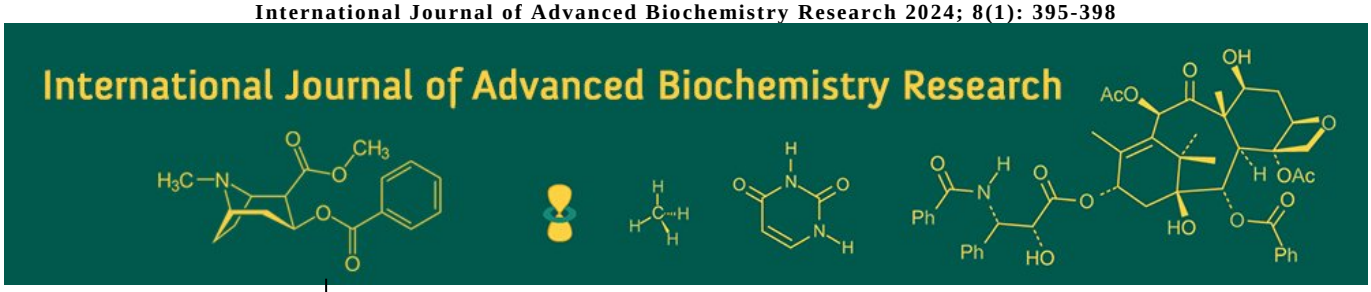

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Jyoti Choudhary
College of Veterinary and Animal Science, RAJUVAS, Biknaer, Rajasthan, India

Mahendra Milind
College of Veterinary and Animal Science, RAJUVAS, Biknaer, Rajasthan, India

Nareshi Meena
College of Veterinary and Animal Science, RAJUVAS, Biknaer, Rajasthan, India

BN Shringi
College of Veterinary and Animal Science, RAJUVAS, Biknaer, Rajasthan, India

Corresponding Author:
Jyoti Choudhary
College of Veterinary and Animal Science, RAJUVAS, Biknaer, Rajasthan, India

Sequence identification of toll like receptor 6 in dromedary camel (*Camelus dromedarius*)

Jyoti Choudhary, Mahendra Milind, Nareshi Meena and BN Shringi

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Abstract
The present study was conducted for the mRNA sequence identification of Toll-like receptor 6 (TLR 6) gene of *Camelus dromedarius*. It was made up of 2496 nucleotide-long open reading frame (ORF) and the encoded protein was 831 amino acid-long. The sequence was submitted to the NCBI with accession no. MN807291. TLR 6 gene was found to be located on chromosome 2. It consists of total three exon in which second and third exons were involved in expression of gene. The 3D structure of the gene revealed a curved or horseshoe-shape. The amino acid (aa) sequence had four leucine-rich repeat (LRR) domains followed by one C – terminal LRR domain, a transmembrane membrane domain, and an intracellular Toll–interleukin (IL)-1 receptor (TIR) domain.

Keywords: Amino acid, camel, sequence, TLR 6

Introduction
The innate immune system employs germline-encoded pattern-recognition receptors (PRRs) which are expressed on the host immune cells that recognize conserved molecular structure on the surface of the microbial species called as pathogen-associated molecular patterns (PAMPs) (Nie *et al*., 2018) [9]. Toll-like receptors (TLRs) are most well characterized PRRs and expressed on the surface of immune cells (Mukherjee *et al*., 2014) [8]. TLRs belong to the type I transmembrane glycoprotein receptor family from insect to vertebrate present on the cell surface or membrane compartments of immune and non-immune cells (e.g. epithelial cells) (Barton *et al*., 2009) [2]. They are main component of innate immune system and recognize conserved molecular pattern in infectious agents and initiate intracellular signal transduction pathways that trigger the production of pro-inflammatory cytokines and type I interferons leading to both innate and adaptive immune responses which defend itself against the invading pathogens (Wallet *et al*., 2018) [12].
The TLR paralogs have been organized by phylogenetic analysis into six major groups, in which TLR 6 belongs to TLR2 group. Amongst different TLRs, the TLR2 group (TLRs 1, 2, 6, 10 and 14), a unique family within the TLR not only share high levels of homology and recognize related agonists but also the only TLRs to heterodimerize upon ligand recognition (Roach *et al*., 2005; Matsushima *et al*., 2007) [10, 7]. TLR2/TLR6 which exist as heterodimers on the cell surface and detect diacylated lipoproteins lipopeptides from Gram-positive bacteria and mycoplasma. (Uematsu and Akira, 2008*;* Kawai and Akira, 2010) [11, 6].
In my knowledge analysis of TLR 6 of dromedary camels are still scarce, and has not been investigated so far. The aim of the present study was to identify the sequence of TLR 6 for understand the role in immune system of camel.

Materials and Methods
1. Ethics committee approval
The care and use of animals were conducted according to the guidelines which is established by the Institutional Animal Ethics Committee (IAEC) with permit number i.e. CVAS/IAEC/CPCSEA-2044/ GO/Re/SL/18/2019/05.

2. Designing of oligonucleotide primers
Primers were designed by used the predicted sequence of camel TLR 6 was available on NCBI along with accession number XM_010980396.1. The PRIMER3 (http://frodo.wi.mit.edu/primer3/), and Oligo Analyzertool

(https://www.idtdna.com/pages/tools/oligoanalyzer) software were used for designing of primers. After that, these were synthesized at Integrated DNA Technologies (IDT), India (Table 1).

Table 1: Primers used for PCR amplification of TLR 6 gene of camel (*Camelus dromedarius*)

Sr. No.	primers	Sequence of primes	Amplicon Size	Annealing temp. (ºC)
1.	TLR6-1F	CGGGAAGGGATCACTCAAG	600	58
	TLR6-1R	TTGCCAAATTCCTTGCAGAT		
2.	TLR6-2F	CAAGTTCAACCAGGATTTGGA	460	56
	TLR6-2R	TGGCCCTCTAGTGAGTTCTGT		
3.	TLR6-3F	CCAATTGCTCACTTGCATCT	563	52
	TLR6-3R	GGTACTTGCGGACAAAGCA		
4.	TLR6-4F	TTGGCCCAAACCAATAGAAT	476	54
	TLR6-4R	CCACTATACTCCCAACCCAAA		
5.	TLR6-5F	TGATGTTAACCATATCAGACACATTT	474	54
	TLR6-5R	CACATCCAGGAAGGTGGACT		
6.	TLR6-6F	TGCATTTGGGTTGGGAGTAT	489	58
	TLR6-6R	CAGAGCTGTGTTGCAGGATAA		
7.	TLR6-7F	TGTGGTACCTTTAGCAGCCTTT	501	54
	TLR6-7R	CTTCACCCAGGCAGAATCAT		
8.	TLR6-8F	CCGTGACCGTCCTCTGTATC	461	58
	TLR6-8R	GAGAGCCCTCAGCTTGTGAT		
9.	TLR6-9F	TCTTGTCTCCCAACTTTGTTCA	456	56
	TLR6-9R	CAAGTCTGAGCACCACCTCA		

3. Collection of blood samples and extraction of RNA

For RNA isolation, blood samples were randomly collected from the jugular vein of *Camelus dromedarius* admitted to the veterinary clinical complex of the College of Veterinary and Animal Sciences Bikaner. Approximately 5 ml of blood was collected in anticoagulant containing vacutainers and immediately transported to the laboratory on ice and processed the same day. Total RNA was extracted from blood by using a Gene Jet Whole blood RNA purification kit, following the manufacturer's instructions given as described in the user's manual.

4. Complimentary DNA (cDNA) synthesis

The cDNA was synthesized from the isolated RNA using RevertAid™ First Strand cDNA Synthesis kit (Thermo scientific). This kit is designed for preparation of full-length first strand cDNA from RNA templates. This allows the synthesis of full-length cDNA. The reaction mixture contained total RNA-8µl, Oligo (dT) 18 primer (0.5µg/µl)-1µl and DEPC-treated water to make total 12µl in amount. The contents were mixed gently and centrifuged briefly in a micro centrifuge and incubated at 72 ºC for 7 min to linearise the RNA removing secondary structure followed by snap chilling for 10 min. The tube was placed on ice and the components were added in this order to made 20µl final volume: 5X reaction buffer- 4µl, RiboLock™ Ribonuclease inhibitor (20units/µl)- 1µl, 10mM dNTP mix- 2µl, RevertAid™ - 1µl and Nuclease free water. The Contents were gently mixed and mixture was incubated at 25 ºC for 5 min followed by 60 min at 42 ºC for reverse transcription and the termination of reaction was done by heating at 94 ºC for 10 min and stored at -70 ºC, for further use.

5. Amplification of camel TLR5

The cDNA of camel was taken as a template and amplification of ORF was done in fragments by using gene specific primers (Table 1). Gradient PCR was attempted for primers to determine their exact annealing temperature. The concentrations of various reaction components and cycling conditions were optimized. The following reaction mixture was found to be optimum for PCR amplification in a 25µl reaction volume (Table 2).

Table 2: Composition of reaction mixture for amplification

PCR components	Volume added in each reaction
5X PCR assay buffer	5.00 µl
1.5 mM MgCl2	2.50 µl
dNTP mix (10 mM)	1.00 µl
Primer (F) (10 pmoles/µl)	1.00 µl
Primer (R) (10 pmoles/µl)	1.00 µl
Taq DNA polymerase (5 U/µl)	0.30 µl
Template cDNA	4.00 µl
Nuclease free water (NFW)	10.20 µl

The above reaction mixture was subjected to PCR amplification with the following PCR cycling conditions: The initial denaturation at 94 ºC for 5 min followed by 38 cycles at 94 ºC for 1 min, the annealing temperature 52 to 58 ºC for 30s, extension at 72 ºC for 1 min and a final extension at 72 ºC for 10 minute. To confirm PCR amplification, 3 µl of PCR product was mixed with 0.5µl of 6X gel loading dye from each tube and electrophoresed on 1.5% agarose gel at a concentration of 0.5 µg/ml.

6. Sequence analysis

The obtained PCR products were sequenced at the sequencing facility of Delhi University (South Campus). BioEdit is an easy-to-use biological sequence alignment editor. The sequences were edited in Bio Edit Sequence Alignment Editor according to the height of the peak in the chromatogram and checked in a BLASTn search to verify their identity. A nucleotide sequence is compared to all nucleotide sequences in the database in a BLASTn search. After the quality check, Contig Assembly program (CAP) of BioEdit software was used to create contigs of sequences in order to make complete sequence assembly of TLR 6. Nucleotide sequences were translated into amino acid through the Expert protein analysis system (ExPASY) software.

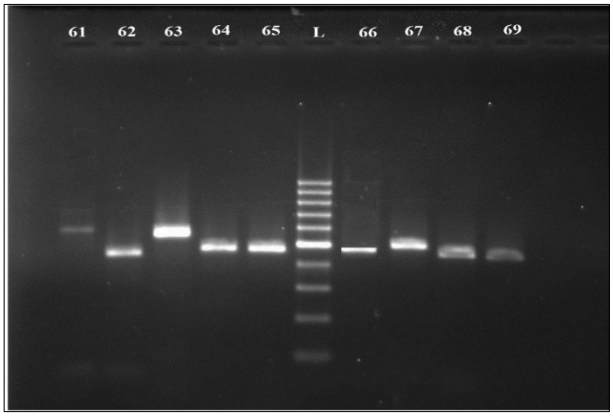
7. Determination of domain architecture of TLRs

The protein sequence was segmented into ectodomain (containing LRR motifs and trans-membrane region) and endo-domain (consisting of TIR). Deduced TLRs protein

domains were featured by results from Simple Modular Architecture Research Tool (SMART) databases.

Results and Discussion

The amplification of listed (Table 1) fragments were reliable with the target, which was depicted by a single band in 1.5% agarose gel by electrophoresis in parallel with 100 bp DNA marker. These primers pairs amplified the fragments of 600 bp, 460 bp, 563 bp, 476 bp, 474 bp, 489 bp, 501 bp, 461 bp, 456 bp length (Fig. 1).



(**L** = DNA marker-100bp)

Fig 1: PCR amplification of overlapping fragments of camel TLR6 gene coding region

Product name	61	62	63	64	65	66	67	68	69
Product size (bp)	600	460	563	476	474	489	501	461	456

After the amplification of fragments of coding region (cds) of gene by internal primers they were sequenced at Delhi University South Campus and finally, it was assembled into 2683 base pair (bp) long contigs. These contigs had 2496 bp long open reading frame (ORF) regions. In the camel, TLR 6 gene had been described by genome data viewer and mapped to genomic regions on 2nd chromosomes with total three numbers of exons. Exon second and third were involved in expression of this gene. Similar finding was obtained by Wille *et al*., (2014) [13] and Clop *et al*., 2016 [3]. Amaral *et al.* (2008) [1] found that TLR 1, 6 and 10 were closely linked and present on the same chromosome. This might have arisen through gene duplication which was evident by their presence on the same chromosome and it is striking that they belonged to the same TLR family. It was the first report when the full-length cDNAs sequences of TLR 6 of the dromedary camel had been submitted to Gene Bank at NCBI with accession numbers MN807291. After the analyses of sequence demonstrate that TLR6 belong to the three-domain subfamily; which bind and activated by hydrophobic ligands (Jeen and Lee, 2008) [5]. The ORF translated into protein sequences by ExPASY software which contained 870 amino acids (aa). It was found to be made up of 20 different types of amino acids in which leucine (Leu) was maximum in number whereas, the least one was tryptophan (Trp) (Table 3).

Table 3: Amino acid composition of coding region of camel TLR 6

S. No.	Amino acid (AA)	Number of amino acid (%)
		TLR 6
1.	Leu** (L)	119 (14.3%)
2.	Ser (S)	64 (7.7%)
3.	Asn (N)	57 (6.9%)
4.	Glu (E)	43 (5.2%)
5.	Phe (F)	53 (6.4%)
6.	Val (V)	57 (6.9%)
7.	Ile (I)	52 (6.3%)
8.	Gln (Q)	35 (4.2%)
9.	Lys (K)	47 (5.7%)
10.	Thr (T)	46 (5.5%)
11.	Ala (A)	24 (2.9%)
12.	Asp (D)	46 (5.5%)
13.	Arg (R)	27 (3.2%)
14.	Gly (G)	24 (2.9%)
15.	His (H)	29 (3.5%)
16.	Pro (P)	34 (4.1%)
17.	Met (M)	15 (1.8%)
18.	Tyr (Y)	23 (2.8%)
19.	Cys (C)	23 (2.8%)
20.	Trp* (W)	13 (1.6%)

** amino acid in highest number
* amino acid in lowest number

We successfully retrieved the complete coding sequence of TLR 6 from dromedary camel. Then, according to SMART (Simple Modular Architecture Research Tool), assigned the predictions of the amino acid sequence (Fig. 2).

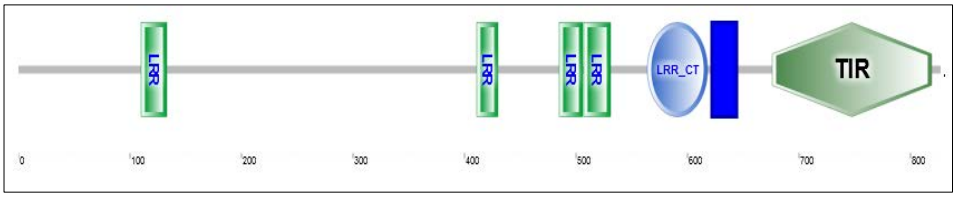


Fig 2: Domain architecture of TLR6 in *Camelus dromedarius* [Green box-LRRs, Blue box-TM domain, TIR]

The TLR 6 consisted by four Leucine rich repeats domains (residue 110-531aa) tracked by one C – terminal LRR domain of around 55 aa (LRR-CT, residues 564-618) in the ectodomain region. The Transmembrane region was composed of 23 aa (residue 622-644), and a intracellular Toll–interleukin (IL)-1 receptor (TIR) domain of 144 aa (residues 676-819) was in the cytoplasmic-region. (Table 4).

Table 4: Transmembrane structures of *Camelus dromedarius* TLR6 predicted domain, repeats and motifs done by using SMART tool

Name	Start	End	E-value
LRR	110	133	0.286
LRR	411	430	221
LRR	485	506	57.3
LRR	507	531	16.2
LRR-CT	564	618	8.11e-11
Transmembrane region	622	644	N /A
TIR	676	819	5.53e-39

The 3D structure of the camel TLR 6 (ECD) revealed a curved or horseshoe-shape in which an LRR residue is a turn of solenoid (Fig.4.23-Fig.4.29). Our finding was agreed with Gao and wang (2017) [4], who also reported similar shape of TLR 6 in other species.

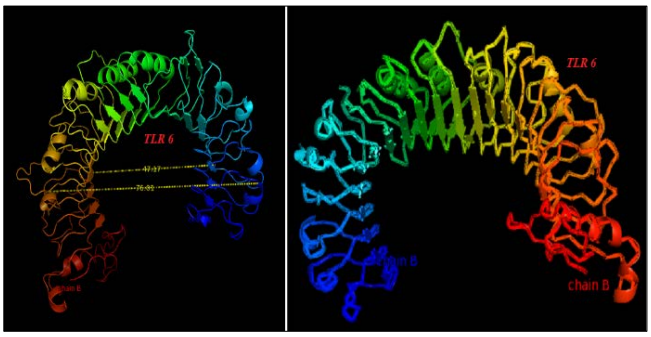


Fig 3: 3D structure model of camel TLR6 protein

The mean inner diameter, outer diameter and mean thickness of these models were evaluated by Pymol software (Fig. 3). In my findings, outer diameter, inner diameter, thickness of studied gene was 76.01 Å, 47.17 Å, and 20.47 Å respectively. The quality of the constructed model was good enough because it had a significant amount of sequence identity between the target and templates which was 65.90%.

The sequence and structure analysis of dromedary camel TLR 6 is first time found and this may be helpful in further studies about role in immune system and disease.

Conclusion

Dromedary camels are domestic animals and well adapted in extreme ecosystem in desert. They have relatively more resistance to wide variety of pathogens compared to other species from same region. So, there has been increased interest in the immune system of camel but still lack of knowledge about most of camel TLRs, which plays very important role in immune system. In my knowledge, it is first report regarding to camel TLR6. It may be useful for evolutionary and phylogenetic analysis as well as role of TLR6 in camel immune system.

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