

Molecular characterization and analysis of TLR-1 in rabbit tissues

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Abstract

The rabbit has great commercial importance as a source of meat and fur, as well as its uses as a laboratory animal for the production of antibodies, used to detect the presence or absence of disease and for research in infectious diseases and immunology. One of the most critical problems in immunology is to understand how the immune system detects the presence of infectious agents and disposes the invader without destroying the self-tissues. Genetic characterization of Toll-like receptors has established that innate immunity is a skillful system that detects invasion of microbial pathogens. Our work aimed to identify, clone and express the *Oryctolagus cuniculus* (rabbit) TLR-1 mRNA and its encoding protein. We cloned the complete mRNA sequence of *Oryctolagus cuniculus* TLR-1 and deposit it in the GenBank under accession number (KC349941), which has 2388 base pair and it encodes an open reading frame (ORF) translated into 796 amino acids mRNA and consist of 20 types of amino acids. The analysis of amino acid sequence revealed that the rabbit TLR-1 has a typical protein components belonging to the TLR family. Rabbit TLR-1 was expressed in a wide variety of rabbit tissues, which indicate an important role in immune system in different organs.

Key words: rabbit, Toll-like receptor, cloning, molecular characterization.

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Introduction

Knowledge of the rabbit's immune system is of vital importance where rabbit is used as a meat source, fur source and as a model animal for infectious diseases and other inflammatory responses [1]. The rabbit has been a model for immunology studies since 1960s [2, 3]. In the last years, several studies have been performed in the European rabbit and other leporid's innate immune system [4-9]. However, only one was about TLRs [6]. Toll-like receptors in fishes, birds and mammals have been evolving under positive selection [10-18]. However, relatively little immunological knowledge exists for the rabbit, compared to that available for the human or mouse. Innate immunity is believed to be essential for the removal of invading microbes as well as the control of their systemic spreading. Mammalian toll-like receptors are members of the pattern recognition receptor (PRR) family and play a major role in the initiation of innate immune responses and the subsequent adaptive immune responses against the invading

pathogens [19, 20]. Members of the TLR family are necessary for the immune function through the sensing of pathogenic agents and initiation of a proper immune response. More specifically, tissue and cell specific TLR expression patterns have been correlated with the ability to respond to different pathogenic challenges [16, 21-23]. Detection of pathogens by the acquired immune system requires their prior recognition and presentation by cells of the innate immune system, so it plays a major role during outbreaks of new infectious threats mainly when pathogens are able to avoid the immune detection [24]. TLRs help the body to distinct foreign from self-tissue, so their function begins at the earliest stages of immune development [16, 25-30]. In addition, TLRs are able to differentiate between pathogenic agents and commensals. Factors such as the site and amount of each TLR are mainly involved in this process [16, 21, 31]. Recognition of foreign molecules is based on the detection of molecular patterns. These pathogen-associated molecular patterns include molecules such as flagellin,

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lipopolysaccharide (LPS), lipoproteins, bacterial DNA and unmethylated dinucleotide. The ability of TLRs to recognize a broad spectrum of microbial molecules enables the host to detect the presence of pathogens rapidly, before a more widespread infection occurs [32-38]. Several studies had proved an association between the TLR gene polymorphisms and the occurrence of the disease [15-17, 39-41]. In addition to their role in both bacterial and viral infections, TLRs also recognize and are activated by endogenous molecules associated with damaged cells and tissues [42-44]. TLR1 is a member of the Toll-like receptor family of pattern recognition receptors of the innate immune system. TLR1 recognizes the pathogen-associated molecular pattern with a specificity for gram-positive bacteria. TLR1 has been designated as CD281 [20]. The TLR2/TLR1 form a heterodimer that recognizes a variety of lipoproteins, including those from mycobacteria and meningococci, TLR1-deficient macrophages show normal responses to triacyl lipopeptides but not to diacyl lipopeptides [45, 46].

Our work was aimed to identify, characterize, clone and detect tissue expression of the rabbit (*Oryctolagus cuniculus*) TLR-1.

Material and methods

Samples

Spleen, duodenum, lung, ileum, colon, kidney, liver, skin, cecum, heart and brain samples were collected from live mature rabbits from common farms in Egypt and kept under inspection for 5 days to be sure that it is free from any clinical infection. The samples were collected and stored at -80°C . RNA was extracted from organs with Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions; both the quantity and quality of total RNA were assessed at OD260 and OD280 using a NanoDrop ND-1000

spectrophotometer (NanoDrop Technologies, Wilmington, USA). Preparation of cDNA library from RNA was performed by AMV reverse transcriptase (Bioer, China) according to the manufacturer's instructions. The PCR was performed to amplify the target gene using specific primers using Ex Taq polymerase (Takara Bio, Japan) according to the manufacturer's instructions.

Primers

To identify the TLR-1 from rabbit tissue; degenerative primers were designed based on the alignment of the protein sequence of the ortholog TLR-1 gene; of *Pongo pygmaeus* (Bornean orangutan, accession no. AB445621), *Macaca mulatta* (rhesus monkey, accession no. AC204076), *Homo sapiens* (human, accession no. DQ012261). We used iCODEHOP v1.1 (interactive program for creating Consensus Degenerate Hybrid Oligonucleotide Primers) web-based software, at University of Pittsburgh website, to detect the conservative sites between the different species and then to design primers based on it. The degenerative primers (d-rTLR-1 forward and d-rTLR-1 reverse) were designed based on the conserved sites to the clone short sequence. RACE primers designed toward 3' end (r-rTLR-1 sense) and 5' end (r-rTLR-1 antisense) were followed by nested PCR primers (nr-TLR-1 sense, nr-TLR-1 antisense) to use in the RACE system to get the full sequence. Primers were to detect the expression level by qRT-PCR (q-rTLR-1 Forward, q-rTLR-1 Reverse); all the primers were designed by primer premier 5.1 software (PREMIER Biosoft, Palo Alto, CA, USA) (Table 1).

Cloning of rabbit TLR-1(rTLR-1) cDNA sequence

The PCR product was purified and ligated into pMD18-T easy vector, transformed into the competent

Table 1. Primers used for the rabbit (*Oryctolagus cuniculus*) TLR-1 cloning and expression analyses

Primer	Accession no.	Sequence (5' to 3')	Primer type or use
GAPDH Sense primer	L23961	GAATCCACTGGCGTCTTCAC	Housekeeping gene primers
GAPDH Anti-sense primer		CGTTGCTGACAATCTTGAGAGA	
d-rTLR-1 Forward	-	ATGCTGTAGTAGGCTCAG	Degenerative primers
d-rTLR-1 Reverse	-	ATTACATCTGCGTGGTCAT	
r-rTLR-1 Forward	KC349941	TATAAACCAATTTTGCAGTAGTACGCT	RACE primers
r-rTLR-1 Reverse		TGGGAGGTAAACATCTGAAAACAGAGTC	
nr-rTLR-1 Forward		AGTCTTTCCCAAGGTAGCATCTACG	
nr-rTLR-1 Reverse		CGTGTCCGTCAAGAGGTTGTTGG	
q-rTLR-1 Forward		AATGCGTTTATGCTCTGCC	Tissue expression primers
q-rTLR-1 Reverse		CCTCCGTGCCATGAGGGTTT	
rTLR-1 Forward		TGACCATCACTATTCTAGCAGCTT	Full length primers
rTLR-1 Reverse		CCATGTTTAGTCTTCTCCTTCGGC	

Escherichia coli DH5a cell and plated on the LB-agar Petri dish. Positive clones containing the expected-size inserts were screened by colony PCR. Plasmid DNA was extracted using an Axyprep plasmid miniprep kit (Axygen Biosciences, USA) according to the manufacturer's instructions. Three representative plasmid DNAs were sequenced. Rapid amplification of cDNA ends (RACE) system, was done by SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) to get the full length of the required sequences according to the manufacturer's instructions.

Sequence analyses and alignment

The BLAST tool (<http://www.ncbi.nlm.nih.gov/blast>) was used to detect sequence homology. The translated amino acid sequences were analyzed with the Expert Protein Analysis System (<http://www.expasy.org/>) and the protein domain features in the translated amino acids were predicted by Simple Modular Architecture Research Tool (SMART) (<http://smart.embl-heidelberg.de/>). Phylogenetic and molecular evolution analysis was conducted by MEGA 5 software [47] and optimized manually.

Rabbit TLR-1 expression

The real-time quantitative polymerase chain reaction was used to quantify the rTLR-1 gene expressions using an ABI Prism 7000 Sequence Detection Systems and TaqMan 2x PCR Master Mix Reagents Kit following the manufacturer's instructions (Applied Biosystems; Life

Table 2. Illustrate transmembrane structure of *Oryctolagus cuniculus* TLR-1 showing confidently predicted domains, repeats and motifs done by SMART analysis web based application

Name	Start	End	E-value
Signal peptide	1	28	N/A
LRR	72	95	7.36
LRR	375	397	33.6
Low complexity	428	437	N/A
LRR	471	494	52.7
LRRCT	528	582	0.00000654
Transmembrane region	586	608	N/A
TIR	640	783	1.45e-42

Table 3. The site of variation in the encoded polypeptide sequence for the cloned rabbit TLR-1 (UniProt ID: M9T155) and the predicted rabbit TLR-1 (UniProt ID: G1TIU2)

Amino acid sites	3	10	20	22	44	84	180	181	204	231	262	302	312	325	339	368	377	459	567	568	740
Cloned rabbit TLR-1 M9T155	I	Q	L	S	G	T	R	V	N	E	S	C	C	E	R	T	A	S	N	R	H
Predicted rabbit TLR-1 G1TIU2	N	H	V	R	P	R	G	E	K	D	W	S	S	K	G	I	G	N	K	G	Q

Technologies, USA). The housekeeping gene (GAPDH) primers [48] were used as an internal control for cDNA normalization where the unit number showing relative mRNA levels in each sample was determined as a value of mRNA normalized against GAPDH. The expression data obtained from three independent biological replicate, RT-PCR data were analyzed by using the 2^{-ΔΔCT} method as described [49].

Nucleotide sequence deposition

The BankIt tool was used to deposit the analyzed sequence (rTLR-1) in the GenBank (<http://www.ncbi.nlm.nih.gov/genbank/submit/>).

Results and discussion

Identification and characterization of rTLR-1

The complete mRNA sequence of *Oryctolagus cuniculus* TLR-1 was deposited in the NCBI GeneBank database under accession no. KC349941. While it consists of 2388 nucleotides and the consensus cDNA sequence showed identity with the TLR-1 mRNA sequences Chinese hamster TLR-1 (*Cricetulus griseus*), of 69%; nine-banded armadillo TLR-1 (*Dasypus novemcinctus*) – 47% identity, cattle TLR-1 (*Bos taurus*) – 39% identity; mice (*Mus musculus*) – 65% identity.

The encoded polypeptide consists of 796 amino acids; we detect the site of variation in the encoded polypeptide sequence for the cloned rabbit TLR-1 (UniProt ID: M9T155) and the predicted Rabbit TLR-1 (UniProt ID: G1TIU2) (Table 2). The identity degree in the encoded polypeptide amino acids sequences around 97% between the cloned rabbit TLR-1 and the predicted rabbit TLR-1, where the variation occurs in 24 amino acids (Table 3). While the cloned rabbit TLR-1 showed a degree of identity with the rodents; where it shows 73% identity with the Mole rat, 71% identity with the guinea pigs, 68% identity with the house mouse and 65% identity with the rat.

Analysis of the rTLR-1 sequence

The predicted protein encoded by *Oryctolagus cuniculus* TLR-1 mRNA sequence is composed of 796 amino acids and shows a typical toll-like receptor structure. The *Oryctolagus cuniculus* TLR-1 domain structure has been estimated using the SMART web tool. The rTLR-1

started by signal peptide (28 amino acids from 1-28) followed by three leucine rich repeat (LRR) domains (at position 72-95, 375-395, 471-491) and one C-terminal LRR domain around 54 amino acids (LRR-CT, residues 528-582) in the extracellular region, then transmembrane region around 22 amino acids (at position 586-608) and a TIR domain around 143 amino acids (residues 640-783) in the cytoplasmic region as shown in Fig. 1, Table 3 and Fig. 2. The encoded amino acids vary among different

available amino acids where it has 20 different amino acids; the highest amino acid encoded is leucine while the lowest is tryptophan as shown in Fig. 2.

We detect the site of variation in the encoded polypeptide sequence for the cloned rabbit TLR-1 (UniProt ID: M9T155) and the predicted Rabbit TLR-1 (UniProt ID: G1TIU2). The identity degree in the encoded polypeptide amino acids sequences around 97% between the cloned rabbit TLR-1 and the predicted rabbit TLR-1, where the

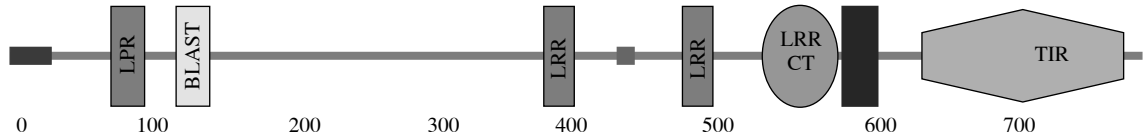


Fig. 1. The transmembrane structure of rabbit TLR-1; started by signal peptide (28 amino acids from 1-28) followed by three lucien rich repeat (LRR) domains (at position 72-95, 375-395, 471-491) and one C-terminal LRR domain around 54 amino acids (LRR-CT, residues 528-582) in the extracellular region then transmembrane region around 22 amino acids (at position 586-608) and a TIR domain around 143 amino acids (residues 640-783) in the cytoplasmic region

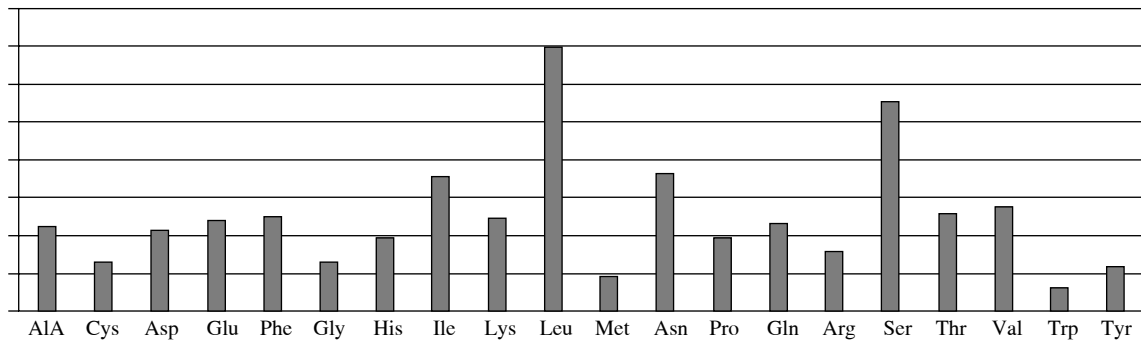


Fig. 2. *Oryctolagus cuniculus* TLR-1 amino acid composition and percentage; where it has 20 different amino acids; the highest amino acid encoded is leucine while the lowest is tryptophan

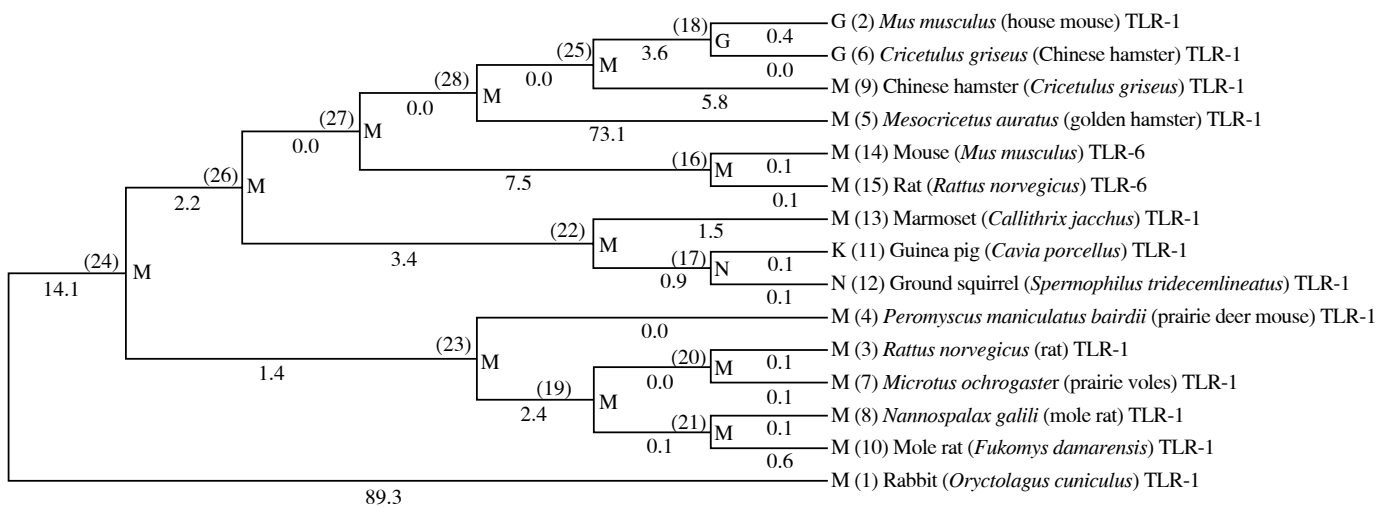


Fig. 3. Phylogenetic tree of rabbit TLR-1 against the available rodents TLR-1 sequence in the Genebank

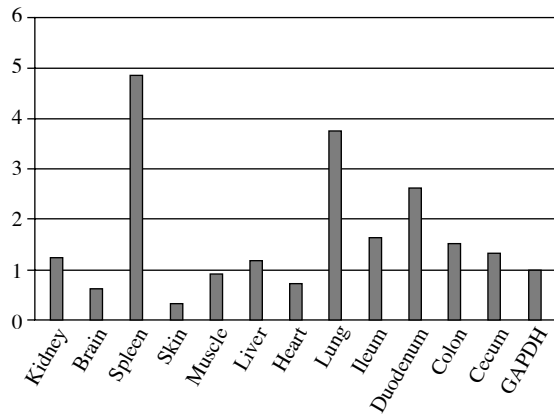


Fig. 4. rTLR-1 expression throughout the tissue cells (spleen, skin, duodenum, lung, ileum, colon, kidney, liver, cecum, heart and brain). TLR expression counts were normalized to GAPDH using the formula $2^{-(TLR-GAPDH)}$. All values represent mean \pm standard error of the mean of triplicate from quantitative real-time PCR assays

variation occurs in 24 amino acids (Table 3) while showed 73% identity with the Mole rat, 71% identity with the guinea pigs, 68% identity with the house mouse and 65% identity with the rat.

Phylogenetic analysis with other TLRs

Two methods were used to construct a phylogenetic tree (neighbour-joining and maximum parsimony) based on the amino acid of TLR-1, which was downloaded from the GenBank. The phylogenetic analysis performed using the translated *Oryctolagus cuniculus* amino acid sequence with almost all the known amino acid sequences found in the GenBank, both phylogenetic methods provided almost the same results, where the phylogenetic analysis showed that *Oryctolagus cuniculus* TLR-1 is closely related to mole rat, guinea pig and house mouse TLR-1. The structure of *Oryctolagus cuniculus* TLR-1 amino acids is in general similar to other identified TLR1 sequences in different mammals and birds as shown in Fig. 3.

Expression pattern of *Oryctolagus cuniculus* TLR-1

The rabbit TLR-1 mRNA was constitutively expressed in all selected tissues, with higher levels detected in spleen, duodenum, and lung; moderate levels in ileum, kidney, liver, cecum, colon; and low levels in heart, skin and brain; as shown in Figure 4 and the level of expression measured by RT-PCR as shown in Table 4 which similar to the distinct distribution of specific tissue expression in mice, mouse, human, chicken and fish TLR-1 models.

Table 4. rTLR-1 expression throughout the tissue cells (spleen, skin, duodenum, lung, ileum, colon, kidney, liver, cecum, heart and brain). TLR expression counts were normalized to GAPDH using the formula $2^{-(TLR-GAPDH)}$. All values represent mean \pm standard error of the mean of triplicate from quantitative real-time PCR assays

Tissue	TLR-1 relative expression level (mean \pm SD)
Kidney	1.23 \pm 0.33
Brain	0.64 \pm 0.03
Spleen	4.87 \pm 0.78
Skin	0.32 \pm 0.01
Muscle	0.92 \pm 0.15
Liver	1.20 \pm 0.25
Heart	0.71 \pm 0.04
Lung	3.76 \pm 0.34
Ileum	1.64 \pm 0.17
Duodenum	2.62 \pm 0.31
Colon	1.52 \pm 0.29
Cecum	1.32 \pm 0.27
GAPDH	1.00 \pm 0.08

Conclusions

This is the first study to make actual cloning and characterization to the rabbit toll-like receptor type 1. The rTLR-1 encodes an open reading frame (ORF) translated into 796 amino acids mRNA and consists of 20 types of amino acids. The structure of *Oryctolagus cuniculus* TLR-1 amino acids is in general similar to other identified TLR1 sequences in different mammals and birds. Rabbit TLR-1 was expressed in a wide variety of rabbit tissues, which indicates an important role in the immune system in different organs.

The authors declare no conflict of interest.

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