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Molecular evolution and structural analysis of Caprine CD14 deduced from cDNA clones

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ABSTRACT

The study was conducted to characterize CD14 gene of goat (*Capra hircus*), an important molecule for innate immunity that can act against a wide range of pathogens. Cloning and sequence analysis of CD14 cDNA revealed 1, 119 nucleotide long open reading frame encoding 373 amino acids protein and 20 amino acids signal peptide. Caprine CD14 peptide (gene bank protein ID: ABE 68725.1) exhibited a high percentage of peptide identity (54.2–87.2%) with the corresponding mammalian homologs. In caprine CD14 68 distinguished nucleotide changes were identified and the site for mutational hot-spot was detected in caprine CD14 gene. Non-synonymous substitutions exceeding synonymous substitutions indicated the evolution of this protein through positive selection among domestic animals. Predicted protein structures obtained from deduced amino acid sequence indicated caprine CD14 molecule to be a receptor with horse shoe-shaped structure when exists as dimer and many grooves responsible for ligand binding.These studies revealed CD14 molecule of goat as a receptor with amino terminal pocket as the site for LPS binding. The sites for LPS binding, LPS signalling, leucine-rich repeats, putative N-linked glycosylation, O-linked glycosylation, glycosyl phosphatidyl inositol anchor, disulphide bridges, alpha helix, beta strand, leucine rich nuclear export signal, leucine zipper and domain linker were predicted. Most of leucine and cysteine residues remain conserved across the species.

Key words: 3-D structure, Bioinformatics, CD14 gene, Goat, Molecular evolution, SNP, Trans-species polymorphism

CD14 is the most important molecule known so far for immunity, binds mainly with LPS (lipopolysaccharide), lipotechoic acid, arachidonic acid and thus releases various cytokines which act for body's defense (Yu *et al.* 1997). Future application for CD14 gene includes Recombinant protein as therapeutic agent; cloned gene for somatic gene therapy, transgenic disease resistant animals, detection of SNP and marker assisted selection. It has been considered as an important molecule influencing various diseases, like mastitis (Lee *et al.* 2003), treponemiasis (Schroder *et al.* 2000) and glomerulonephritis (Yoon *et al.* 2003). India with 126 million goats (14.31% of the world of goat population), ranks second in the world (Annonymous 2011). Black Bengal goat is an important breed of West Bengal with the unique trait of high fecundity, good meat quality and excellent skin quality, but are susceptible to disease incidences, especially PPR, which causes economic losses of 1, 800 million Indian rupees (US$39 million) every year in India (Singh 2011).

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Keeping the above points in view, the present investigation was planned to clone and sequence the CD14 gene of goat, study of CD14 derived peptide using bioinformatics tools, to compare the sequence homology with other species and to identify the SNP of CD14 gene of Black Bengal goat.

MATERIALS AND METHOD

Molecular cloning and characterization of CD14 gene of goat

Animals, sample collection, and RNA isolation: Goat mRNA was isolated from liver tissue of Black Bengal breed of goat, using oligotex mRNA isolation kit. The liver tissue from the Black Bengal goat was collected during the slaughter of goat from village of Dubrajpur Block, Birbhum District, West Bengal, India. The first strand cDNA from liver was synthesized from purified mRNA by RT-PCR (reverse transcription PCR) using oligo dT primer using reverse transcriptase kit.

PCR amplification: To amplify the full length open reading frame (ORF) of CD14 gene sequence, a specific primer pair was designed based on the CD14 mRNA sequences of cattle (Accession no. AF141313) by DNASIS MAX software. The fragment for CD14 gene was amplified with ATGGTCTGCGTGCCCTACCTG as forward primer

and GGAGCCCGAGGCTTCGCGTAA as reverse primer. PCR reaction mixture and cycling conditions were similar to that followed in CD14 gene of CB cattle (Pal *et al.* 2011).

cDNA cloning and sequencing: PCR amplicons verified by 1% agarose gel electrophoresis were purified from gel using Gel extraction kit and ligated into pGEM-T easy cloning vector using the procedure followed by Pal *et al.* (2011). CD14 gene fragment insert in recombinant plasmid was sequenced by automated sequencer (ABI prism) using dideoxy chain termination method with T7 and SP6 primers.

Sequence analysis: The nucleotide sequence so obtained was analyzed for protein translation, sequence alignments, and contigs comparisons by DNASTAR Version 4.0. Novel sequence was submitted to the NCBI Genebank and accession number was obtained which is available in public domain now.

Study of predicted goat CD14 protein using bioinformatics tools

Predicted peptide sequence of CD14 gene of goat was derived by Edit sequence Programme of Lasergene Software (DNASTAR). Predicted peptide sequence of CD14 gene of goat was then aligned with the CD14 peptide of other livestock and avian using Megalign sequence Programme of Lasergene Software (DNASTAR). Prediction of signal peptide of CD14 gene was conducted using the software, Signal P 3.0 Sewer-prediction results. Leucine percentage was calculated manually from predicted peptide sequence. Prediction of di-sulphide bridges between cysteine residues were conducted by homology search with other species (Kim *et al.* 2005, Pal *et al.* 2011) and suitable software (http: //bioinformatics.bc.edu/clotelab/DiANNA).

Protein level study was carried out with specific software (www.expasy.org./tools/blast) for determination of leucine rich repeats (LRR), leucine zipper, N-linked glycosylation sites, detection of leucine rich nuclear export signals (NES) and detection of the position of GPI anchor. Detection of leucine rich nuclear export signals (NES) was carried out with NetNES 1.1 Server. Analysis of O-linked glycosylation sites were carried out using NetOGlyc 3.1 server (www.expassy.com), whereas N-linked glycosylation site were detected by NetNGlyc 1.0 software ((www.expassy.com)). Regions for alpha – helix and beta sheet were predicted using NetSurfP- protein surface accessibility and secondary structure prediction. Domain linker prediction was done according to the software developed by expasy proteomics server. LPS binding site as well as LPS signaling sites were predicted based on homology studies with other species. 3-D model of CD14 polypeptide was predicted based on Swissmodel repository.

Molecular evolution and SNP study of caprine CD14 gene: SNP study in CD14 gene in goat has been conducted based on the CD14 sequences of other ruminants using DNASTAR software.

RESULTS AND DISCUSSION

Molecular cloning and characterization of CD14 gene of goat: After sequencing of a selected representative clone, the DNA insert of CD14 gene was found to be exactly 1122 bp with ATG as start codon followed by an open reading frame of 1119 nucleotides and TAA as stop codon (Fig. 1). Similar findings were observed for cattle (Pal *et al.* 2011) and buffalo (Pal 2006). GC content of goat CD14 gene was high (62.2%), almost similar to buffalo (62.3%) and less than cattle (62.75%) and human (62.94%) to some extent. CD14 gene of goat is much less than dog (64.07%) and much higher than rat (56.12%)

Study of predicted goat CD 14 peptide using bioinformatics tools: Goat CD14 derived peptide (Mol wt. 39929.42 Da) is characterized by 35 strongly basic, 31strongly acidic, 146 hydrophobic and 90 polar amino acids (Fig. 2). Derived CD14 peptide of goat has molecular weight of 39929.42 D, which is slightly higher than that of buffalo (Pal 2006) and slightly lower than that of crossbred cattle (Pal *et al.* 2011).

Prediction of amino acid sequence from CD14 gene sequence revealed 373 amino acids precursor which corresponds to the coding sequence of the gene and a signal peptide consisting of 20 amino acids (Figs 2, 3). This is similar to bovine CD14 peptide consisting of 373 amino acids (Pal *et al.* 2011). This is different from mouse (351aa) and human (356 aa) as reported by Setuguchi *et al.*(1989) and rat (300 aa) as reported by Takai *et al.* (1997). However, rat contains a predicted 17- amino acid signal peptide (Takai *et al.* 1997).

Comparison of goat CD14 peptide (Table 1, Fig. 3) with buffalo CD14 and cattle revealed that buffalo CD14 has 6 leucine rich repeats (Pal 2006), which is less than goat, whereas cattle had nine LRR (Pal *et al.* 2011). Thus, the present finding is in agreement with the reports of LRR in other species such as human, mouse and cattle. These LRRs are considered to participate in receptor and ligand interactions and have various cellular functions during early phases of the immune response. Sufficient variabilty in the pathogen recognition and receptor binding site, which is primarily comprised of LRR region of the CD14 molecule may explain between species variability for the number of LRR. Although there are no reports regarding the number of LRR and its relation to disease susceptibility, a trend has been observed with more the animal is resistant, less the number of LRR. Buffaloes were reported to have least number of LRR among the species under study. As reported from a separate study, buffalo is most resistant (Pal and Chatterjee 2010b) among common farm animals, viz. cattle, goat, sheep and buffalo. So, it may be possible that lesser the species possess number of LRR, more it is resistant to diseases. However further studies are needed for its confirmation.

Goat CD14 molecule was predicted to contain 15.5% leucine. This is much less than mouse (17.66%, Setuguchi *et al.* 1989). However leucine percentage was reported to be

Table 1. Leucine rich repeats detected in derived peptide sequence of CD14 gene of goat

repeat	from	to	fragment	score
LRR	86	107	KALRVRRLKLGAA. QVPAQLLVA	1456
LRR	114	138	YSRLKELTLEDLEV TGPTPPAPLEA	1502
LRR	168	191	KPGLRALNIAQAHSL AFPCAGLST	1600
LRR	192	221	FEALTTLDLSDNPSL GDSGLMAALCPNKFR	1910
LRR	245	270	AARVQPQNLDLSHNS LRVTAPGATRC	1950
LRR	273	294	PSAISSLNCSFAGLEQ VPKGLP	1740
LRR	295	318	PKLSVLDLSCNKLSR EPRRDELPY	2290

17.2% for buffalo (Pal 2006). The alignment report suggested that most of leucine residues were conserved among all the species (Fig. 3). Similar report was also in CD14 gene of rat (Takai *et al.* 1997) and cattle (Pal *et al.* 2011).

Cysteine residues present in goat CD14 gene are also conserved mostly, except amino acid position 190, where cysteine is replaced by threonine in dog and amino acid position 309, where cysteine is replaced by tyrosine in mouse and rat (Fig. 3).

The predicted peptide sequence of CD14 gene of goat (gene bank protein ID: ABE 68725.1) revealed 86.9%, 87.2, 57.9, 55.9, and 54.2% amino acid identity with buffalo, cattle, dog, mouse and rat.

Disulphide bridges are responsible for protein folding. Protein folding in turn gives rise to groove formation for binding site for ligand. Five disulphide bridges were predicted among cysteine residues in CD14 polypeptide of goat of which only 4 were visualized in Fig. 3. Since Cys287 is a tyrosine in the mouse CD14 sequence, in the homology study of CD14 sequences for different species (Fig. 3), the fifth disulphide bond is not visualized. Number of di-sulphide bonds in cattle (Pal *et al.* 2011) and mice (Kim *et al.* 2005) were reported to be 5 and 4 respectively.

Four LPS binding site as depicted from 29th -32nd, 44th–47th, 55th–59th and 75th –80th amino acid positions (Fig. 3). Three LPS signalling sites were predicted for amino acid positions as 27th -33th, 109–119th, 169–171th, based on homology studies with other species. Similar LPS binding site and LPS signaling site were reported in cattle (Pal *et al.* 2011) and mice (Kim *et al.*2005). LPS binding site is in agreement to present study that N-terminal region of CD14 molecule is responsible for LPS binding (Viriyakosol *et al*. 1995). LPS binding site is contained in the pocket formed at amino terminal end of CD14 molecule of goat.

GPI anchor is vital for the molecule as the basic function of GPI in mammalian cell system is to make a bridge between CD14 and cell surface. In goat CD14, a glycosyl phosphatidyl inositol (GPI) anchor was located at C-terminus near 344th position of the CD14 molecule. GPI anchor was located at C-terminus near 353th and 345th position of the CD14 molecule in buffalo (Pal 2006) and cattle (Pal *et al.* 2011) respectively. GPI- anchor is a feature particular to mammals. However in case of avains, CD14 is trans-membrane rather than GPI anchored (Wu *et al.* 2009).

N-linked glycosylation is vital for the molecule as it supports the molecule to be present either in membranous or soluble form and the folding of eukaryotic proteins. The CD14 derived peptide sequence of goat contained 3 putative N-linked glycosylation sites (Table 2, Fig. 1; suppl.). There is report of 3, 5, 5 and 4 glycosylation sites in cattle (Pal *et al.* 2011), mice (Julius *et al.* 2002), rat (Takai *et al.* 1997) and buffalo (Pal 2006) respectively. There was no change at the position of N-linked glycosylation sites of goat CD14 predicted peptide sequence with other species, otherwise differences in the position of N-linked glycosylation might lead to functional alterations.

Glycosylation is needed when hydrophilic clusters of carbohydrates alter the polarity and solubility of protein or protein folding. Five sites for O-linked glycosylation were detected at amino acid positions 128, 131, 139, 144 and 145 (Fig. 2; Suppl.). Similar 5 sites for O-linked glycosylation were detected in cattle (Pal *et al.* 2011).

Leucine zipper pattern was found at amino acid position 279th to 300th amino acid position in goat CD14 peptide (Fig. 3, Fig. 3; Suppl.). A leucine zipper or leucine scissors, is a common 3-dimensional structural motif in proteins. This gives an indication that in functional form CD14 molecule of goat exists as a dimer and acts as receptor. Similar dimer form of CD14 molecule of mice (Kim *et al.* 2005) and cattle (Pal *et al.* 2011) was reported which acted as receptor.

Leucine rich nuclear export signals (NES) were detected at amino acid positions 15, 16, 17, 20, 117, 122, and 127 of goat CD14 peptide (Fig. 4; Suppl.). A nuclear export signal (NES) is a short amino acid sequence of 4 hydrophobic residues in a protein that targets it for export from the cell nucleus to the cytoplasm through the nuclear pore complex. Similar studies were reported in cattle (Pal *et al.* 2011).

In CD14 polypeptide of goat, 5 sites of alpha helix were predicted for amino acid positions 4–18, 46–52, 81–84, 241–244 and 361–370.Twelve sites for beta strand were predicted for amino acid positions 26–27, 34–37, 59–60, 118–122, 145–147, 173–176, 181–182, 197–199, 225–228, 252–254, 299–301, 321–323 and rest region as coil (Fig. 4). However in mice, 7 alpha helix and 13 beta sheets were identified (Kim *et al.* 2005), whereas in cattle, 5 regions were detected for a helix, 11 regions were identified for ß strand conformation were identified (Pal *et al.* 2011).

Domain linker site was predicted for amino acid positions 121–150 and 283–334 (Fig. 5). Domain linker sequences are loop sequences connecting two structural domains and linker

Table 2. N-linked glycosylation sites for derived CD14 peptide of goat

Nucleotide position	Nucleotide in goat	Nucleotide in other ruminants	Type of mutation	Remarks	Resulting amino acid change
37	T	C	Transition		S/P
40	G	C/T	Transversion	C in goat, sheep and Buffalo T in cattle	A/P in sheep & buffalo, S in cattle
55	A	T	Transversion		T/S
65	A	C	Transversion		K/T
68	G	C	Transversion		R/T
87	T	C	Transition		Synm
88–91	CCAC	GACG	Transversion		P/D
93–94	GC	CG	Inversion		Q/DH/D
102	C	T	Transition	C in goat and sheepT in cattle and buffalo	Synm
358	C	T/C	Transition	T in sheepC in cattle and buffalo	Synm
401	G	A	Transition	A/T in sheep and cattleK in buffalo	
453	G	A/G	Transition	A in sheep, cattle and buffaloG in big horn sheep	Synm
457	G	T	Transversion		G/W
459	C	G	Transversion		G/W
460	A	G	Transition	G in sheep and buffaloA in cattle	T/A in sheep and buffalo. T in cattle
494	C	A	Transversion		P/Q
495	A	G	Transition		P/Q
518	C	T	Transition	T in cattle and buffaloC in sheep	A/VV in cattle and buffalo A in sheep
519	G	C	Transversion	G in cattle and buffalo, C in sheep	A/VV in cattle and buffalo A in sheep
555	C	G	Transversion		synm
662	G	C	Transversion		R/P
668–669	CT	TC	Inversion		P/L
676	T	C	Transition		Synm
703	G	A/T	Transition	A in cattle and sheepT in buffalo	A/T in sheep and cattle, S in buffalo
706	G	C	Transversion		A/P in cattle and sheep, L in buffalo
710	C	G	Transversion		T/S
712	C	G	Transversion		R/G
715	C	G	Transversion		P/V
716	C	T	Transition		P/V
724	C	G	Transversion		P/A
755	A	G	Transition		N/S
825–826	CA	AC	Inversion		Synm, I/L
842–843	TG	GT	Inversion		C/L
873	A	G	Transition	G in cattle and buffaloA in sheep	synm
952	T	G	Transversion		Y/E
954	T	G	Transversion		Y/E
970	G	C	Transversion		V/L
973	A	G	Transition		N/D
1033	C	G	Transversion		R/G
1035	T	C	Transition		R/G
1036	A	G	Transition		M/V
1039	A	G	Transition		I/V
1041	T	C	Transition		I/V
1046	A	C	Transversion		D/A
1048	C	T	Transition		R/C
1053	C	G	Transversion		synm
1055	C	G	Transversion		P/R
1056	C	T	Transition		P/R
1057	C	T	Transition		P/S in buffalo and sheep, F in cattle
1059	A	G/T	Transition/ Transversion	T in cattle and buffalo G in sheep	
1060	T	G	Transversion		S/A
1062–1063	TC	CT	Inversion		S/A, synm
1066–1067	GT	AC	Transition		V/T
1071	A	G	Transition		I/M
1091	T	C	Transition		V/A
1092	C	G	Transversion		V/A
1093	T	C	Transition		Synm
1096	T	C	Transition		Y/L
1097	A	T	Transversion		Y/L

are likely to act as a scaffold to prevent unfavourable interactions between folding domains (George and Heringa, 2002).

CD14 molecule of goat is predicted to be mostly expressed on cell membrane (Fig. 5; Suppl.). Since CD14 is receptor molecule and it contains GPI anchor for the attachment with cell membrane, it is obvious to be expressed on cell surface. CD14 exist in 2 forms as membranous and soluble form (Wang *et al.* 2002). Since in the present study, CD14mRNA was isolated from liver cells, it is obvious to be in membranous form.

3-D model of CD14 molecule of goat was predicted from amino acid position 24 to 331 (Fig. 6; Suppl.), with horseshoe shaped structure with alternating alpha helix and beta chains. The finding is similar to study conducted by Kim *et al.* (2005) and Pal *et al.* (2011) where they studied crystal structure of CD14 molecule of mice and cattle respectively. The amino terminal pocket along with grooves is responsible for ligand binding. Since CD14 is a receptor, ligand binding is an important factor.

Molecular evolution and SNP study of caprine CD14: Phylogenetic analysis of goat with other species revealed that goat was evolutionary closest to other small ruminants, namely sheep and bighorn sheep (Fig. 7; Suppl.). Nucleotide identities of Caprine CD14 were reported to be 97.7, 93.5, 92.7, 92.0, 90.5% with respect to *Ovis aries, Ovis orientalis, Bos taurus, Bubalus bubalis, Bos taurus* × *Bos indicus*. Similar evolutionary closeness of goat with sheep was also depicted while studying growth hormone gene (Pal and Chatterjee 2010a).

SNP study in CD14 gene in goat was conducted based on the CD14 sequences of other ruminants (Table 3). A high degree of nucleotide sequence variability with transitions, transversions and nucleotide substitutions which was considered as mutational hot spot (Table 3). Non synonymous exceeding synonymous indicates evolution of goat through positive selection. At nucleotide position 1059, both transition and transversion mutations were observed.

At nucleotide positions 102 and 518, specific nucleotides were present for small ruminants. Nucleotide change at amino acid position 102 lead to synonymous change. Nucleotide change at amino acid 518 leads to alanine in sheep and goat, whereas valine in cattle and buffalo (Table 3). Apart from these at nucleotide positions, alleles specific for small ruminants and large ruminants have been identified (Table 3). Hypervariable site or mutational hot spot identified for nucleotide positions 703–724, 952–973, 1033–1071 of caprine CD14 gene.

CD14 molecule being a receptor, nucleotide variability may lead to diversity in ligand binding. Nucleotide sequences from 102 to 357 were identical to ruminant concensus, indicating this region as conserved across the species in ruminants. Trans-species polymorphism were detected in ruminants (Table 3), where specific alleles were detected for small ruminants (goat and sheep) and large ruminants (cattle and buffalo). Trans-species polymorphism (TSP) is the occurrence of similar alleles in related species. Similar higher degree of SNP was identified in cattle (Pal *et al.* 2011) and buffalo (Pal 2006).

The predicted peptide sequence of CD14 gene of goat (gene bank protein ID: ABE 68725.1) revealed 86.9%, 87.2, 57.9, 65.2, 55.9, and 54.2% amino acid identity with buffalo, cattle, dog, human, mouse and rat.

Goat was observed to be evolutionary closest to other small ruminants, sheep and bighorn sheep. Nucleotide identity of Caprine CD14 ranges from 97.7 to 90.5%, and 68

Table 3. Distinguished nucleotide changes (68) in Caprine CD14 in comparison to ruminants

1	atggtgtgcg	tgccctacct	gctgctgctg	ctgctgtccg	cactgctgcg	tgtgactgcg
61	gacaaaagag	agccctgcga	gctggatcca	cagcatttcc	gctgtgtctg	caacttcacg
121	gatccgaagc	ctgactggtc	tagcgccgtt	cagtgtatgg	ttgccgtcga	ggtggagatc
181	cgtggcggcg	gccacagcct	ggaccagttt	ctcaagggag	ccaacaccga	cccgaagcag
241	tatgctgaca	caatcaaggc	tctgcgcgtt	cggcgactca	agctgggcgc	tgcacaggtt
301	cctgctcagc	ttctggtcgc	cgttctgcgc	gcgctcgggt	actctcgtct	caaggaactg
361	acgcttgagg	acctggaggt	aactggccca	acgcccccgg	cgcctctgga	agccactggg
421	cctgcgctca	ccaccctcag	tctccgtaac	gtgtcgggca	caacaggagg	tgcctggctc
481	ggcgaactgc	agccatggct	caagcctggg	ctcagggcgc	tgaacattgc	ccaagcacac
541	tcgcttgcct	ttccctgcgc	agggctctcc	accttcgagg	cgctcaccac	cctagacctg
601	tctgacaatc	ccagtctcgg	cgacagcggg	ctgatggcgg	ctctctgtcc	gaacaagttc
661	cgggcccctc	aatatttagc	actacgcaac	gcggggatgg	aggcggcgac	ccgcccgtgc
721	gcgccgctgg	cggcagcgag	ggtgcagccc	caaaacctgg	acctcagcca	caactcgctg
781	cgcgtcaccg	ccccgggcgc	tacccgatgt	gtctggccca	gtgccataag	ctctctcaat
841	tgttcgttcg	ctgggctgga	gcaagtgcct	aaaggactgc	cgcccaagct	cagcgtgctt
901	gatctcagct	gcaacaagct	aagcagggag	ccgcggcgag	acgagctgcc	ctatgtaaat
961	gtcctgactg	tgaacggaaa	tccctttctg	gaccctggag	ccctccagca	ccaaaatgac
1021	ccgatgatct	cccgtatgat	tccagaccgt	gccccccccat	ctctggtcat	aggggtgtca
1081	ggagccctgg	tcttgtatca	aggagcccga	ggcttcgcgt	aa	

nucleotide changes (with 6 synonymous nucleotide substitutions) were detected in caprine CD14 with respect to other ruminants. Primary and secondary structures of caprine CD14 deduced from cDNA clones along with the predictions for the sites of post-translational modifications and 3-D structure of CD14 molecule are reported here for the first time. Characterization of CD14 peptide gives an insight of its function as an immune response gene. However, further studies are needed for the determination of functional relationship of species-wise differences number of repeats or other structures or differences in other post-translational modification sites. This study revealed CD14 molecule of goat as a receptor with amino terminal pocket as the site for LPS binding. Further research need to be directed to find out the association of allelic variants with traits related to disease incidences to establish it as marker. Gene insert containing the resistant variety of CD14 gene of goat can be used for somatic gene therapy, particularly against mastitis. Transgenic animal production with goat CD14 gene insert may provide the scope for future research to develop disease resistant stock

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