Veterinary Immunology and Immunopathology 140 (2011) 252–258

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Sequence analysis of Toll-like receptor genes 1–10 of goat (*Capra hircus*)

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ARTICLE INFO

Article history:
Received 24 September 2010
Received in revised form 3 January 2011
Accepted 6 January 2011

Keywords:
Toll-like receptors
Sequencing
sequence analysis
Simple modular architecture research tool
Phylogenetic tree
Multi dimensional scaling

ABSTRACT

This study involved cloning and sequencing of the coding regions of all 10 Toll-like receptor (TLR) genes of goat. Goat TLR 1–10 gene sequences revealed a high degree of nucleotide identity with sheep and cattle sequences (>90%) and 75–85% with pig, mouse and human sequences. At the amino acid level, 85–99% similarity was observed with sheep and cattle and 60–85% with pig, mouse and human. TLR9c DNA of goat showed the highest amino acid identity to that of sheep (99%) while TLR8 cDNA showed the lowest identity of 88.7% to that of sheep. Variations were seen in the number of leucine rich repeats (LRRs) of goat TLRs as compared to other ruminant species with maximum differences in the TLR3 gene. Phylogenetic analysis through molecular evolution and genetic analysis (MEGA) software and multi dimensional scaling revealed a high degree of conservation of goat TLRs with those from other species. However when the TIR domain of all the TLRs were compared, goat TLR7 TIR alone showed a high divergence of 19.3 as compared to sheep sequences. This is the first report of the full-length cDNA sequences of all the 10 TLR genes of goats which would be a useful tool for the study of evolutionary lineages and for phylogenetic analysis.

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1. Introduction

During the recent past, there has been rapid progress in understanding the innate immunity against microbial components and its critical role in host defense against infection. The early concept was that the innate immune system of the host nonspecifically recognized microbes. However, following the discovery of Toll-like receptors (TLRs) in the mid-1990s it has been clearly shown that pathogen recognition by the innate immune system is broadly specific, relying on germline-encoded pattern-recognition receptors (PRRs) that have evolved to detect relatively conserved components of pathogens referred to as pathogen associated molecular patterns (PAMPs) (Akira et al., 2001; Janeway and Medzhitov, 2002). The PAMPs recognized by TLRs include lipids, lipoproteins, proteins and nucleic acids derived from a wide range of microbes such as bacteria, viruses, parasites and fungi (Akira et al., 2006). TLRs are type I transmembrane proteins with three domains, ecotodomain, transmembrane and Toll-interleukin 1 (IL-1) receptor (TIR) domains. The ectodomain consists of leucine-rich repeats that mediate the recognition of PAMPs, while the cytoplasmic TIR domain mediates downstream signal transduction.

Binding of ligands to TLRs triggers at least two important cell signaling pathways. One pathway involves MyD88, an

Abbreviations: TLR, Toll-like receptor; cDNA, complementary DNA; SMART, simple modular architecture research tool; MEGA, molecular evolution and genetic analysis; TIR, Toll-interleukin 1 receptor domain.

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doi:10.1016/j.vetimm.2011.01.007

adaptor protein shared by most of the TLRs that leads to the activation of the transcription factor NF-κB resulting in the release of pro-inflammatory cytokines. The other pathway involves maturation of antigen-presenting cells (APCs) and increased expression of major histocompatability complex (MHC) molecules, co-stimulatory molecules, CD40 and interferon beta or Type 1 interferon. Both signals are essential for the initiation of T cell-mediated immunity (Pasare and Medzhitov, 2004). To date, the TLR family comprises a total of 13 genes, of which 10 have been identified in human, pig, mouse, cattle and sheep (Chang et al., 2006, 2009; Werling and Coffey, 2007; McGuire et al., 2006). The ligand recognition regions of TLRs for intracellular pathogens (e.g. viruses), TLR3, 7, 8 and 9, are located in the endosomal compartment within the cytoplasm whereas the bacterial and fungal ligand recognition regions on TLR1, 2, 4, 5, 6 and 10 are found on the cell surface. Species-specific differences in recognition of TLR ligands such as single-stranded RNA, bacterial DNA and flagellin have been observed between man and mouse (Farhat et al., 2010; Keestra et al., 2008; Roberts et al., 2005). These differences may be due to distinct selective pressure on each host to adapt to new environments and pathogens.

Significant progress has been made to delineate the TLR's association with disease resistance and susceptibility in man and mouse. TLR genes in other animals are less well defined. With respect to the sequence information in the public database the full-length sequence data has been reported for 10 TLR genes in human, mouse, pig, cattle, sheep and chicken. The TLR10 counterpart of human has not been reported in mouse however it is thought to be a non-functional pseudogene (Takeda and Akira, 2005). There is a paucity of information with respect to goats (*Capra hircus*). Knowledge of the innate immune mechanism and signaling mediated through TLRs could provide more insight into the disease resistance of goats. Esteves et al. (2008) showed that neonatal goats expressed higher levels of TH-1 type cytokines to different TLR ligands than adult goats. There is a recent publication from our group which details the TLR expression profile in different tissues in an Indian breed of goat (Tirumurugaan et al., 2010). In this study, we have generated the full-length sequence information of the TLR genes 1–10 and generated a comparative analysis with respect to other species namely human, mouse, sheep, pig, chicken and cattle.

2. Materials and methods

2.1. Samples

Peripheral blood samples were collected from one-year old apparently healthy Barbari breed of goats maintained at the University Research Farm, Madhavaram, Chennai, India. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by centrifugation over Histopaque 1.077 (Sigma, USA) and resuspended in RPMI 1640 (Sigma, USA) at 1.5×10^6 cells/ml.

2.2. Isolation of RNA and cDNA Synthesis

Total cellular RNA was isolated from the isolated cells using TRIzol reagent (Invitrogen, USA) as per manufacturer's protocol. The extracted RNA was checked for its quantity and purity by Biophotometer (Eppendorf, Germany). The cDNA was synthesized by using High Capacity cDNA synthesis kit (Applied Biosystems, USA) as follows: 1 μg of RNA in 8 μl of DEPC water was mixed with 2 μl of random hexamers and incubated at for 60 °C for 5 min, snap cooled on ice followed by the addition of 10 μl of 2× cDNA master mix. The cDNA was synthesized by incubating at 37 °C for 1 h and the enzyme was inactivated at 70 °C for 10 min.

2.3. Amplification, cloning and sequencing of full-length goat TLR 1–10

The primers for the amplification of the coding sequences of goat TLR genes were designed based on the full-length sequences of bovine and sheep TLR 1–10 genes using FastPCR software available at www.biocenter.helsinki.fi/bi/Programs/download.html and were synthesized at Sigma–Aldrich, India. Each TLR full-length cDNA was synthesized by using the high-fidelity XT polymerase PCR kit (Genei, Bangalore) using the primers and the respective annealing temperatures listed in Table 1. PCR was performed in a thermal cycler (Mastercycler gradient, Eppendorf, Germany), with initial denaturation at 94 °C for 5 min; 35 cycles of denaturation 94 °C for 1 min, respective annealing temperatures for 1 min and extension at 72 °C for 1 min followed by a final extension at 72 °C for 7 min.

The amplified TLR genes were purified by PCR gel extraction kit (M/s. BioBasic, Canada) and cloned into TA vector plasmid (RBC Biosciences, USA) according to manufacturer's instructions. Recombinant plasmid DNA was extracted from 3 colonies for each gene by using the plasmid extraction kit (M/s. BioBasic, Canada). The purified plasmid DNA was checked for the presence of specific TLR gene inserts by PCR and the recombinant plasmids were sequenced using the Big dye terminator cycle sequencing ready reaction kit (Applied Biosystems, California, USA) in an automatic sequencer (ABI Prism 3100, Genetic analyzer, Applied Biosystems, California, USA). Sequencing was performed with overlapping primer pairs with the information generated from an earlier study (data not shown). The sequences were assembled in the SeQman Pro module of the Lasergene software V 7.1 (DNASTAR Inc., USA) to obtain the full-length sequence of each TLR gene.

2.4. Sequence analysis

Sequences of cloned TLR genes were analyzed using the Lasergene software package (DNASTAR, London, UK). MegaBlast was used to identify mammalian TLR nucleotide sequences within the non-redundant nucleotide database (http://www.ncbi.nlm.nih.gov/) by comparison with the goat TLR sequences obtained. The multiple alignment of the TLR 1–10 coding sequences from multiple species was performed using the program ClustalX2. The simple mod-

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