeat, motifs, domains and feature structures in comparison to Japanese flounder, Japanese medaka and fuku rubripes TLR-5

Confidently predicted domains, repeats, motifs and features structure of Nile tilapia TLR-5 in comparison with Japanese flounder, Fugu rubripes and Japanese medaka TLR-5

Predicted Japanese flounder TLR-5



Nile tilapia TLR-5

Fugu rubripes TLR-5

Predicted Japanese medaka TLR-5

Figure 3; transmembrane structure of Nile tilapia TLR-5 showing the size and position of its motifs done by SMART analysis web-based application.

Table contain Nile tilapia TLR-5 Confidently predicted domains, repeats, motifs and features structure

Name	Start ▲	End	E-value
signal peptide	1	20	N/A
LRR	44	68	28.4
LRR_TYP	144	167	0.00389
LRR	285	307	45.8
LRR	308	331	269
LRR	335	356	73.8
LRR	357	380	0.93
LRR	381	405	13.3
LRR_TYP	507	530	0.0667
LRR	531	554	5.26
LRRCT	587	638	1.26e-7
transmembrane region	651	673	727
TIR	702	851	6.59e-14

Table contain Fugu rubripesTLR-5 confidently predicted domains, repeats, motifs and features

Name	Start ▲	End	E-value
signal peptide	1	21	N/A
low complexity	22	37	N/A
LRR	45	67	261
LRR	93	116	31.8
LRR	143	166	0.133
LRR	311	334	60.6
LRR	337	359	176
LRR_TYP	360	383	0.00632
LRR	384	404	51.2
LRR	406	428	32.7
LRR_TYP	511	534	0.000908
LRR	535	558	7.57
LRR	559	580	103

Table contain Japanese medaka TLR-5 confidently predicted domains, repeats, motifs and features

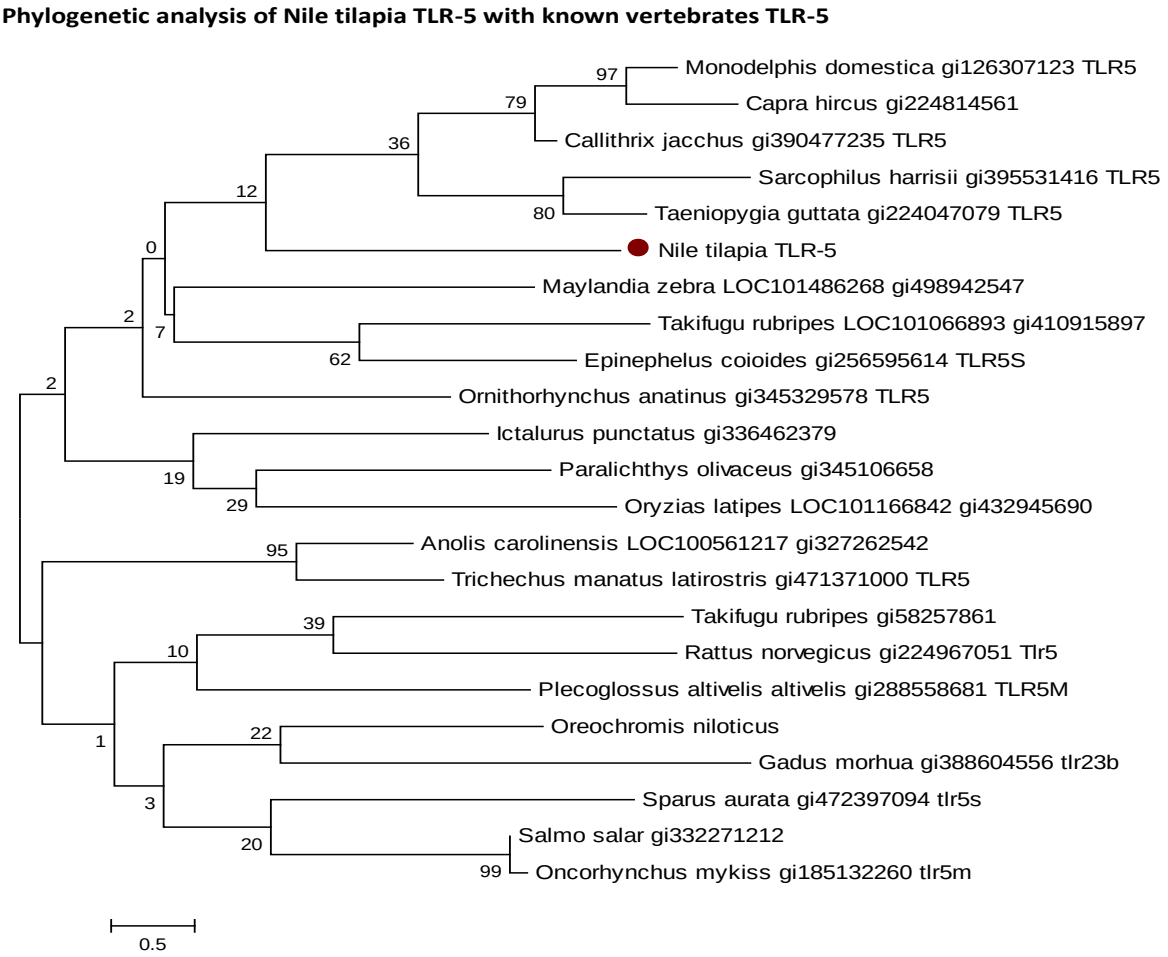
LRRNT	19	50	3.07
LRR	46	68	55.7
LRR_TYP	144	167	0.0472
LRR	169	192	75.9
LRR	289	311	51.2
LRR	312	335	18.1
LRR	338	360	5.88
LRR_TYP	361	384	0.0236
LRR	385	405	32.7
LRR	407	430	89.8
LRR_TYP	514	537	0.0136
LRR	538	561	7.36
LRR	562	583	48.4
LRRCT	592	643	0.0492

3.3 Phylogenetic analysis

Depending on the amino acid sequence of TLR-5, which was retrieved from NCBI database, we used two techniques to create phylogenetic trees (Neighbour-joining and maximum parsimony). By using translated Nile tilapia amino acid sequence and almost all of the recognised amino acid sequences found in NCBI database, the phylogenetic study was carried out. The phylogenetic analysis showed that Nile tilapia TLR-5 is closely related to

Fugu rubripes TLR-5, Japanese flounder TLR-5 and Takifugu rubripes TLR-5. The two phylogenetic approaches yielded almost the same results. The composition of the TLR-5 amino acid sequence of Nile tilapia is generally identical to that of other known TLR-5 sequences, with 36-48 percent identity to different vertebrate sequences; 71 percent identity to rainbow trout (Oncorhynchus mykiss), Japanese flounder (orange-spotted grouper), Paralichthys olivaceus (Japanese flounder) and Larimichthys crocea (large yell crocea); TLR-5; 68% identity to *Takifugu rubripes* (*Fugu rubripes*) TLR-5; and 39% identity to chicken TLR-5 sequences, as shown in Fig 4.

Figure 4: phylogenetic analysis of Nile tilapia TLR-5 against available vertebrates TLR-5 models

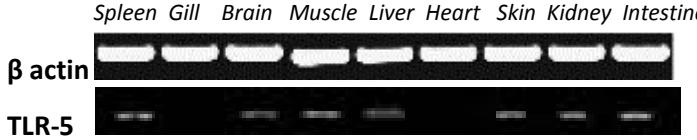


3.4 Expression of Nile tilapia TLR-5

Highly expressed in the kidney, brain, spleen, intestine, muscle, liver, gills, and heart and skin was the transcript of Nile tilapia TLR-5. After reverse transcription, semi-quantitative PCR revealed variations in the level of expression between the tissues studied, with the highest expression in the spleen, muscle, liver, kidney, and intestine, and lower levels of expression in the gill and heart, as shown in Figure 5.

Figure 5: Expression of the Nile tilapia TLR-5

Figure 5; tissue specific expression of Nile tilapia TLR-5; total RNA were extracted in various tissues from three healthy fish, then cDNA were equally mixed from the three samples in the corresponding tissues

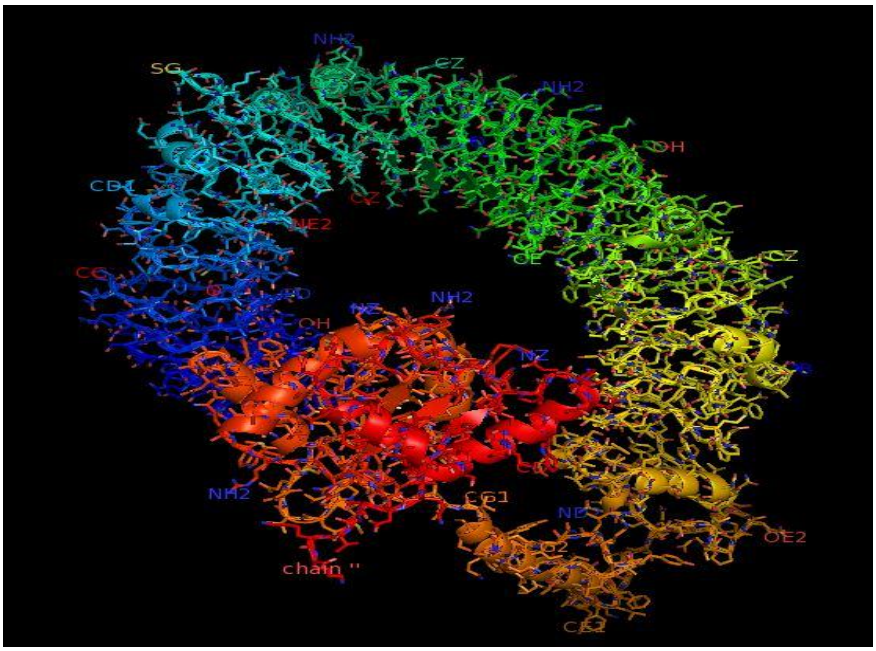


Tissue specific expression of Nile tilapia TLR-5; total RNA were extracted in various tissues from three healthy fish, then cDNA were equally mixed from the three samples in the corresponding tissues

3.5 The predicted 3D crystal structures of the extracellular domain of the protein

Here, we present the crystal structures of the extracellular domain of Nile tilapia TLR-2, which were predicted by the CPHmodels 4.0 Server (Fig 6).

Figure 6: the crystal structures of extracellular domain in Nile tilapia TLR-5



4. DISCUSSION

Since many studies have concentrated on non-fish vertebrates, this is the first study to classify Nile tilapia TLR-5. Tilapia fish TLR-5, which is considered a homolog for Fugu rubripes, Japanese flounder, Japanese medaka, orange-spotted grouper, zebrafish and other TLR-5 vertebrate proteins, is mentioned in our findings. The molecular analysis revealed that with 71 percent identity, the Nile tilapia receptor is very similar to the Japanese flounder and Japanese medaka receptors, and closer to the Fugu rubripes receptor than the Japanese flounder receptor; those data agreed on with previous reports focus on the variation in Nile Tilapia TLR-1/3 with other species [17, 18]. The Nile tilapia receptor transmembrane composition analysis showed that the Japanese flounder and Japanese medaka proteins

contain one motif that is absent in tilapia, while tilapia has one special motif, and at the beginning of the gene, zebrafish, Japanese flounder, and Japanese medaka share the same signal peptide (Fig 3) those structure match with the previous degree of identity between other fish species [44]. There are 143 amino acids in the Nile tilapia TIR domain, while 139 amino acids are in the Japanese flounder series, which could be due to uneven selective pressure throughout evolution. In addition, the duck sequence revealed a special LRR domain absent in rainbow trout and orange-spotted grouper at position 128-150, as shown in Fig 1 which similar to the main common structure to the murine and human toll like repceptor-5 models as previous described [45-47]; which confirm that the newly cloned receptor is orthologue to the vertebrate TLR-5 not paralogue . The variation in immune responses to microbes among Nile tilapia and other fish species may play a role in this domain. The expression of Nile tilapia TLR-5 is highly expressed in the kidney, brain, spleen, intestine, muscle, liver, gills, heart and skin, but differs between the various tilapia organs, as shown in Fig 3.

5. CONCLUSION

This study provides the first ever report on the existence of TLR-5 in Nile tilapia, including its transmembrane structure, amino acid composition and distribution in the tissues of fish, is given in this research. Based on its phylogenetic similarity to vertebrate models, Nile tilapia TLR-5 is considered to be a fully functional orthologue of the vertebrate protein.

CONFLICTS OF INTEREST

All authors have no conflicts of interest.

6. ACKNOWLEDGEMENTS

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