

Molecular characterization of toll-like receptor 2 gene in Bikaneri camels (*Camelus dromedarius*)

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Received: 25 June 2022; Accepted: 16 May 2023

ABSTRACT

Toll-like receptors (TLRs) are an important family of receptors constituting the first line of defense system. Toll-like receptors play crucial roles in the innate immune system by recognizing pathogen-associated molecular patterns derived from various microbes. Present investigation was carried out to characterize the coding sequence of *TLR2* gene in Indian dromedary camels. Blood was collected from five Bikaneri camels and genomic DNA was isolated. Eight pairs of overlapping primers were designed for amplification of *TLR2* gene coding region. All the fragments were successfully amplified using Polymerase chain reaction (PCR). The PCR products were purified and subjected to Sanger's sequencing. *TLR2* gene fragment of 3213 bp consisting of 2287 bp of coding sequence of exon 2 and 926 bp of 3'untranslated region (UTR) was successfully amplified. The sequence generated was submitted to the NCBI with accession number MW557315.1. The encoded amino acids codes for 12 leucine rich repeats (LRR) (Accession no. UBN09134.1). At the nucleotide level, the Bikaneri camels' *TLR2* gene showed highest similarity with camelids (99.30 to 99.59%) followed by vicugna pacos (97.54%). The nucleotide sequence similarity was lowest with *Homo sapiens* (82.37%). The amino acid sequence based phylogenetic tree revealed close relationship between camelid species followed by pig (86.2%), whereas human, gorilla, horse and donkey were distantly placed on the tree.

Keywords: Characterization, Indian Camel, *TLR2* Gene, Toll-like receptors

Toll-like receptors (TLRs) are important pattern recognition receptors that play a crucial role in innate immunity of vertebrates (Kawai and Akira 2011) by recognizing pathogen-associated molecular patterns (PAMPs) derived from various microbes. The receptors of the innate immune system recognizing PAMPs are called as pattern recognition receptors (PRRs). The most common PRRs are TLRs that binds with PAMPs of microorganism and help in host defense (Beutler *et al.* 2006). TLRs have a unique ability to distinguish between self and non-self and therefore recognise the first signs of infection (Mukherjee *et al.* 2016). TLRs are evolutionary conserved proteins and characterization of their ligands has contributed to the understanding of their function and the host defense against infections (Janssens and Beyaert 2003). TLRs are type-1 trans-membrane receptors, structurally composed of a large extracellular domain responsible for binding of PAMPs, a trans-membrane domain, and an intracellular Toll Interleukin-1 Receptor (TIR) domain, which binds signaling molecules and initiates innate cellular immune

responses (Muzio *et al.* 2000). The extracellular domains are highly variable between different species (Bella *et al.* 2008) and are composed of leucine-rich repeats (LRRs) motifs. The vertebrates TLRs are rich in LRRs and have been reported to be ranging from 16 to 28 in number (Matsushima *et al.* 2007).

TLR2 is essential for the recognition of a variety of PAMPs from Gram-positive bacteria, including bacterial lipoproteins, lipomannans, and lipoteichoic acids (Miyake 2007). *TLR2* gene plays a vital role in infections and inflammatory diseases in livestock species, making them potential therapeutic targets to study host-parasite interplay. Camel *TLR2* gene maps to chromosome 2 and consists of two exons. The exon 2 of *TLR2* gene is finally translated into a 785 amino acid protein.

Dromedary camels are inhabitants of desert regions with poor grazing resources. India has 4 main breeds of camel, viz. Bikaneri, Jaisalmeri, Kachchhi and Mewari, of which Bikaneri camels are highest in number and predominantly used by camel breeders/rearers in Bikaner and nearby districts of Rajasthan. Bikaneri camels are heavily built with attractive look and are well-known for draught potential and endurance (Mehta and Sahani 2007). Considering the prime place Bikaneri camels have among Indian camel breeds, current study was planned to draw the genetic architecture of the *TLR2* gene of Bikaneri camel.

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MATERIALS AND METHODS

Sample collection: About 10 ml of blood was collected aseptically through jugular vein from five Bikaneri camels maintained at ICAR-National Research Centre on Camel, Bikaner farm in vacutainer tubes containing EDTA and it was stored in -20°C till isolation of genomic DNA. Prior approval of Institutional Animal Ethics Committee (IAEC) of institute was taken for the collection of blood from selected animals (NRCC/PSME/6(141)2000-Tech/).

DNA extraction, primer designing and PCR amplification: Genomic DNA was extracted from blood cells using standard phenol/chloroform/isoamyl alcohol extraction protocol (Sambrook *et al.* 1989). Quality and quantity of gDNA was checked on agarose gel electrophoresis and spectrophotometer. A total of 8 pairs of overlapping primers were designed using Primer 3 software, to amplify the camel *TLR2* gene partial coding sequence and 3' UTR. Primers were designed based on the *Camelus dromedarius* reference sequence (NC_044512.1, region 9864093-9867305) available at NCBI. The eight primers pairs namely TLR2.1, TLR2.2, TLR2.3, TLR2.4, TLR2.5, TLR2.6, TLR2.7 and TLR2.8 were designed to successfully amplify the eight fragments of 449 bp, 579 bp, 466 bp, 1012 bp, 734 bp, 373 bp, 330 bp and 385 bp size, respectively (Table 1). The PCR reaction was carried out in 25 µl of total volume, containing ready to use GoTaq Green PCR master mix -13 µl (Promega, USA), 1 µl of each primer with concentration of 10 pM, 1 µl of 75-125 ng camel genomic DNA and nuclease free water to make total volume up to 25 µl. Amplification was performed in Mastercycler® Gradient (Eppendorf AG, Hamburg, Germany) programmed for initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing (Ta) ranging from 54.8°C to 59.8 °C for 45 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. PCR products were checked for amplification by electrophoresis on 1.5% agarose gel (Sigma-Aldrich, Life Science), in parallel with 100 bp DNA marker (Thermo

Scientific, USA). The amplified PCR products were purified using gel extraction kit (Qiagen).

Sequence analysis: The purified products were sequenced using Sanger's dideoxy chain termination method by Eurofin Genomics. The forward and reverse sequences obtained from all the amplified fragments were assembled and manually edited using codon code aligner software (Codon Code Corporation, USA) and contigs were prepared for each of the eight amplified fragment. The eight contigs were assembled and after removal of overlapping sequences a consequence sequence was arrived which was submitted to NCBI repository and accession number was obtained. The coding sequence was translated into protein using bioinformatics resource portal Expasy (Duvaud *et al.* 2021). All sequences were analyzed with the help of reference sequence already available at NCBI database. The nucleotide BLAST program at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used for sequence homology searches in public databases. Multiple sequence alignment of the obtained sequences was done using ClustalW multiple alignment program (<http://www.ebi.ac.uk/Tools/clustalw2>). The nucleotide and protein sequences of *TLR2* gene were aligned to build the phylogenetic trees in MEGA 6.0 (<http://www.megasoftware.net>) software and the neighbour-joining (NJ) algorithm was used to build the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Kumar *et al.* 2018) and were shown in the units of the number of amino acid substitutions per site.

RESULTS AND DISCUSSION

The PCR amplification of eight fragments were consistent with the target ones, which was clearly depicted by a single band in gel electrophoresis on 1.5% agarose in parallel with 100 bp DNA marker (Fig. 1, 2 and 3). The eight primers pairs successfully amplified the fragments of 449 bp, 579 bp, 466 bp, (Fig. 1), 1012 bp, (Fig. 2) and 734 bp, 373 bp, 330 bp and 385 bp size (Fig. 3). The annealing

Table 1. Details of primers, annealing temperature, amplified regions with amplicon size

Primer name	Primer sequence (5-3')	Amplified size	Annealing temperature (Ta)
TLR-2.1	F-TCAGGCCCATCTCTGTCTT R- GCCCAGAGAAATCCTTTTCC	449 bp	58°C
TLR-2.2	F-TTGAGGCTGGGGTCCAATG R- CAGCAGTTTCACCAGCTCAC	579 bp	55°C
TLR-2.3	F-CTGCGAGATACTGATCTGAACAC R- GCTCACAGGCTGAGTTTCC	466 bp	58°C
TLR-2.4	F-TCGTTAGAATATTTGGATCTCAG R-TGTGGCTCTTTCAATGCAG	1012 bp	54.8°C
TLR-2.5	F-TGGCCAGAAACTACCTGTG R- AGTGAGACCGAAACCACCAC	734 bp	58.3°C
TLR-2.6	F-TTGAGAGCTGCAATAAAGTCC R- CCCAGTTCACATGAAACACG	373 bp	58.3°C
TLR-2.7	F-TCCACTAGGAAACAGCATAAAGG R- GGAGGCAGACCTGAAGACAG	330 bp	59.7°C
TLR-2.8	F-GAAGCTCTGACTGGGGTCTG R- GCCACTTCCTGTCTATGTGTG	385 bp	59.8°C

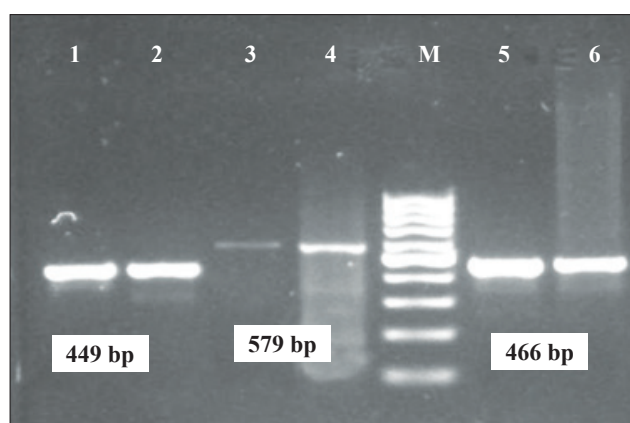


Fig. 1. Amplification of *TLR2* gene partial exon fragments of Bikaneri camel. Lane 1 & 2 - *TLR2.1* fragment of 449 bp, Lane 3 & 4 - *TLR2.2* fragment of 579 bp. Lane 5 & 6 - *TLR2.3* fragment of 466 bp, M - Molecular marker (100 bp).

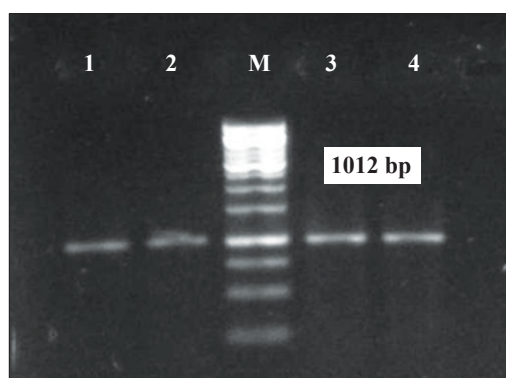


Fig. 2. Amplification of *TLR2* gene partial exon fragment of Bikaneri camel. Lane 1 & 2 and 3 & 4 - *TLR2.4* fragment of 1012 bp, M - Molecular marker (1kb).

temperature ranging from 54.8-59.8°C (Table 1) was found optimal for amplification of the targeted regions. After the assembly of the eight *TLR2* gene fragments and removal of the overlapping sequences a consensus sequence of 3213 bp were generated and sequence was submitted with accession number MW557315.1. The *TLR2* gene consensus sequence consisted of partial coding sequence of 2287 bp spanning exon-2 and 3' UTR of 927 bp. The present study characterized the partial coding sequence of 2287 bp encoding for 761 amino acids. After *in-silico* analysis we found that ORF of Dromedary camel is of 2358 bp coding for 785 amino acids which is similar to our findings. In contrast to present study Dahiya *et al.* 2014 reported lower ORF of 1857 bp long encoding for 618 amino acids in Dromedary camel. However, Dahiya *et al.* (2014) reported similar *TLR2* gene ORF for Bactrian camel. The ORF and encoded amino acids for *TLR2* gene of cattle (McGuire *et al.* 2006) and buffalo (Tantia *et al.* 2012) also depicted similar pattern indicating highly conserved nature of this gene across all mammalian species. The encoded amino acid (Accession no. UBN09134.1) coded for 12 LRR. The LRR were found from region 55-501 which were at amino acid position 31-54, 55-78; 79-102; 103-127; 128-142; 153-176; 339-365, 366-391, 392-414,

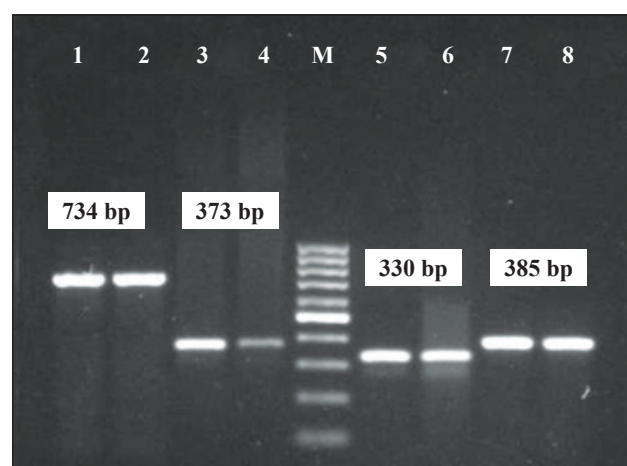


Fig. 3. Amplification of partial exon fragments and 3' UTR of *TLR2* gene in Bikaneri camel. Lane 1 & 2 - *TLR2.5* fragment of 734 bp, Lane 3 & 4 - *TLR2.6* UTR region of 373 bp, Lane 5 & 6 - *TLR2.7* UTR region of 330 bp, Lane 7 & 8 - *TLR2.8* UTR region of 385 bp, M - Molecular marker (100 bp).

436-455, 456-477 and 478-501, respectively. The TLR family proteins are characterized as a trans-membrane receptor with an extracellular domain with Leucine-rich repeats (LRR) participating in ligand recognition and an intra-cytoplasmic domain containing a Toll/ interleukin-1 receptor homology (TIR) domain, critical to mammalian TLR signaling (Werling *et al.* 2003).

When *TLR2* gene sequence of Bikaneri camel were aligned and compared with available *TLR2* nucleotide sequences of camelid family, the sequence shared more than 99% identity with Camelids and more than 97% similarity with Vicugna (Table 2). At the nucleotide level *TLR2* gene sequence identity of Bikaneri camel with that of *Sus scrofa*, *Bubalus bubalis*, *Capra hircus*, *Bos taurus*, *Bos indicus*, *Ovis aries*, *Equus caballus* and *Homo sapiens* was 86.18, 85.80, 85.75, 85.70, 85.65, 85.51, 85 and 82.37%, respectively. Similarly, the *TLR2* gene of the Dromedary camel also had the highest nucleotide and amino acid identity with pig, i.e. 86.18% and 82.79%, respectively in our report. However, Dahiya *et al.* (2014) reported nucleotide and amino acid identity of Dromedary Camel *TLR2* gene with pig to be 66.8% and 59.6%, respectively. In this study Dromedary and Bactrian camels shared 99.3% nucleotide and 98.95% amino acid identity with each other (Table 2). These findings were in accordance to the previous study that showed homologous family of Toll like receptors exists in all the vertebrates sharing some conserved regions (Medzhitov *et al.* 1997).

Phylogenetic analysis based on nucleotides sequence of *TLR2* gene revealed that all the sequences of Camelid family were grouped to single branch with different sub branch. Hence it can be concluded that our sequence was more closely related to dromedary camel, followed by bactrian and ferus camel. Study also revealed close ancestral relationship for *TLR2* gene between Bikaneri camel (*Camelus dromedarius*) and Vicugna.

Phylogenetic analysis based on amino acid sequences

Table 2. Comparison of nucleotide sequence of TLR2 gene of Bikaneri camel (MW557315.1) with other vertebrates

Species	NCBI accession no. (Nucleotide)	Per cent identity (Nucleotide)	NCBI accession no. (Protein)	Per cent identity (Amino acid)
<i>Camelus ferus</i>	XM_032465196.1	99.59	XP_03232108.1	99.47
<i>Camelus dromedarius</i>	JQ979305.1	99.47	AFK8006.1	98.92
<i>Camelus bactrianus</i>	JX453495.1	99.30	AGI51670.1	98.95
<i>Vicugna pacos</i>	XM_015246998.2	97.54	XP_015102484.1	97.63
<i>Sus scrofa</i>	AB208696.2	86.18	BAD91799.1	82.79
<i>Bison bison</i>	EU580539.1	85.94	ACB72728.1	80.95
<i>Bubalus bubalis</i>	HM756161.1	85.80	ADO51627.1	81.08
<i>Bos grunniens</i>	KF878304.1	85.78	AIA59622.1	80.95
<i>Capra hircus</i>	JQ911706.1	85.75	AFM80032.1	81.60
<i>Bos taurus</i>	NM_174197.2	85.70	NP_776622.1	81.21
<i>Bos indicus</i>	EU413951.1	85.65	ABY90177.1	80.81
<i>Ovis aries</i>	AM981300.1	85.51	CAQ37821.1	80.81
<i>Equus caballus</i>	NM_001081796.1	85.00	NP_001075265.1	81.47
<i>Homo sapiens</i>	NM_001318796.2	82.37	NP_001305725.1	78.76

of *TLR2* gene in various livestock species depicted three major branches. One branch had *Bos grunniens*, *Bos mutus*, *Bos frontalis*, *Bos indicus*, *Bison bison*, *Bos taurus*, *Bos indicus* × *Bos taurus*, *Bubalus bubalis*, *Capra hircus*, *Ovis aries*. The other branch contained *Sus scrofa* and Camelid family, whereas the third branch had *Gorilla gorilla*, *Homo sapiens*, *Equus asinus* and *Equus caballus*. These findings suggested the closest phylogenetic relationship of Bikaneri camel *TLR2* gene is with camels, vicugna followed by pigs and most distant with humans (Fig. 4).

In this study *TLR2* gene fragment of 3213 bp was successfully amplified which spanned over 2287 bp of coding sequence of exon-2 and 926 bp of 3' UTR. The partial coding sequence encoded for 761 amino acids. The encoded amino acid (Accession no. UBN09134.1) coded for 12 leucine rich repeats (LRR). Phylogenetic analysis of Bikaneri camel *TLR2* gene sequences showed close relationship between dromedary and bactrian camel followed by vicugna, llama and pigs. However they were found distantly related to humans.

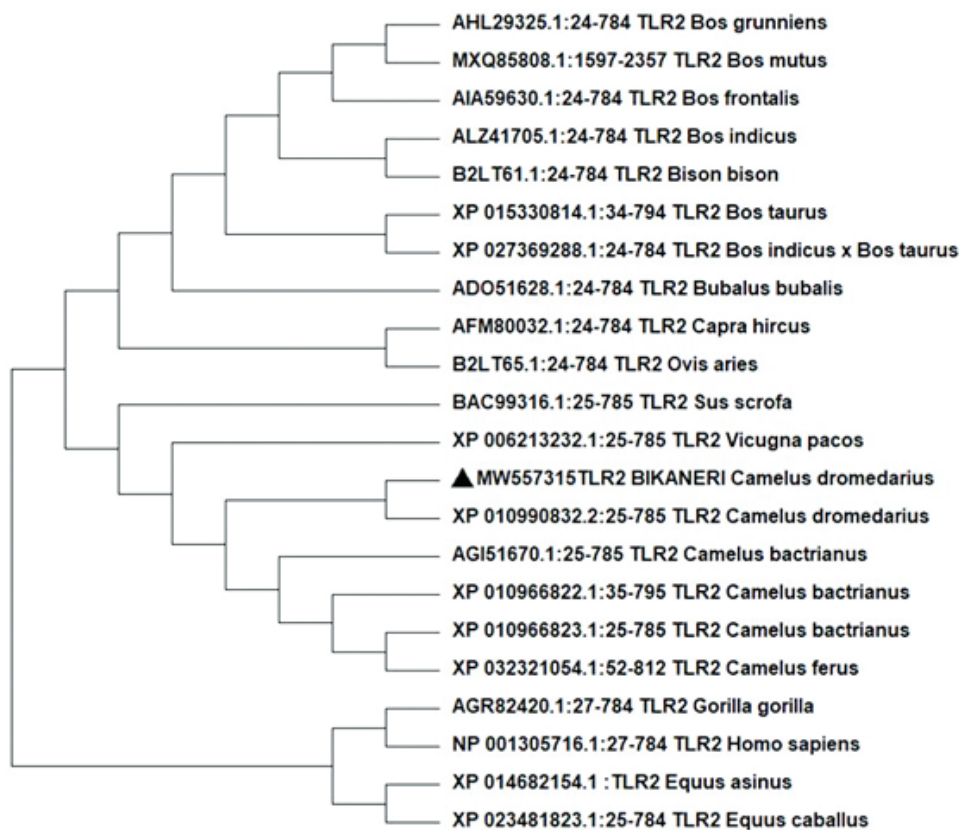


Fig. 4. Phylogenetic tree of camel *TLR2* gene was constructed using MEGA 6.0 software based on amino acid sequences. The optimal tree sum of branch length is 0.61. The analysis involved 22 amino acid sequences of different vertebrates.

ACKNOWLEDGEMENT

Authors are thankful to Director of ICAR-National Research Centre on Camel, Bikaner for providing financial support for this study.

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