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Research paper

Characterization of ovine TLR7 and TLR8 protein coding regions, detection of mutations and Maedi Visna virus infection

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ABSTRACT

Toll-like receptors (TLRs) 2, 3, 4, 7, 8 and 9 play a crucial role in the recognition of viral entities and modulation of the innate immune system. This work presents sequence analysis of ovine TLR7 and TLR8 genes, depicts novel mutations and describes frequencies of mutations in Maedi Visna infected and healthy sheep. Totally 48 samples of the breed Tsigai were analyzed for the presence of mutations. Within 20 mutations, 14 were silent whereas 6 were missense. The frequencies of missense mutations in the Maedi Visna infected compared to non-infected sheep were: Lys115Glu (P - 0.766, F-test), Asn117 (P - 0.380) and Lys818Arg (P - 0.739). These three mutations were localized in extra LRR (lucine rich repeat) region of TLR7, while mutation Ile73Leu (P - 0.498) was located within LRR2 motif. Both mutations in TLR8, Asn165Lys (P - 1.0) and Tyr349His (P - 0.700), were present in extra LRR region. The secondary structure analysis of ovine TLR7 and TLR8 revealed conserved LRR motif structure, however with some irregularities compared to cattle and human. Transmembrane domains of TLR7 and TLR8 showed 100% homology between sheep and cattle wherein no mutations were found. In both TLRs TIR domains were highly conserved with occurrence of 4 silent mutations. Mutations in TLR7 and TLR8 may play an important role as predisposition factor for Maedi Visna infection. Considering the sequence homology among sheep, cattle and human genes encoding TLR7 and TLR8, we predict their similar function, localization and downstream signaling.

1. Introduction

Mammalian toll-like receptors (TLRs), a group of pattern recognition receptors, consist of at least 14 TLR members (Kawai and Akira, 2008). TLR2, TLR3, TLR4, TLR7, TLR8 and TLR9 are known to trigger antiviral defense mechanisms (Alexopoulou et al., 2001; Bieback et al., 2002; Burzyn et al., 2004; Dahlstrom et al., 2004; Dalod et al., 2003; Diebold et al., 2004; Heil et al., 2004; Horikawa et al., 2003; Krug et al., 2004; Kurt-Jones et al., 2000; Lund et al., 2003; Rassa et al., 2002; Tabeta et al., 2004). Different bacterial products induce various responses, e.g. lipopolysaccharide triggers TLR4, peptidoglycan activates TLR2, double-stranded RNA stimulates TLR3, single-stranded RNA (ssRNA) stimulates TLR7 and TLR8, and CpG motifs of bacterial and viral origin stimulate TLR9 response (Akira and Takeda, 2004; Bowie and Haga, 2005; Diebold et al., 2004; Hemmi and Akira, 2005). Mutations in the TLRs may cause reduced pathogen recognition and hampered innate immune activation (Franchimont et al., 2004; Hawn et al., 2007;

Abbreviations: TLR, Toll-like receptors; OR, Odd's ratio; LRR, leucine rich repeats; MV, Maedi Visna; SRLV, small ruminant lentivirus; nBLAST, nucleotide BLAST; ECD, ectodomain; TM, transmembrane; TIR, Toll/interleukin-1 receptor; PAMP, pathogen associated molecular patterns.

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Henckaerts et al., 2007; Johnson et al., 2007; Morre et al., 2003; Schroder and Schumann, 2005). Till to date, a number of studies have shown the association between mutations in TLR genes and the host susceptibility to diseases like malaria or pneumococcal disease (Khor et al., 2007; Malley et al., 2003), tuberculosis (Ben-Ali et al., 2004; Thuong et al., 2007), encephalitis caused by herpes simplex virus (Zhang et al., 2007), hepatitis caused by hepatitis C virus (Schott et al., 2008); AIDS (Oh et al., 2009, 2008), etc.

Maedi Visna virus (MVV), a member of *Retroviridae* family, is the prototype virus of *Lentivirus* subfamily (Coffin, 1996), which causes two different manifestations in sheep namely maedi and visna (Thormar, 1965). The virus shares many characteristics with the human immunodeficiency virus (HIV), including the establishment of persistent infection (Blacklaws et al., 1994), while MVV does not induce severe immunodeficiency (Ryan et al., 2000; Thormar, 2005). The primary host cells for MVV are monocyte/macrophage lineage (Gendelman et al., 1986; Gorrell et al., 1992; Narayan et al., 1982) and dendritic cells (Ryan et al., 2000).

The role of TLRs 3, 7, 8 and 9 in the host's defense against viruses makes them suitable markers for exploration of the linkage between mutations in the genes of innate immunity and increased susceptibility to viral infections. TLR3 and TLR9 deficient mice showed normal IFN-α response to influenza virus that was abolished in the absence of TLR7 (Diebold et al., 2004). This indicates that the TLR7 (but neither TLR3 nor TLR9) plays a pivotal role in the immune response against influenza virus. Moreover, cells expressing human TLR8 but not TLR7 were activated in response to ssRNA of HIV (Heil et al., 2004). This indicates that both TLR7 and TLR8 are essential for cell signaling against viral infections. The studies have revealed that ssRNA of HIV and influenza virus trigger TLR7 and/or TLR8 downstream signaling (Diebold et al., 2004; Heil et al., 2004). Taking into account the genetic similarity between MVV and HIV it can be proposed that both viruses may trigger TLR7 and TLR8 in similar fashion.

Hitherto, no study reporting mutations in the ovine TLR7 and TLR8 genes is available. A linkage study reporting association between a lentiviral infection and mutation in the innate immunity genes is also missing. Here we present detailed characterization of ovine TLR7 and TLR8 genes, *in silico* prediction of the secondary structure of encoded proteins and frequencies of the mutations in Maedi Visna infected and non-infected sheep.

2. Materials and methods

2.1. Animals and isolation of DNA

3–4 ml blood samples (in duplicate) were collected from 1150 Tsigai sheep (all females of similar age). Two sheep farms, with the history of MVV prevalence, located in the close vicinity in the eastern region of Slovakia were included in this study. None of the animals exhibited clinical manifestation of the disease at the time of sample collection. One tube of the blood sample containing 2 ml of 1.5% EDTA was used for the isolation of DNA and the second one for serum separation. DNA isolation was performed according to the method described previously (Sambrook et al., 1989).

2.2. Maedi Visna diagnosis

For detection of antibodies against MVV, commercial ELISA kit (POURQUIER® ELISA; Pourquier, France) was used. Simultaneously, samples were subjected to PCR based diagnosis of pro-viral DNA in white blood cells as described earlier (Eltahir et al., 2006). Relatively conserved *pol* gene was targeted for nucleic acid amplification with degenerated primers with the ability to cover 85 SRLV (small ruminant lentivirus) isolates. For the strain identification, PCR products were cut from the agarose gel and sequenced on ABI Avant-3100 (Applied Biosystem, Bratislava, Slovakia). Sequence homology analysis was performed by nBLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The animals of similar age, sex and breed were selected for further mutation detection and linkage study (24 positive and 24 negative sheep for MVV infection). Endemic and chronic presence of MVV infection in both farms assured the equal pressure of pathogen in the sheep population.

2.3. TLR7 and TLR8 gene fragment amplification

Primers were designed (Primer3, http://frodo.wi.mit.edu/) to amplify ectodomain (ECD) except start codon, transmembrane (TM) helix and Toll/interleukin-1 receptor (TIR) regions of TLR7 and TLR8 from genomic DNA. Fifteen partially overlapping primer pairs to amplify TLR7 (Table 1) and ten primer pairs for TLR8 (Table 1) were used. PCR was run in total volume of 10 μl. The reaction mixture contained 1x LightScanner Master Mix (Idaho, USA), 0.20 μM of each primer and 15 ng of DNA. Twenty microliters of mineral oil was overlaid (Sigma, Bratislava, Slovakia) and amplified on DNA Engine Dyad Peltier Thermal cycler (Bio-Rad, Bratislava, Slovakia). Cycling conditions for all reactions were similar except annealing temperature: initial denaturation at 95 °C for 3 min, followed by 33 cycles of denaturation for 35 s at 95 °C, annealing for 10 s (temperatures are depicted in Table 1) and extension for 30 s at 72 °C. Samples were denaturated at 95 °C for 40 s, cooled at 20 °C and subsequently high resolution melting was performed.

2.4. High resolution melting (HRM) analysis for mutation detection

Melting-curve analysis was performed as described previously (Kennerson et al., 2007) with a Light-Scanner software. Melting curves were normalized by selecting linear regions before (pre-denaturation) and after the melting transition (post-denaturation). These regions were defined for each curve, with an upper (100%) fluorescence and lower (0%) baseline being common for all curves. To eliminate slight temperature errors between samples, the normalized melting curves were temperature shifted by moving the curves along the *X*-axis to bring them through a common temperature that facilitates clustering into groups. To avoid false negatives, we performed this procedure at a temperature at which the entire mixture of

Table 1
Primers used for amplification of TLR7 and TLR8 protein coding regions.

Primer denotation	Sequence 5′–3′ (F/R)	Annealing temp.
TLR7/ECD-1	AAATTCTGGATCTTGGTGTATTTT/TGCACACGTTGTCTTTTGG	60 °C
TLR7/ECD-2	AATGCCACCAACCTTACCC/GCCAGTTCTGTTAGATTCTCCTTC	63 °C
TLR7/ECD-3	GGATCTTCCTCCCAGCTTACA/GGCAAAACAGTGGGGACA	64 °C
TLR7/ECD-4	TTAAAATTGCTCTCCCTAAAAGATAA/GGTGTGCAAGGAAATGGAA	62 °C
TLR7/ECD-5	AGTTCTTGATCTGAGTGGAAATTG/GTTGACAAGGTTATGAAGAAGATG	62 °C
TLR7/ECD-6	GCCAAAGAAATTGGGGATG/TCATGTTAGAGCAGAAACCACCT	51 °C
TLR7/ECD-7	TGAAGTTCTTGATCTTGGCACTA/TGAGGAAAGAAAGATGCTGGA	61 °C
TLR7/ECD-8	CCGGTCCAAGAGCAAAGAGC/CAAGCCGATTGTTAGAGAAGTCCA	66 °C
TLR7/ECD-9	GAATTTCAGCCTTTAGTGGAGTTG/GCTGGTGGAGGTAGCAATGT	64 °C
TLR7/ECD-10	GGTTCTGAGGAAACTGATGATG/GAGTCTTTAGATTTGGAGGCATACT	64 °C
TLR7/ECD-11	CTCTGAGTTTCTTACCTTTGGGAGT/ACTGGAAAGCACCTTGGAGAA	64 °C
TLR7/ECD-12	CGCAGCCTCAAGAAACTCATAC/CCACCAGACAAACCACACAG	66 °C
TLR7/TM	TTTGTTTCTGCATCACAATCG/CGTCGATACCCTTTTATTTTGG	61 °C
TLR7/TIR-1	TCTCTATTTCTGGGATGTGTGG/AGCCTCTGATGGGATAAGTAAAA	62 °C
TLR7/TIR-2	TTATGTCTTGAGGAAAGAGACTGG/ACATTCGGCAAAGAAGGG	62 °C
TLR8/ECD-1	CATTGTGACTCTACTTTGATTTTCCT/ATTTCCACTCTGGGACTTGG	52 °C
TLR8/ECD-2	TCTGACTAAAATCAACCTGAACCA/GGGGATTAAAAGATAATGACAGCA	59 °C
TLR8/ECD-3	GGAGCATTCCAAAACCTTACC/CCTTCAGATTGTGCATGTTGT	55 °C
TLR8/ECD-4	CCCAACTTCGCTACCTAAACC/CAGTGGAAAATGCTAAAATCAATC	53 °C
TLR8/ECD-5	CGACTATCAACTTGGGCGTTA/AGCATGAAAATTCCGTTCCA	55 °C
TLR8/ECD-6	TTTTGGCAACATTTCCTGTTT/CTTGAGCATTCCACAGAAGGT	58,5 °C
TLR8/ECD-7	ACTTTAACAGAAACACAACTGAAAAG/TGGCTCCAGAAAGAAAATCA	57 °C
TLR8/TM	CGAAACAGAATTTCCCACCT/CCAAGCATCCCAGTAAAACC	59 °C
TLR8/TIR-1	AACCTTTTCTGTCACCATCTC/TTCTCCTCCATTAGCCTCTG	54,5 °C
TLR8/TIR-2	CAGAGCATCAACCAAAGCAA/GACAGCTAAGAACAAAAGGAGCA	57 °C

duplexes had melted. Fluorescent difference curves were generated from normalized temperature-shifted data by selecting a wild type for comparison and subtracting the fluorescence of all other melting curves.

2.5. DNA sequencing

Representative samples from each genotype were sequenced on ABI Avant-3100 (Applied Biosystem). Forward and reverse sequences were aligned and mutations were validated using SeqScape v.2.1 software (Applied Biosystems). Sequences were submitted to the Genbank (Rockville Pike, USA) under the accession numbers: TLR7 - GQ175927, GQ175928, GQ175929, GQ175930, GQ175931, GQ175932, GQ175933, GQ175934, GQ175935; TLR8 - GQ175936, GQ175937, GQ175938, GQ175939, GQ175940, GQ175941, GQ175942, GQ175943, GQ175944, GQ175945, GQ175946, GQ175947, GQ175948, GQ175949, GQ175950, GQ175951, GQ175952, GQ175953, GQ175954, GQ175955, GQ175956, GQ175957.

2.6. Secondary structure prediction and in silico analysis

Based on the HRM analysis coupled with sequencing we identified a homozygote individual for TLR7 and TLR8. Overlapping sequences of TLR7 and TLR8 of these homogygotes were assembled and contig was created (DNASTAR software, Madison, WI). Sequences were further translated into amino acid sequences (BioEdit software, Carlsbad, CA). Human and cattle TLR7 (AF245702.1, ABQ52582.1) and TLR8 (NP619542, NP001029109) amino acid sequences were retrieved from Genbank (USA). Pairwise percent similarity and divergence between cattle, human and sheep TLRs were calculated using MegAlign (Lasergene software, Madison, USA). LRR motifs were outlined according to the method described earlier (Matsushima et al., 2007) using SSpro4.0 server (Cheng et al., 2005). TM helix was predicted with TMHMM software (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

2.7. Statistical analysis

Potential linkage between TLR mutations and increased incidence of MVV in sheep population was calculated by Odd's ratio (OR) along with 95% confidence interval (CI) using Win episcope software (CLIVE, UK). Further, Fisher's exact test was applied to assess the significance (both side *P*-value) of observed mutation linked with Maedi Visna susceptibility.

3. Results

3.1. Prevalence of MVV infection

The prevalence of MVV infection detected by ELISA was 79.1% ($n = 910$) in the years 2006–2007, which is in accordance with the previous seroprevalences in these farms (data not shown). 2.7% ($n = 17$) of seronegative animals were found PCR positive for MV infection. nBlast analysis of the *pol* sequence showed 86% (*e*-value: 4e−115) sequence homology with Visna virus strain 85/34 isolated in USA.

3.2. Prediction of secondary structure

We sequenced whole protein coding regions (except start codon) of the ovine TLR7 and TLR8 genes. When nucleotide sequences were translated *in silico* and subjected to the secondary structure prediction, total 27 LRR motifs were found in each TLR7 and TLR8 ectodomain (Figs. 1–3). The LRR structure prediction was based on the

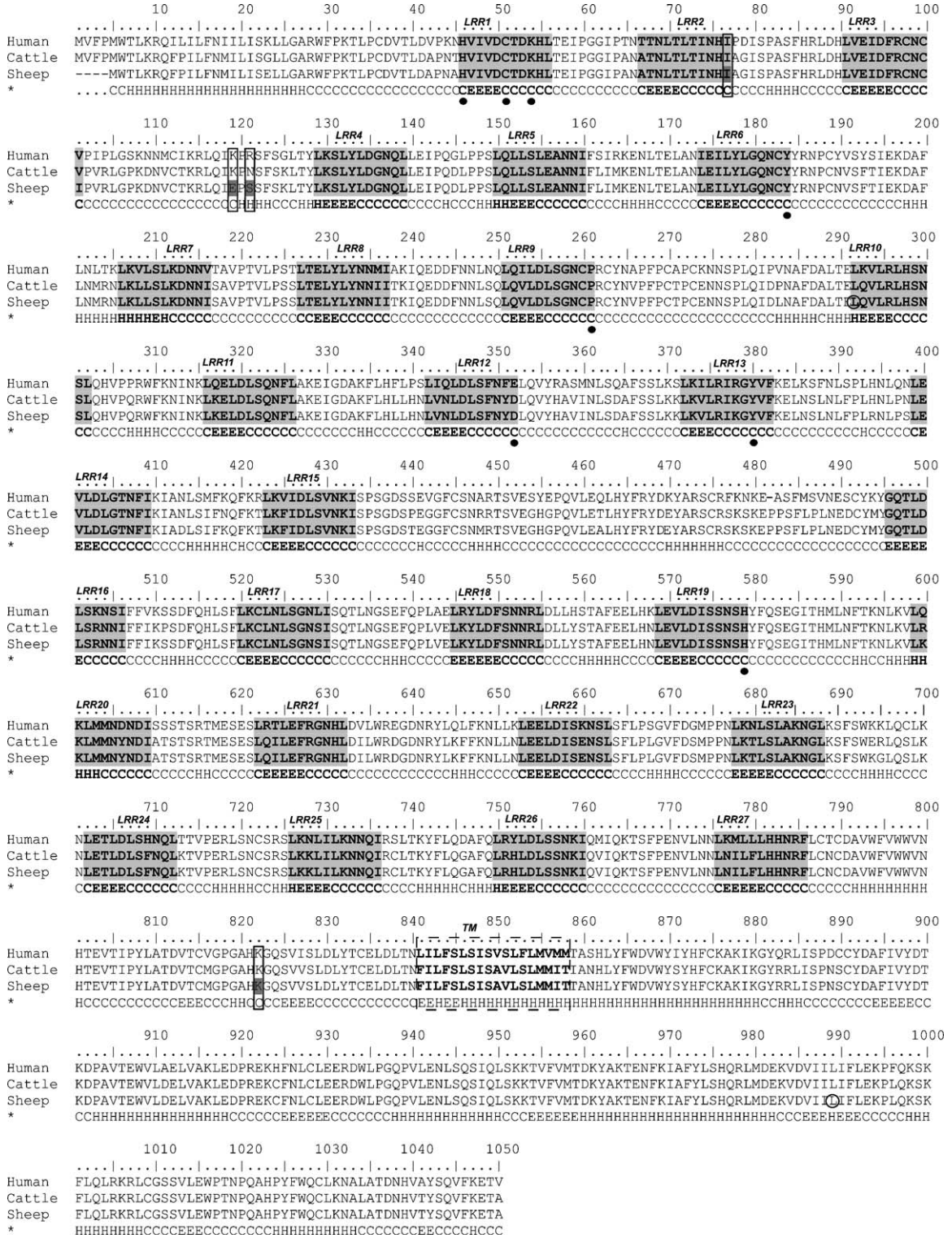

Fig. 1. Ovine TLR7 protein coding sequence, its secondary structure, irregularities in LRR and observed mutations. LRR, leucine rich repeats (grey coloured); TM, transmembrane domain (dashed bracket); irregularity in the LRR (black circles); silent mutation (hollow circles) and missense mutation (bracketed).

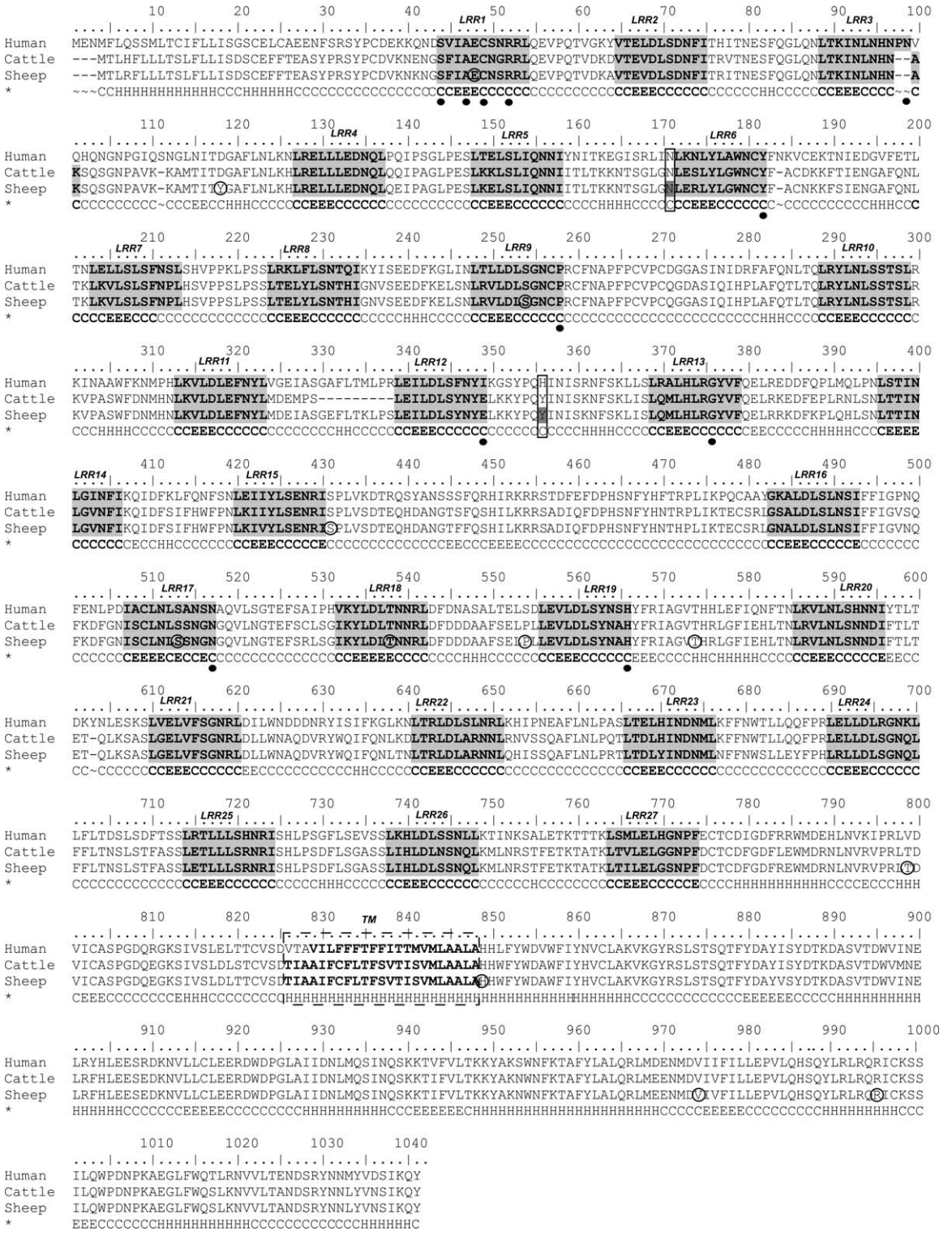

Fig. 2. Ovine TLR8 protein coding sequence, its secondary structure, irregularities in LRR and observed mutations. LRR, leucine rich repeats (grey coloured); TM, transmembrane domain (dashed bracket); irregularity in the LRR (black circles); silent mutation (hollow circles); and missense mutation (bracketed).

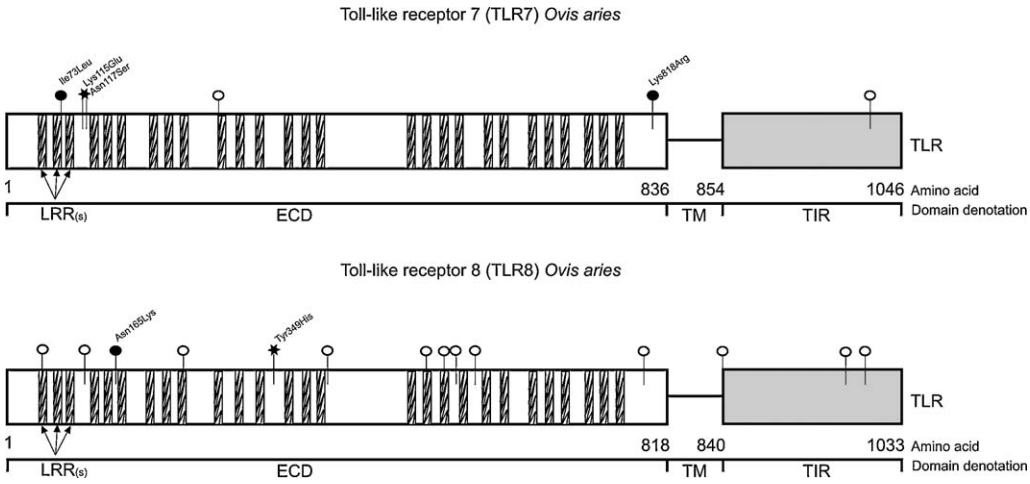

Fig. 3. Schematics of mutations found in TLR7 and TLR8 ovine genes. LRR(s), leucine rich repeat(s); ECD, ectodomain; TM, transmembrane domain; TIR, Toll/interleukin-1 receptor domain; hollow circles indicate silent mutation; black circles indicate missense mutations and star indicates missense mutation with possible association to Maedi Visna infection.

beta sheet that shows at least 85% identity (85% cutoff) with LxxLxLxxNxL motif structure (Matsushima et al., 2007). Interestingly, LRR20 of TLR7 had no beta sheet. Deviation from the LxxLxLxxNxL structure (structure of LRR motif is described in the discussion section) was considered as irregularity. Eight irregularities were found in LRR of TLR7, while in TLR8 there were eleven. The eleventh residue in the LRR motif had the highest tendency of indiscretion in both TLR7 and TLR8. Four LRR motifs in TLR7 (LRR6, 9, 12 and 19; Fig. 1) and six in TLR8 (LRR3, 6, 9, 12, 17 and 19; Fig. 2) were irregular at 11th position ('L'). Irregularity at 9th position ('N') of LRR motif was noticed in LRR1 and LRR13 in both TLRs. LRR1 was the most irregular motif in both TLR7 and TLR8.

Ovine TLR7 protein coding motif was the shortest (1046 residues) as compared to bovine and human (1050 and 1049 amino acid residues, respectively). Similar disparity was also observed in the length of ovine (1033 residues), bovine (1024 residues) and human (1041 residues) TLR8 protein coding motifs. No insertions and deletions were found in both TLR7 and TLR8. In the percent similarity matrix (MegAlign – Lasergene software), 86% amino acid sequence similarity was found between sheep and human TLR7. The similarity between ovine and bovine TLR7 was 98%. The divergence between sheep and human TLR8 amino acid sequences was 26%, while it was only 5% between sheep and cattle. The start codon of ovine TLR7 was 4 residues offset of human and bovine TLR7 start codons (Fig. 1). Similarly, the start codon in both ovine and bovine TLR8 was 3 residues offset than human (Fig. 2). The membrane-spanning regions of ovine TLR7 and TLR8, predicted by TMHMM server, were comprised of 18 and 23 amino acid residues, respectively. Interestingly the sequence homology of TM region between human and sheep was only 61% in case of TLR7 and 52% in case of TLR8. 100% homology between ovine and bovine TM domains of both TLRs was found (Figs. 1 and 2).

3.3. Point mutations in TLR7 and TLR8 genes

Four missense mutations were found in ovine TLR7: Ile73Leu, Lys115Glu, Asn117Ser, Lys818Arg (Table 2). Three of them were located in the extra-LRR regions, namely Lys115Glu and Asn117Ser were situated 8 and

Table 2
Missense mutations in TLR7 and TLR8 genes.

Gene	Nucleotide substitution	Amino acid change	Genotype frequency of the WT amino acid	Genotype frequency of the mutated amino acid in homozygous state	Genotype frequency of the mutated amino acid in heterozygous state	MV positive samples with mutated amino acid (%)	MV positive samples with heterozygous amino acid (%)
TLR7	A217M	Ile73Leu	0.958	0	0.021 (–)[a]	0	$4.16^{P-0.498}$
TLR7	A343R	Lys115Glu	0.582	0.042 (1)0.009–102	0.188 (1.43) 0.35–5.78	2.083^{P-1}	$20.83^{P-0.766}$
TLR7	A350R	Asn117Ser	0.541	0.042 (1)0.009–102	0.208 (2) 0.51–8.02	2.083^{P-1}	$25^{P-0.380}$
TLR7	A2453R	Lys818Arg	0.729	0.021 (–)[b]	0.125 (0.64) 0.32–8.00	0	$10.41^{P-0.739}$
TLR8	C495S	Asn165Lys	0.980	0	0.010 (–)[b]	0	2.08^{P-1}
TLR8	T1045Y	Tyr349His	0.833	0	0.083 (1.84)0.28–14.61	0	$10.41^{P-0.700}$

Figures in the parenthesis depict Odd's ratio value. Underlined figures indicate confidence interval (95%) for Odd's ratio, *P*-linkage between TLR mutation and susceptibility to MV infection assessed with *F*-test (two side *P*-value is indicated).

[a] All samples were MV positive with this mutation.

[b] All samples were MV negative with this mutation.

Table 3
Silent mutations found in TLR7 and TLR8 gene in sheep.

No.	Gene	Nucleotide substitution	Amino acid
1	TLR7	*G864R*	Leu288
2	TLR7	*G2955R*	Leu985
3	TLR8	*A135R*	Glu45
4	TLR8	*C336Y*	Tyr112
5	TLR8	*C741Y*	Ser247
6	TLR8	*G1272R*	Ser424
7	TLR8	*T1518Y*	Ser506
8	TLR8	*A1593R*	Thr531
9	TLR8	*G1641R*	Pro547
10	TLR8	*G1701R*	Thr567
11	TLR8	*T2373Y*	Thr791
12	TLR8	*C2523Y*	His841
13	TLR8	*G2898R*	Val966
14	TLR8	*G2961R*	Arg987

10 residues downstream to LRR4, respectively, while Lys818Arg was located in the C-terminal end of the TLR7 ectodomain. (Figs. 1 and 3). Two missense mutations, both of them extra-LRR, were found in TLR8 gene. Mutation at 165 amino acid position changing asparagine to lysine and mutation at 349 amino acid position changing tryptophan to histidine were located one amino acid residue downstream of LRR6 and seven amino acids upstream of LRR12, respectively (Table 2; Figs. 2 and 3). Silent mutations observed in TLR7 and TLR8 are presented in Table 3 and Figs. 1 and 2. Any missense mutation was observed neither in TM nor in TIR domain.

3.4. TLR mutations and susceptibility to MV infection

From total 48 targeted subjects, 37.6% were carrying Lys115Glu mutation (TLR7), from which 55.55% (*P* - 0.766) were MVV positive (OR - 1.43, CI - 0.35–5.78, in heterozygous form). Another mutation in TLR7 changing aspagarine to serine at 117 amino acid position was found in 41.7% (*P* - 0.380) of sheep of which 60% were found positive for MVV (OR - 2.0, CI - 0.51–8.02, in heterozygous form). We found no MVV negative animal carrying Ile73Leu in its homozygous or heterozygous state, thus it was not possible to calculate Odd's ratio. Only one MVV negative sheep carried Lys818Arg in homozygous state, whereas, among twelve sheep carrying this mutation in the heterozygous state 5 (41.66%, *P* - 0.739) was MVV positive. In case of TLR8 two missense mutations were found at the positions 165 (changing aspagarine to lysine) and 349 (changing tyrosine to histidine). Mutation Try349His was linked (OR - 1.84, CI - 0.28–14.61 in heterozygous form) with Maedi Visna infection (62.5%).

4. Discussion

Mutations trigger many disease conditions and alter the predisposition to several infections (Bochud et al., 2003, 2008; Fukusaki et al., 2007; Hong et al., 2007; Khor et al., 2007; Lakatos et al., 2005; Malley et al., 2003; Schroder and Schumann, 2005; Tabel et al., 2007; Thuong et al., 2007). Mapping of mutations is crucial in human medicine especially against familial genetic disorders. In veterinary medicine, mutation mapping can be employed in the breeding programs to increase the natural resistance of the herd. Hitherto, several studies reported mutations in TLR7 and TLR8 linked with disease conditions (Chen et al., 2008; Davila et al., 2008; Moller-Larsen et al., 2008; Oh et al., 2009, 2008; Schott et al., 2008; Ziaei et al., 2008). Till to date, no study reports comprehensive analysis of ovine TLR7 and TLR8 and mutations mapping.

Similar to ovine TLR7 and TLR8, 27 LRR motifs were also reported in other mammals (human, mouse, dog, pig and Takifugu rubripes) (Matsushima et al., 2007). Although irregularities were found within the LRR motifs of ovine TLR7 and TLR8 (Figs. 1 and 2), the residue change did not differ significantly from human and cattle. The first LRR at N-terminus was the most irregular motif in both ovine TLR7 and TLR8 with motif structure – (S/H)xx(A/L)xCxx(R/K)xL (Figs. 1 and 2). Interestingly this structure was also found in other animals (Matsushima et al., 2007).

LRR motif forms a loop structure and the juxtaposition of several loops produces solenoid-like shapes of ECD. The LRR consensus motif forms the inner core of horseshoe shaped ECD, while extra LRR regions form convex surfaces (Bell et al., 2003). Irregularities in the convex surface, for example a mutation at the residue downstream from the LRR motif, may affect PAMP (pathogen associated molecular pattern) binding onto the TLR horseshoe (Bell et al., 2003). Here we report, Lys115Glu and Asp117Ser, both extra-LRR mutations in ovine TLR7 and Tyr349His as extra-LRR mutation in ovine TLR8 (Table 2).

From the data obtained in the linkage analysis (Odd's ratio and exact Fisher's test), it can be concluded that the putative linkage found in our study is not definitive enough, while larger cohort should be examined to obtain conclusive association between mutations in TLRs and susceptibility to Maedi Visna infection.

Transmembrane motif structure is responsible for the localization as well as interaction with the accessory molecules like UNC93B for downstream signaling (Brinkmann et al., 2007; Nishiya et al., 2005). It is predicted that dog TLR4 has no transmembrane region (Matsushima et al., 2007). Here we have shown the presence of transmembrane region in both ovine TLR7 and TLR8. The transmembrane region is predicted to be a highly conservative region. So far, very few mutations were found in human (Beghdadi et al., 2009), rat and bovine TM region (Cargill and Womack, 2007). We found no mutation in the transmembrane region of the ovine TLR7 and TLR8, however, a predicted domain of TLR8 comprised of the 23 amino acid residues, 3 residues more than in human TLR8 TM. Multiple regions including ECD, TM and TIR of bovine TLR8 are involved in determining its localization in cellular endoplasmatic reticular compartment (Zhu et al., 2009). Considering the 100% sequence homology of transmembrane regions in ovine (predicted by us) and bovine TLR7 and TLR8 (Zhu et al., 2009) we propose their similar endosomal localization.

Functional studies confirmed that TIR domain plays a crucial role in MyD88 mediated downstream signaling. The TIR structure has a large conserved patch region (Xu et al., 2000). Mutations in TIR conserve region may abolish immune response (Ben-Ali et al., 2004; Berdeli et al., 2005; Xu et al., 2000). We found no missense mutation in TIR region (Figs. 1–3).

The differential susceptibility of sheep to Maedi Visna infection (maedi form of MVV) was observed already in 1947 in Iceland, where the crosses between Icelandic ewes and Border Leicester rams were less susceptible to this infection (Thormar, 2005). Moreover, Karacul rams did not show any signs of disease in Germany and Iceland (Straub, 2004). The comparative studies on purebred and crossbreed sheep showed distinct breed dependent susceptibility to the disease (Campbell et al., 1994; Lamontagne et al., 1983; Simard and Morley, 1991). Although, a number of studies have attempted to explain the differential resistance against Maedi Visna infection, none of them used genetic basis of the resistance. Based on the Maedi Visna prevalence found in this study it is evident that Tsigai sheep are relatively susceptible to MVV. Furthermore, our study indicates that individual susceptibility exists within the breed that may be defined by presence of the polymorphism in TLR genes.

It is important to note that sheep breeding programs are focused mainly on quantitative trade loci (production markers). Inbreeding is one of the choices frequently used to stabilize these markers in the population, what may have also resulted in the stabilization of deleterious recessive genes (Griffiths et al., 2007). Moreover, artificial insemination helps in spreading these deleterious markers.

Considering the highly conserved nature of TLRs the data acquired from *in silico* analysis of ovine TLRs, mutation mapping and linkage study may be the valuable benchmark for similar studies in other mammals including humans.

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