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Short communication

Sequence and functional variability of Toll-like receptor 9 gene in equines

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ABSTRACT

Significant structural differences in the extracellular domain of toll-like receptor 9 (TLR9) account for species-specific recognition of its ligand CpG-ODN sequences. TLR9 is extensively studied in human, mice and some domestic animals. The recognition ability appears to be utilized differently by various species and breeds, but so far no comprehensive study exists about the equine TLR9 gene. We characterized TLR9 sequences of Marwari and Zanskari breeds of horses and Poitu donkey. We sequenced and identified the protein coding regions of equine TLR9 and compared with other animals and human beings. Furthermore, we also analyzed the amino acid substitutions and their likely implications on functions. The analysis revealed 14% evolutionary divergence between equine and human TLR9, while it was 1% between the *Equus caballus* and *Equus asinus* and less than 1% within *Equus caballus*. In phylogenetic analysis of predicted amino acids, the indigenous equines grouped with thoroughbred *Equus caballus*, while human, cattle, dog, sheep, mice, and buffalo formed separate clades. Furthermore, we also analyzed the amino acid substitutions and their likely implications on functions by sorting intolerant from tolerant (SIFT) analysis and predicted two substitutions of amino acids (D80N and S822P) in Marwari horses in leucine rich repeat 1 (LRR1) without any functional effects. The substitutions (V214A and Y579C) in LRR 3 and LRR11 in Zanskari horses were predicted to have functional consequences. Out of overall 8 substitutions, three substitutions (I420V, S970R and R1001C) were found in *Equus asinus* in LRR7, LRR 13, and toll interleukin receptor (TIR) domains, while the substitution G649S is observed in Poitu donkey only. We report for the first time that despite the conserved residues, the striking effect of substitutions, found within the TLR9 genes of different equine breeds/species may have possible implications.

1. Introduction

Toll-like receptors (TLRs) are a family of proteins that constitute a phylogenetically ancient system that is expressed in both vertebrate and invertebrate species. The ligands for these receptors are components of pathogenic microbes called Pathogen Associated Molecular Patterns (PAMPs). Non-methylated CpG motifs present in viral and bacterial DNA are one of the PAMPs recognized by the mammalian innate immune system. TLR9 has been characterized as a receptor that recognizes unmethylated CpG motif and signaling by this receptor triggers a pro-inflammatory cytokine response that influences both innate and adaptive immune responses. It has been observed that subtle differences exist in different species in response to CpG-ODN. The sequences on either side of the transcribed region in CpG-ODN and the cellular patterning of TLR9 expression can impact the species-specific response to CpG-ODN (Griebel et al., 2005). Previously, we reported that CpG-stimulated horse peripheral blood mononuclear cells (PBMC) induce the expression of interferon-α (IFN-α), tumor necrosis factor-α (TNF-α), TLR9 and enhance immune response (Manuja et al., 2014a). We have also observed the augmented expression of proinflammatory cytokines IL-1, TNF-α, IFN-gamma and TLR9 when donkey's PBMC were stimulated with its ligand CpG-ODN C suggesting it as a potent inducer of innate immune response in donkeys as well. The cellular pattern of TLR9 expression and recruitment of specific cell population may be important factors contributing to interspecies differences in innate and adaptive immune response following CpG-ODN stimulation (Griebel et al., 2005). Significant structural differences in the extracellular domain of TLR9 have been implicated for species-specific recognition of CpG ODN sequences (Griebel et al., 2005). We also observed the dose-dependent proliferative responses of the horse PBMC upon stimulation with CpG-ODN and *Trypanosoma evansi* antigen (Manuja et al., 2014a).

TLR9 is extensively studied in human, mice and in some domestic animal species but there is no report of TLR9 characterization of Indian breeds of horses and donkeys. Zanskari horses have their origin in Great Himalayan range (India) and more adaptable for higher altitude whereas, the natural habitat of the Marwari breed is the Marwar region of the Rajasthan. The donkey or ass (*Equus asinus*) is a tamed animal of the family *Equidae* (Anon., 2018a, [Anon,2018b]b). Poitu, is a breed of

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Table 1
Primers for reverse transcription-polymerase chain reaction for amplification of equine Toll-like receptor 9 gene.

Gene	Primer	Sequence 5′-3′
TLR91	Forward	CTGATGAAGGGACTGCGA
TLR91	Reverse	TGGTAATTGAAGGACAAGTTGA
TLR92	Forward	CACTTTCCTGGCTGTACC
TLR9 2	Reverse	TCTCTGGCTGGACTGTTA
TLR9 3	Forward	CTCGGCATCTTCAAGGAC
TLR9 3	Reverse	AGCAGGAAGTCCACAAAG
TLR9 4	Forward	CAGGAACAGCATCATCTTC
TLR9 4	Reverse	CCAGTGGCATTTATTGAGT

donkey originating in the Poitu region of France and is one of the largest donkey breeds. Breed enthusiasts use them for agricultural work, driving, and riding (Svendsen, 1997). Although horses and donkeys belong to the same family *Equidae* and genus *Equus*, their phenotypic and genetic characteristics differ due to variations in gene sequences. Characterization and expression variability of TLR9 amongst different species of equines would help to understand the biological responses to its ligand (Anon., 2018c). Therefore, the aim of the present study was to analyze the polymorphism in TLR9 gene and variations in predicted amino acid profiles of horses and donkeys. We report for the first time, the substitutions indicating intraspecies polymorphism and their functional implications predicted by sorting intolerant from tolerant (SIFT) analysis. The possible underlying mechanisms for functional changes like change in polarity, charge, post translational modifications and structural architecture of functional domains like leucine rich repeats (LRRs) and toll-interleukin receptor (TIR) have been discussed.

2. Material and methods

2.1. Ethics statement

Sample collection from equines was performed strictly, as specified by Institutional Animal Ethics Committee of National Research Centre on Equines, Hisar, Haryana, India with minimal stress to animals with the approved study “Characterization of TLR9 and CpG immunomodulation in Equines”.

2.2. Isolation of PBMCs, RNA extraction and reverse transcription

Blood samples were collected from Marwari & Zanskari breeds of horse and donkey (Poitu). PBMCs were separated by density gradient centrifugation on histopaque (Sigma, St Louis, USA) as per manufacturer’s instructions. The cells were washed thrice with PBS by centrifuging at 200 *g* for 10 min at room temperature. The viable cells were counted by trypan blue dye exclusion technique, resuspended at 10^7 cells per ml in PBS and used for RNA extraction. Total RNA was extracted from 1×10^7 PBMCs using RNeasy kit (QiagenGmBH, Hilden, Germany), as per manufacturer’s instructions and treated with DNase I (Fermentas, Lithuania) onto the column in order to remove genomic DNA. The quality and quantity of RNA were determined by spectrophotometer by taking the absorbance at 260 nm and 280 nm. The RNA samples having A_{260}/A_{280} ratios from 1.9 to 2.1 were used in further experiments. Total RNA was used for first-strand synthesis using oligodT with reverse transcriptase enzyme as per manufacturer’s instructions.

2.3. Amplification, cloning, and sequencing of TLR9 transcripts

Overlapping sets of consensus primers were designed by aligning reported TLR9 mRNA sequences to amplify TLR9 sequence to get mRNA complete codons of equine TLR9 (Table 1). Amplicons from TLR9 of Marwari and Zanskari breeds of horse and donkey were cloned into pGEM-T easy vector and subsequently clones were screened by colony PCR. The plasmids obtained from positive clones were got sequenced using universal primers and submitted the sequences to GenBank database.

2.4. Amino acid sequences and predicted proteins

The nucleotide sequences of TLR9 of different species and the deduced amino acid sequences were retrieved from GenBank. The sequences were aligned by ClustalX and phylogenetic tree was constructed by the neighbor-joining method using MEGA 5. The evolutionary divergence between the nucleotide sequences of TLR9 and deduced amino acid sequences of related species were estimated (Tamura et al., 2011). The probability of substitution from one base to another base was also determined (Tamura et al., 2004). Amino acid substitutions were analyzed by multiple alignments in ClustalX and

Table 2
Estimates of evolutionary divergence between sequences of TLR9 gene. Percent distances of amino acid sequences of TLR9 gene of different species retrieved from GenBank.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
gi|AB445676| *Gorilla gorilla*																		
gi|DQ019999 *Homo sapiens*	0.01																	
NM_001081790 *Equuscaballus*	0.14	0.14																
NM_014638427.*Equus asinus*	0.14	0.14	0.01															
KT427379*Equuscaballus*Marwari	0.14	0.14	0.00	0.01														
KR052243 *Equuscaballus*Zanskari	0.14	0.14	0.00	0.01	0.00													
KR052244 *Equusasinus*DonkeyPoitu	0.14	0.14	0.01	0.00	0.01	0.01												
gi|AY859724| *Feliscatus*	0.15	0.14	0.11	0.10	0.11	0.11	0.10											
gi|AY859723|*Canisfamiliaris*	0.15	0.15	0.11	0.11	0.11	0.11	0.11	0.09										
XM_010985362 *Camelusdromedarius*	0.13	0.14	0.11	0.10	0.11	0.11	0.11	0.10	0.11									
XM_010969076*Camelusbactrianus*	0.14	0.14	0.11	0.11	0.11	0.11	0.11	0.10	0.11	0.00								
XM_006179985*Camelusferus*	0.14	0.14	0.11	0.11	0.11	0.11	0.11	0.10	0.11	0.00	0.00							
gi|GU451250| *Ovisaries*	0.17	0.17	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.12	0.12	0.12						
gi|EU747825| *Capra hircus*	0.18	0.17	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.12	0.12	0.12	0.02					
gi|EU747827| *Bubalusbubalis*	0.18	0.18	0.15	0.15	0.15	0.15	0.15	0.15	0.16	0.14	0.14	0.14	0.04	0.05				
gi|GQ922105| *Bosindicus*	0.18	0.17	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.14	0.14	0.14	0.04	0.04	0.03			
gi|NM_183081| *Bostaurus*	0.18	0.17	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.13	0.13	0.13	0.04	0.04	0.03	0.00		
gi|NM_213958| *Susscrofa*	0.15	0.15	0.12	0.12	0.12	0.12	0.12	0.13	0.13	0.10	0.10	0.10	0.13	0.13	0.15	0.14	0.14	
gi|AY859725| *Rattusnorvegicus*	0.26	0.26	0.26	0.26	0.26	0.26	0.26	0.26	0.25	0.26	0.26	0.26	0.28	0.28	0.30	0.29	0.29	0.26

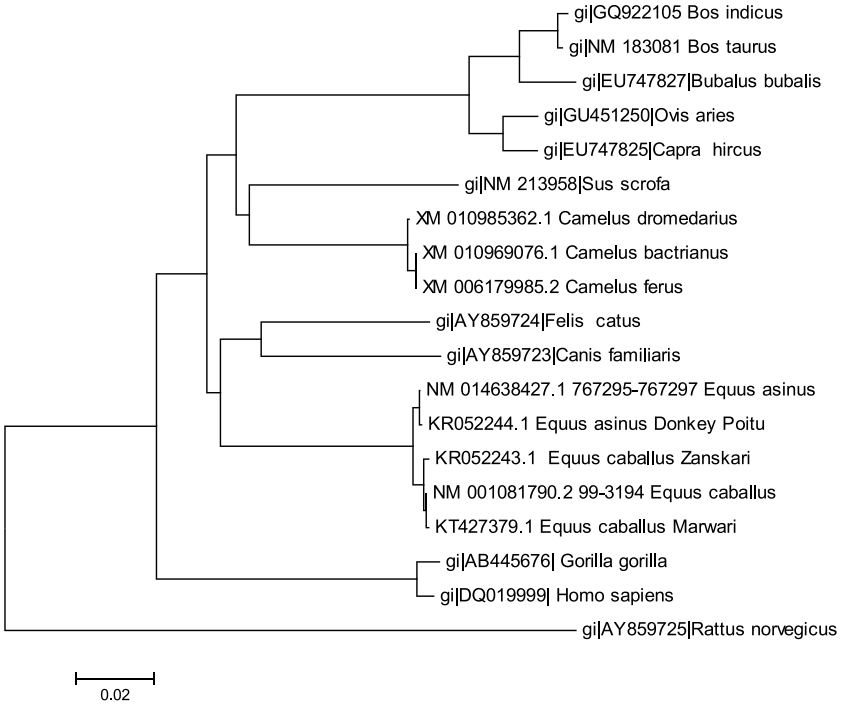

Fig. 1. Phylogenic tree of deduced amino acid sequences of TLR9 mRNA of *equine species* indicating genetic relationship with TLR9 gene sequences of different species retrieved from GenBank database (*Bos indicus*; *Bos taurus*; *Bubalus bubalis*, *Ovis aries; Capra hircus*; Sus scrofa; *Camelus dromedaries; Camelus bacterians; Camelus ferus; Felis catus; Equus asinus; Equus caballus; Gorilla gorilla*; *Homo sapiens*; *Rattus norvegicus.* Distances and groupings were determined using the MEGA 5 software by neighbor joining method. The veracity of these trees was studied using the bootstrapping method by executing 500 replicates.
The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.67415513 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Table 3
Maximum-Composite-Likelihood Estimate of the Pattern of Nucleotide Substitution.

	A	T	C	G
A	–	*3.07*	*5.35*	**20.99**
T	*2.56*	–	**22.96**	*4.64*
C	*2.56*	**13.18**	–	*4.64*
G	**11.6**	*3.07*	*5.35*	–

Each entry shows the probability of substitution (r) from one base (row) to another base (column)(Griebel et al., 2005). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in **bold** and those of transversionsal substitutions are shown in *italics*. The nucleotide frequencies are 16.41% (A), 19.66% (T/U), 29.69% (C), and 34.24% (G). The analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1589 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Manuja et al., 2014b).

graphically presented using BioEdit program. The online utility expasyprosite (http://prosite.expasy.org) was used for comparative prediction of protein domain architectures.

2.5. Amino acid substitutions and association with functional changes

Sorting intolerant from tolerant (SIFT) algorithm was used to assess the effect of amino acid substitutions on the function of a TLR9 protein from different equine species (Ng, 2003). Association of polymorphisms in TLR9 gene with functional changes was predicted using online prediction based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences through PSI-BLAST.

Predicted functional motifs such as glycosylation, phosphorylation, and cysteine residue sites were determined using the Center for Biological Sequence Analysis prediction service (http://www.cbs.dtu.dk/services/) and generunner program (http://www.generunner.net).

3. Result and discussion

3.1. TLR9 gene amplification and sequence

The representative equid TLR9 samples were cloned into PGEMT vector, sequenced and were submitted to GenBank. The overlapping sequences were joined to make contigs for Marwari horses (KT427379.1), Zanskari horses (KR052243) and Poitu donkey (KR052244.1) respectively. Blast analysis of TLR9 sequences of equines indicated that these are much closer to odd-toed ungulates e.g homo sapiens than to even-toed ungulates (buffalo, cattle etc.) (Supplementary file 1).

3.2. Sequence divergence and phylogenetic analysis

The nucleotide TLR9 sequences of Marwari horse (KT427379.1) and Zanskari horse (KR052243) and Poitu donkey (KR052244.1) and deduced amino acid sequences of TLR9 were obtained and analyzed following alignment as given in materials and method section. The pairwise distances calculated amongst amino acid sequences of *Equus* spp and the evolutionary distances computed using the Maximum Composite Likelihood method are in the units of the number of base substitutions per site. The analysis involved 19 nucleotide sequences. There was a total of 1589 positions in the final dataset.

Estimates /of evolutionary divergence between the sequences of TLR9 revealed *Equus caballus* (Marwari breed) and *Equus asinus* differ from human by 14%. Differences between the breeds are less than 1%. The evolutionary distances are shown in Table 2. Phylogenic tree based

on amino acid sequences of TLR9 from different species of equines in the present study and other species retrieved from GenBank is presented in Fig. 1. The TLR9 proteins from the present study clustered with *Equus caballus* protein sequences, while human, cattle, dog, sheep, mice, and buffalo formed separate clades. The analysis shows conserved sequences and close association of TLR9 proteins within species and high divergence with other species of animals.

Maximum Composite Likelihood estimated the pattern of nucleotide substitution revealing the rates of different transitional substitutions (shown bold) and transversional substitutions (shown *italics)* in Table 3. The nucleotide frequencies are 16.41% (A), 19.66% (T/U), 29.69% (C), and 34.24% (G). The analysis involved 19 nucleotide sequences.

3.3. Prediction of interacting proteins and functional characteristics

TLRs are type I transmembrane glycoproteins consists of an N-terminal signal peptide, an ectodomain containing tandem LRR consensus motifs defined by hydrophobic residues, a single transmembrane and a cytoplasmic region including a linker region, toll-interleukin 1 receptor domain, and a C-terminal tail region (West et al., 2006). TLRs contain multiple repeats that are protected by the special LRR-N-terminal end and LRR-C-terminal end motifs. TLRs bind a wide variety of pathogenic substances through their extracellular domains, which comprise 19–25 tandem copies of LRRs (Matsushima et al., 2007; Manuja et al., 2013). These LRRs are of differing size and abundance and involved in a wide variety of physiological processes. All proteins containing LRRs are thought to be involved in protein-protein interactions. In the present study, the online utility expasyprosite was used for comparative prediction of protein domain architectures. In our study, we found several substitutions within these positions (Fig. 2). Substitutions of amino acids in *Equus caballus* (Marwari and Zanskari breeds), *Equus asinus* compared to reference sequence of *Equus caballus* retrieved from the database are presented in Fig. 2 and Table 4.

The functional changes owing to amino acids substitutions in TLR9 protein were indicated by SIFT analysis using online prediction algorithm (Ng, 2003; Manuja et al., 2014b). As compared to the reference

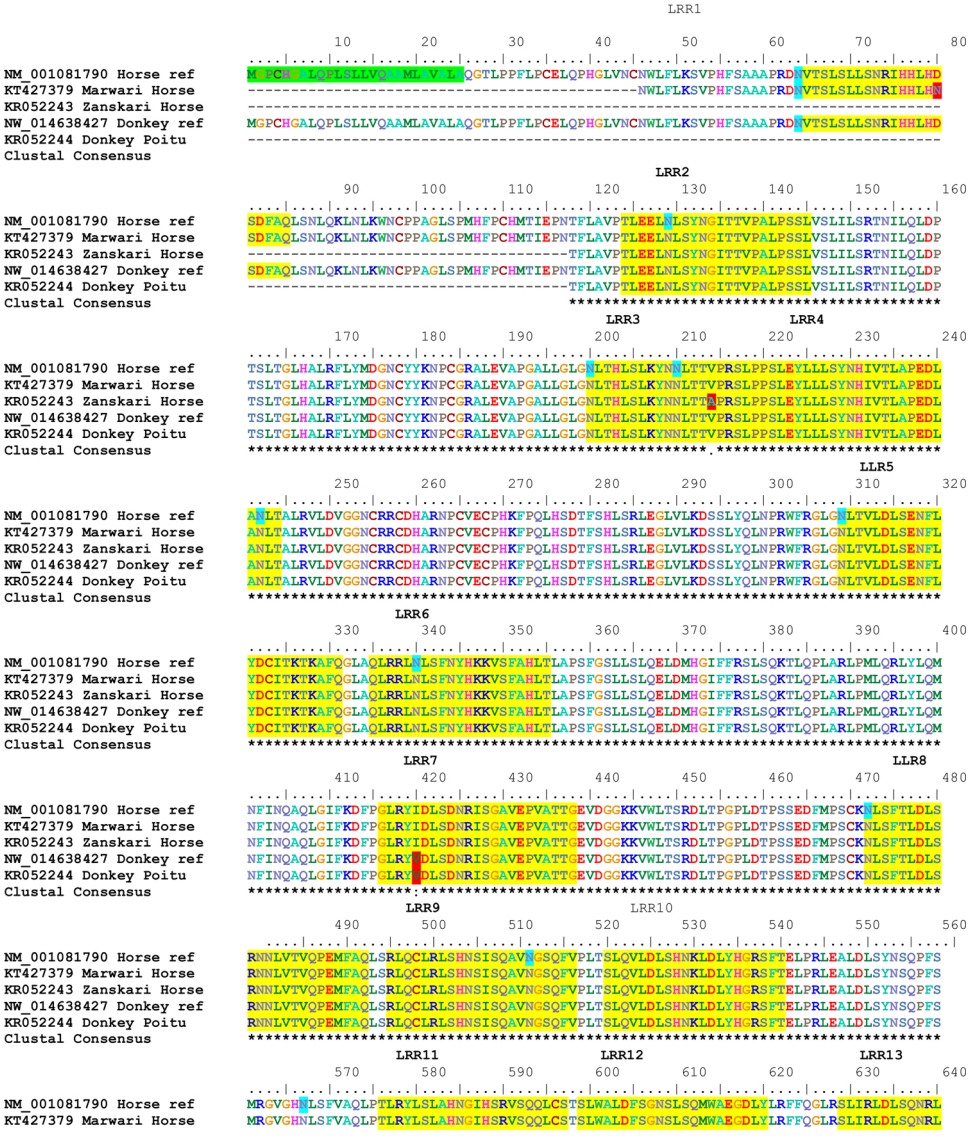

Fig. 2. Comparison of deduced amino acid sequence of TLR9 of Marwari breed, Zanskari breeds of horses (*Equus caballus*), breeds of donkey (*Equus asinus*) as compared to Ref seq (*Equus caballus* TLR9) showing substitution of amino acids (highlighted red). The amino acids coding LRR and TIR regions are highlighted in yellow. N glycosylation highlighted blue shows conserved residues. AA residues1 to 25 highlighted in green show signal transduction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

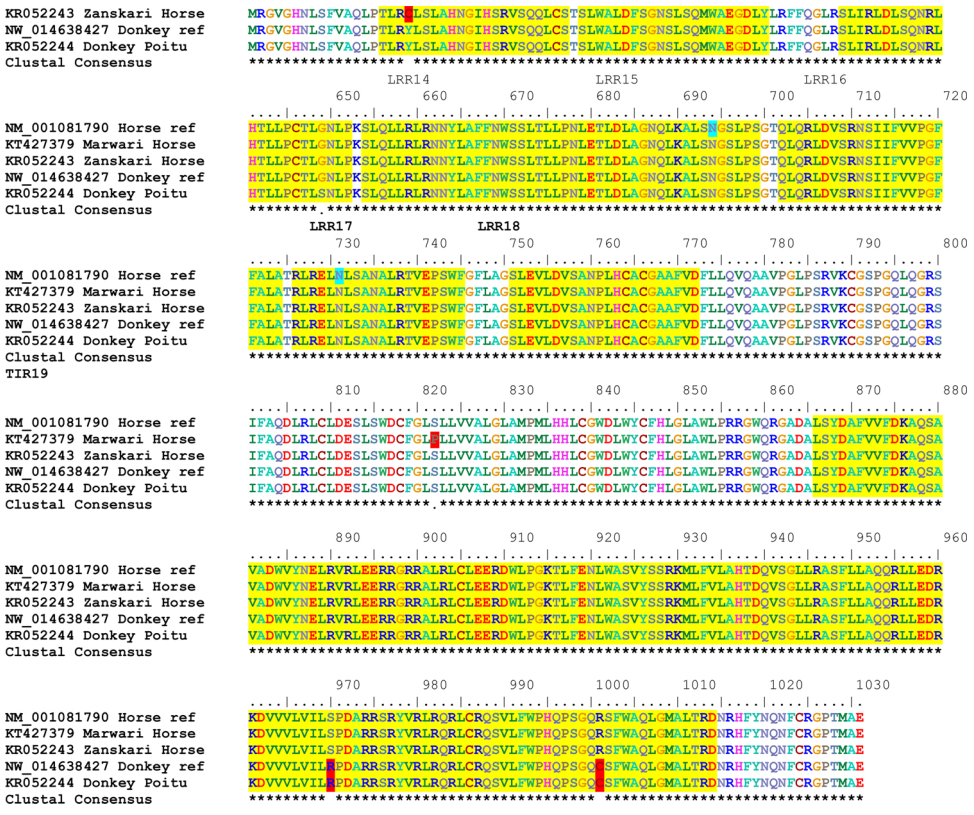

Fig. 2. (*continued*)

sequence of the horse, SIFT analysis predicted 2 tolerated substitutions of amino acids in Marwari horses D80 N in LRR1 and S822 P (Table 4). The 2 substitutions (V214 A and Y579C) in LRR 3 and LRR11 in Zanskari horses have functional consequences on the protein. A total of four substitutions were found in *Equus asinus* Poitu donkey (I420 V, G649S, S970 R and R1001C) in LRR7, LRR 13, and TIR. The three AA substitutions in LRR7 and TIR (I420 V S970R and R1001C) were common in reference *Equus asinus* and Poitu donkey.

3.4. Polarity and charge change

From 8 A A variations amongst reference sequence and other equids, 10 residues were changing their polarity, 2 of which were localized in TIR domain while 3 were localized within the LRR motifs (Table 4). Amino acids were changing from polar to non-polar in 2 cases and from non-polar to polar in 1 case while other changes were either polar to basic polar or acid polar to polar. Hydrophilic conserved residue contributes towards the rigidity of the structure of LRR, on the other hand, hydrophobic residues of LRRs are responsible for ligand binding (Matsushima et al., 2007; Werling et al., 2009; Mikula, 2011). The polarity of the side chain of amino acids has an impact on the hydrophilic or hydrophobic property of a protein and may result in a varied protein structure and protein-protein interactions (Hogg, 2003). In this study, the AA substitutions provide the neutral charge in 10 substitutions (Table 4). We observed neutral to basic pH change only in AA substitution S970R. The substitution S to R has been reported to affect the binding affinity of TLR9. It decreases with the increase in pH (Ohto et al., 2015). Therefore, this substitution suggests enhancing affinity of TLR9 to its ligands.

The amino acid substitution V214 A in Zanskari horse TLR9 led to no change in polarity, class, and charge but the SIFT analysis predicted change in functional characteristics. The substitutions I420 V, S970R and R1001C were observed in reference *Equus asinus* while G649S was observed in *Equus asinus* Poitu donkey only.

3.5. Change in residue/protein potential

The amino acid substitution V214 A in Zanskari breed has a potential to affect the function as predicted by SIFT analysis. Since Valine is hydrophobic, it prefers to be covered in protein hydrophobic cores (Betts and Russell, 2007). Although both valine and alanine are aliphatic, but hydrophobicity intensifies with increasing number of Carbon atoms in the hydrocarbon chain. The change of residue valine to alanine provides more rigidity being less hydophobic as compared to valine due to its short side chain. The aliphatic side chains being non-reactive are hardly involved directly in protein function, however, they play a role in substrate recognition. In particular, hydrophobic amino acids can be involved in binding/recognition of hydrophobic ligands such as lipids (Betts and Russell, 2007). Another amino acid substitution Y579C (tyrosine to cysteine) in Zanskari breed may also affects the function as predicted by SIFT analysis. Being partially hydrophobic, tyrosine choose to be hidden in protein hydrophobic cores (Betts and Russell, 2007). The aromatic side chain can also mean that tyrosine is involved in noncovalent interactions with other aromatic side chains. A common role for tyrosines within intracellular proteins is phosphorylation. Protein kinases often attach phosphates to these three residues as component of a signal transduction process. In a study, single tyrosine mutation (Y888) in the cytoplasmic tail of TLR9 selectively

Table 4
Changes in amino acid residues, polarity, charge, class in equine Toll-like receptor 9 protein in comparison to the reference Equus caballus Toll-like receptor 9 protein.

Amino acid Position	Substitutions	Functional characteristics	Polarity	Class	Charge	species
D80N	Aspartic acid/Asparagine	Tolerated	A P to P	acid to amide	negative to neutral	Marwari horse
S822P	Serine/ Histidine	Tolerated	P to NP	hydroxyl-containing to cyclic	neutral no change in pH	Marwari horse
V214A	Valine/Alanine	Affect protein function	NP (no change)	Aliphatic (no change)	neutral no change	Zanskari horse
Y579C	Tyrosine/ Cysteine	Affect protein function	P to NP	Aromatic to sulfur-containing	neutral no change	Zanskari horse
S970R	Serine/ Arginine	Tolerated	P to Basic polar,	hydroxyl-containing to Basic	neutral to positive pH (basic)	Poitu donkey and donkey (ref seq *Equus asinus*)
I420V	Isoleucine/ Valine	Tolerated	NP (no change)	Aliphatic (no change)	neutral no change	Poitu donkey and donkey (ref seq *Equus asinus*)
R1001C	Arginine/Cysteine	Tolerated	Basic polar to NP	Basic to sulfur-containing	positive pH (basic) to neutral	Poitu donkey and donkey (ref seq *Equus asinus*)
G649S	Glycine/ Serine	Tolerated	NP to P	Aliphatic to hydroxyl-containing	neutral to neutral	Poitu donkey

P; polar; NP; non polar.

impairs TNF production, but not IFN production (Chockalingam et al., 2012). Tyrosine has large side chain which may encounter folding difficulties as compared to cysteine which has small side chains. Moreover cysteine belongs to sulfur containing side chain class and its residues are also related to the formation of disulfide bonds that take part in the regulation of protein folding, stability, and activity (Hogg, 2003; Singha et al., 2015). Cysteines are also frequently involved in protein active and binding sites. Binding to metals can also be crucial in enzymatic functions (e.g. metal proteases) (Betts and Russell, 2007). Cysteine can also act as a nucleophile (i.e. the reactive centre of an enzyme). Probably the best-known example of this occurs within the cysteine proteases, like papains or caspases, where cysteine is the key catalytic residue (Betts and Russell, 2007). Indeed, it provides the insights into possible impact on the functional variations in these species.

3.6. Prediction of glycosylation and phosphorylation

To gain a further insight into the structural and functional conservation of equine TLR9, the positions of glycosylation and phosphorylation motifs were predicted. The biological role of N-linked glycosylation is protein folding, metabolism, transport, and maintenance of cell and protein structure (Hunter, 2000). However, we observed conserved N-linked glycosylation in the different species of equids irrespective of breed and species analyzed in the present study (Fig. 2). Protein phosphorylation on Ser, Thr and Tyr residues is the most ubiquitous post-translational modifications (PTM) and plays a crucial role in regulating protein functions in all living organisms ranging from prokaryotes to eukaryotes (Hunter, 2000; Manning et al., 2002). We have observed lots of phosphorylation sites in the equine TLR9. Fig. 3 shows sequence positions of phosphorylation potential with serine, threonine, tyrosine. Highly conserved glycosylation and phosphorylation sites in the TLR9 protein indicate conservation of tertiary structure and functional integrity.

In conclusion, we characterized TLR9 sequences of horses (Marwari and Zanskari breeds) and donkeys. The analysis shows conserved sequences and close association of TLR9 proteins within species and high divergence with other species of animals. TLR9 sequences of equines are much closer to odd-toed ungulates e.g homo sapiens than to even-toed ungulates like bovines, ovines etc. Regarding interspecies and intraspecies amino acid differences in equids, the substitutions specific to species and breed were determined along with their predicted functional characteristics. One residue change specific to Zanskari breed describing no change in either polarity, class, and charge, however, it affects the functional characteristics as predicted by SIFT. We observed conserved N-linked glycosylation and lots of conserved phosphorylations sites in the different species of equines irrespective of breed and species.

Further work regarding the sensitivity with which TLR9 recognizes its target and its association with diseases need to be explored. The characterization and functional analysis of equine TLR9 in this study may provide the first step to understanding the equine TLR9 system. Moreover, despite conserved residues, the striking effect of substitutions found within the TLR9 genes of different equine breeds/species, may have possible implications on the concluding results, which needs to be elucidated in further studies. Comprehensive validation of these functional changes in terms of how these structural parameters correlate with the TLR9 activation of unique host responses is further required.

Contribution

AM and BKM design the experiment; AM, BKM, HS performed the experiments; AM and BKM analyzed the data; AM wrote the article; BKM edited the article.

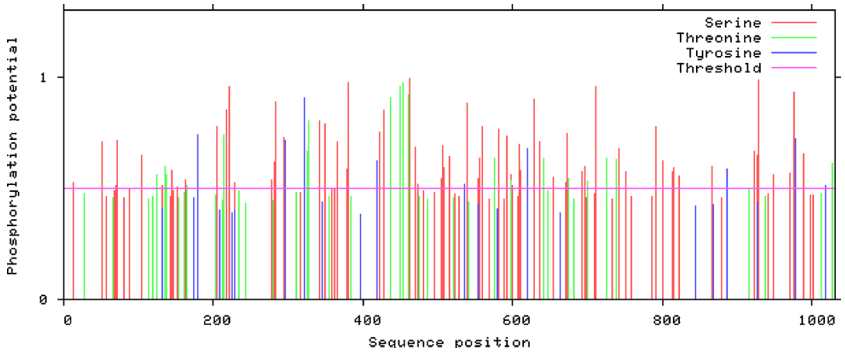

Fig. 3. Predicted potential phosphorylation sites in *Equus* sp. showing amino acid residues serine, threonine and tyrosine.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2018.10.010.

References

http://eol.org/pages/328647/details as Assessed on 2 August 2017.
http://Schools-wikipedia.org/wp/d/Donkey.htm as Assessed on 2 August 2017.
http://nrce.gov.in/downloads/NRCE/AnnualReport/2009-10; 2013-14 as Assessed on 2 August 2017.
Betts, M.J., Russell, R.B., 2007. Amino-acid properties and consequences of substitutions. Bioinf. Genet. 13 (March2), 311–339.
Chockalingam, A., Rose, W.A., Hasan, M., Ju, C.H., Leifer, C.A., 2012. Cutting edge: a TLR9 cytoplasmic tyrosine motif is selectively required for proinflammatory cytokine production. J. Immunol. 15-188 (2), 527–530.
Griebel, P.J., Brownlie, R., Manuja, A., Nichani, A., Mookherjee, N., Popowych, Y., Mutwiri, G., Hecker, R., Babiuk, L.A., 2005. Bovine toll- like receptor 9: a comparative analysis of molecular structure, function and expression. Vet. Immunol. Immunopathol. 108, 11–16.
Hogg, P.J., 2003. Disulfide bonds as switches for protein function. Trends in Biochemical Science 28 (4), 210–214.
Hunter, T., 2000. Signaling—2000 and beyond. Cell 100 (1), 113–127.
Manning, G., Whyte, D.B., Martinez, R., Hunter, T., Sudarsanam, S., 2002. The protein kinase complement of the human genome. Science 2000-298 (5600), 1912–1934.
Manuja, A., Manuja, B.K., Kataria, R., Sethi, R.K., Singh, R.K., 2013. Comparative analysis of molecular structure, function and expression of Buffalo (Bubalus bub alis)toll-like receptor 9. J. Buffalo Sci. 2, 63–71.
Manuja, A., Kumar, P., Kumar, R., Kumar, B., Singha, H., Sharma, R.K., Yadav, S.C., 2014a. CpG-ODN class C-mediated immunostimulation and its potential against *Trypanosoma evansi* in equines. Int. Immunopharmacol. 24-22 (2), 366–370 2014.
Manuja, B.K., Manuja, A., Dahiya, R., Singh, S., Sharma, R.C., Gahlot, S.K., 2014b. Diversity of interferon inducible Mx gene in horses and association of variations with susceptibility vis-à-vis resistance against equine influenza infection. Infect. Genet. Evol. 27C, 142–148.
Matsushima, N., Tanaka, T., Enkhbayar, P., Mikami, T., Taga, M., Yamada, K., et al., 2007. Comparative sequence analysis of leucine rich repeats (LRRs) within vertebrate toll-like receptors. BMC Genomics 8, 124.
Mikula, I., 2011. Characterization of ovine Toll-like receptor 9 protein coding region, comparative analysis, detection of mutations and maedi visna infection. Dev. Comp. Immunol. 35 (2), 182–192.
Ng, P.C., 2003. Henikoff S SIFT: predicting amino acid changes that affect protein function. Nucleic Acids Res. 31 (13), 3812–3814.
Ohto, U., Shibata, T., Tanji, H., Ishida, H., Krayukhina, E., Uchiyama, S., Miyake, K., Shimizu, T., 2015. Structural basis of CpG and inhibitory DNA recognition by Toll-like receptor 9. Nature 30-520 (7549), 702–705.
Singha, H., Goyal, S., Malik, P., 2015. Cloning and sequence analysis of Interleukin (IL)-2, IL-4, IL-10, and IL-18 of Marwari horse. Indian J. Anim. Sci. 85 (6), 621–627.
Svendsen, Elisabeth, 1997. Donkeys abroad. In: Svendsen, Elisabeth (Ed.), The Professional Handbook of the Donkey, 3rd edition. Whittet Books, London, pp. 166–182 ISBN 978-1-873580-37-0.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using mximum likelihood, evolutionary distance and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.
Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbour-joining method. Proc. Natl. Acad. Sci. (U. S. A.) 101, 11030–11035.
Werling, D., Jann, O.C., Offord, V., Glass, E.J., Coffey, T.J., 2009. Variation matters: TLR structure and species-specific pathogen recognition. Trends Immunol. 30, 124–130.
West, P.A., Koblansky, A.A., Ghosh, S., 2006. Recognition and signaling by toll-like receptors. Ann. Rev. Cell Dev. Biol. 22, 409–437.