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# Cloning and sequence analysis of Interleukin (IL)-2, IL-4, IL-10, and IL-18 of Marwari horse

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ABSTRACT

Cytokines play very important roles in the development of protective immune responses against a variety of pathogens. During the last decade rapid progress has been made in the cloning and characterization of cytokines from livestock and companion animals. However, no such work was carried out on cytokines of Indian horse breed. The present work was undertaken with the objective of generating sequence information of cytokines of Marwari horse. The cDNAs encoding interleukin (IL)-2, IL-4, IL-10 and IL-18 of Marwari horse were cloned, sequenced and subsequently compared with respective interleukin sequences of mammalian species including zebra, donkey, cattle, buffalo, sheep, goat, camel and pig available in GenBank database. Horse IL-10 and IL-18 shows nearly 90% sequence homology both at nucleotide and amino acid level with major livestock species. But horse IL-2 and IL-4 had significant sequence divergence with corresponding interleukins of domestic animals. Structural and functional conservation of horse interleukins in respect of cysteine residues, glycosylation, myristoylation, and phosphorylation motifs revealed that IL-10 and IL-18 molecules are evolutionary conserved across species. Nucleotide and amino acid sequences identity and phylogenetic analyses of the 4 cytokine genes in the present study indicated that horse cytokines are closely related to that of pig and camel cytokines.

**Key words**: Cloning, Horse, Interleukin, Sequence

Cytokines are low molecular weight glycoproteins which are produced and active at extremely low (nanomolar to picomolar) concentrations, and exert their actions through binding to specific, high-affinity, cell surface receptors (Pugh-Humphreys and Thomson 1998). Cytokines are indicators of innate and adaptive immunity and are crucial for fighting off infections and in other immune responses. The principal limitations in the field of equine immunology are the lack of key reagents which impede the study of cytokines, immunological pathway analysis, and the identification of antigen-specific immune response in equines. In the past years, some of the equine cytokines like interleukin (IL)-2, IL-4, IL-5, IL-18 and interferon-gamma (IFN-γ) have been cloned, sequenced and expressed in mammalian and prokaryotic system (Dohmann *et al*. 2000, Cunningham *et al*. 2003, Tong *et al*. 2010, Bai *et al.* 2010). However, analysis of deduced amino acid sequences of horse cytokine in terms of functional and structural motif have not been fully demonstrated and compared with companion animals.

The Marwari is a breed of horse from the Marwar (Jodhpur) region of India. Marwari horses are known for its sturdiness, endurance potential and relative disease resistance. Till now no sequence information of cytokines of Indian horse breed is available. In the present study, cDNAs encoding IL-2, IL-4, IL-10 and IL-18 of Marwari horse cytokines were cloned and conservation of structural and functional motifs of these sequence were compared with those of other domestic animals.

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## MATERIALS AND METHODS

The blood sample (5 ml) was collected in ethylene diamine tetra-acetic acid (EDTA) from a healthy Marwari horse and diluted at 1:2 ratios in sterile phosphate buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using Histopaque. PBMCs thus obtained were washed once by PBS-D (PBS-Dextrose) and were seeded at a concentration of $4\times10^5$ cells/ml in 96 well cell culture plate in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 ng/ml streptomycin. Cells were stimulated with Concanavalin A (Con A, 5 µg / ml) and were incubated at 37ºC under 5% $CO_2$. Total RNA was isolated from PBMCs after 4h post stimulation by Trizol reagent and stored in aliquot at –70ºC. An aliquot of total RNA was reverse transcribed using oligo dT primer. The resulting cDNA was used as template to amplify the coding

Table 1. Primer sequences used for PCR amplification of cytokine cDNA

| Cytokine | Primer sequence | Predicted size (bp) | Annealing temperature (°C) |
|---|---|---|---|
| IL-2 | Sense 5´- CGGCGGATCC ATGTACAAGATGCAACTCTTG -3´ | | |
| | Antiense 5´- CGGCCTGCAG TCAAGTCATTGTTGAGAAG -3´ | 450 | 52 |
| IL-4 | Sense 5´-CGGCGGATCCATGGGTCTCACCTACCAACTG-3´ | | |
| | Antiense 5´- CGGCCTGCAGTCAACACTTGGAGTATTTCTC-3´ | 414 | 52 |
| IL-10 | Sense 5´- CGGCGGATCC ATGCACAGCTCAGCACTGCTATG-3´ | | |
| | Antisense 5´- CGGCCTGCAG TCAGTTTTTCATCTTCGTTGTC-3´ | 537 | 52 |
| IL-18 | Sense 5´- CGGCGGATCCTACTTTGGCAGGCTTGAACC-3´ | | |
| | Antisense 5´- CGGCCTGCAGCTAGTTCTGGTTTTGAACAGTG-3´ | 474 | 52 |

sequences of IL-2, IL-4, IL-10 and IL-18 using cytokine specific primers listed on Table 1. The PCR reaction were carried out in 25µl volume containing 10X PCR buffer, 25mM $MgCl_2$, 10mM dNTPs, 1U Taq DNA polymerase. The reaction was carried out in a thermal cycler with thirty cycles of denaturation at 94ºC for 45 sec, primer annealing at 52ºC for 30 sec and primer extension at 72ºC for 1 min followed by final extension at 72ºC for 5 min. The PCR product were visualized by electrophoresis on 1% agarose gels containing fluorescence DNA dye under UV transluminator.

PCR amplified product was eluted from agarose gel by gel extraction kit following manufacturer's protocol. Eluted DNA was ligated to pGEM-T easy cloning vector. The ligation mixtures were transformed into *Escherichia coli* JM109 competent cells. Positive clones were screened and selected by colony PCR and restriction enzyme digestion of recombinant plasmid with *Eco*RI. Two clones of each of the cytokines were commercially sequenced in both directions using universal M13 forward and reverse primer; a third clone was sequenced in case of any discrepancy.

DNA sequence data were first investigated for quality, and identity using BLASTN program available at the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov). Amino acid sequence of horse IL-2, IL-4, IL-10 and IL-18 were deduced using the CLC workbench v. 5.6.1 (www.clcbio.com). Multiple alignment and phylogenetic analysis was carried out with respective interleukin sequences originated from other domestic animals. Phylogenetic trees were constructed using the Neighbour-Joining (NJ) method of Saitou and Nei (1987) using Mega 5.05 software. The robustness of phylogenetic constructions was evaluated by bootstrapping using 1000 replicates and nucleotide distances were estimated from bootstrapped datasets using Kimura's-two-parameter method (Kimura 1980). Predicted functional motifs such as glycosylation, phosphorylation, myristylation 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).

## RESULTS AND DISCUSSION

The cDNA of Marwari horse encoding IL-2, IL-4, IL-10, and mature IL-18 had 450, 414, 537, and 474 nucleotides (Fig. 1), with corresponding protein sequences made up of 149, 137, 178, and 157 amino acid residues, respectively (Fig 2. A-D). The sequences were deposited in the GenBank and assigned accession number JQ432544 (IL-2), JQ432546 (IL-4), JQ432545 (IL-10), and JQ432547 (IL-18). Since the phylogenetic tree patterns constructed with these four cytokines were similar, only that of IL-4 phylogenetic tree is shown in Fig. 3. Indian Marwari horse seqnences clustered together with previously published horse, zebra and donkey sequences in Perissodactyla order.

The deduced amino acid sequences of the cytokine genes and their alignment with the corresponding cytokines originated from other mammals are shown in Fig 2 A-D. Marwari horse IL-2 and IL-4 cytokines were completely identical to those previously reported horse IL-2 sequence,

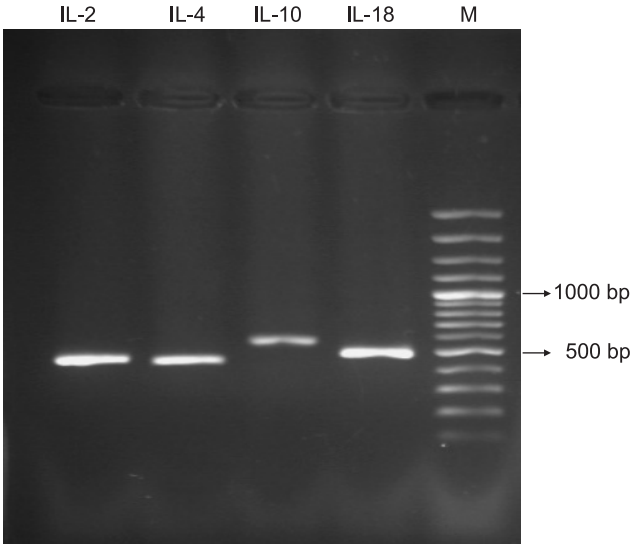
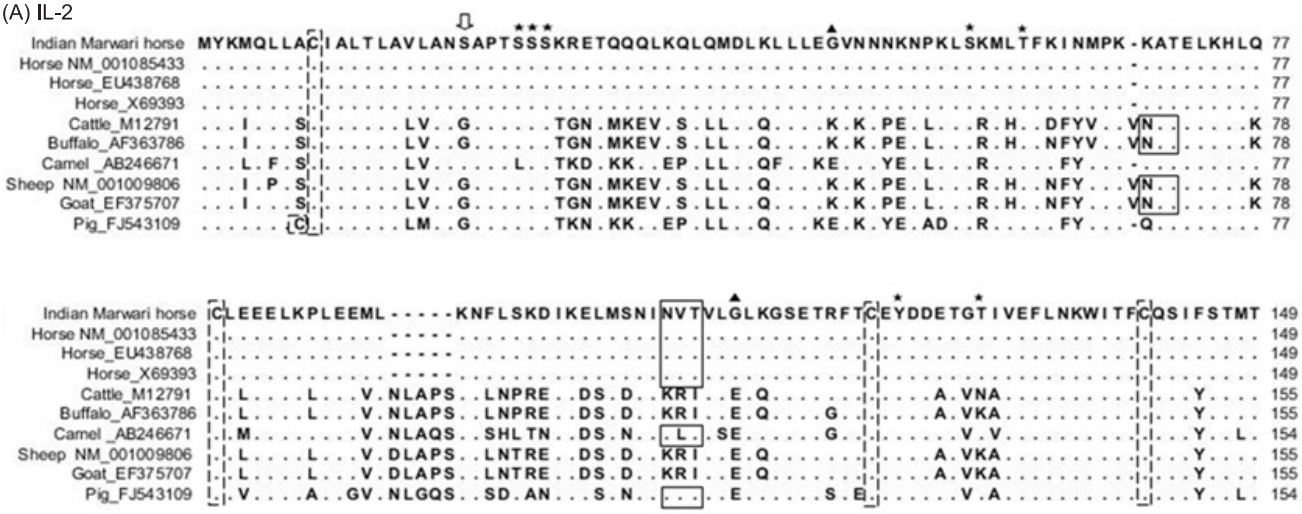



Fig. 1. Agrarose gel electrophoresis of PCR amplified cytokine cDNAs of Marwari horse. Lanes are indicated with corresponding cytokines IL-2 (450 bp), IL-4 (414 bp), IL-10 (537 bp), and mature IL-18 (474 bp). Lane M: 100 bp DNA ladder plus.

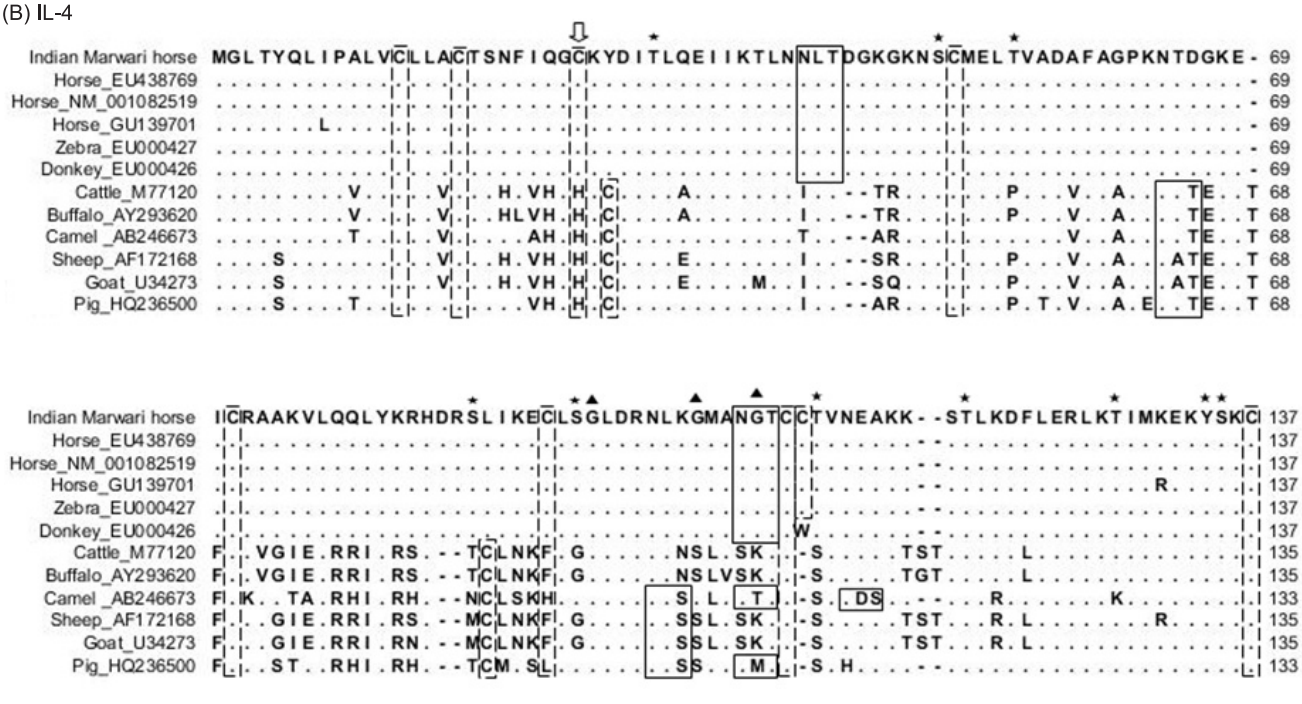
and IL-4 sequence. Marwari horse IL-10 and IL-18 had two nucleotide substitutions at G13T and A26G, T5A and C20A respectively as compared to reference horse sequences. Nucleotide and amino acid sequence identity between horse cytokines and other mammalian species are shown in table 2. Horse IL-10 and IL-18 cDNAs shows high percentage of sequence homology (~ 90%) than IL-2 and IL-4 (~ 82%) with other livestock animals. At the amino acid level, horse IL-2 and IL-4 showed much divergence (63.5–68%, and 71–77.5% sequence identity, respectively) than IL-10 and IL-18 (80–91.5% and 85–91% sequence identity, respectively). Further, Marwari horse IL-10 and IL-18 cytokine showed greatest homology to the respective cytokine sequences of camel and pig, respectively.

To gain a further insight into structural and functional conservation of horse cytokines with other livestock species, position of cysteine residues, glycosylation, myrisoylation, and phosphorylation motifs were compared. Cystein residues are related to formation of disulfide bonds that take part in the regulation of protein folding, stability, and activity (Hogg 2003). Biological role of N-linked glycosylation are protein folding, metabolism, transport and maintenance of cell and protein structure. Protein myristoylation (co-translational linkage of myristic acid (C14:O) to the $NH_2$–terminal Gly residues) has been linked to various biological functions like membrane binding and targeting, signal transduction, apoptosis, or alternative extracellular protein export (Maurer-Stroh *et al*. 2002).

(A) IL-2




(B) IL-4

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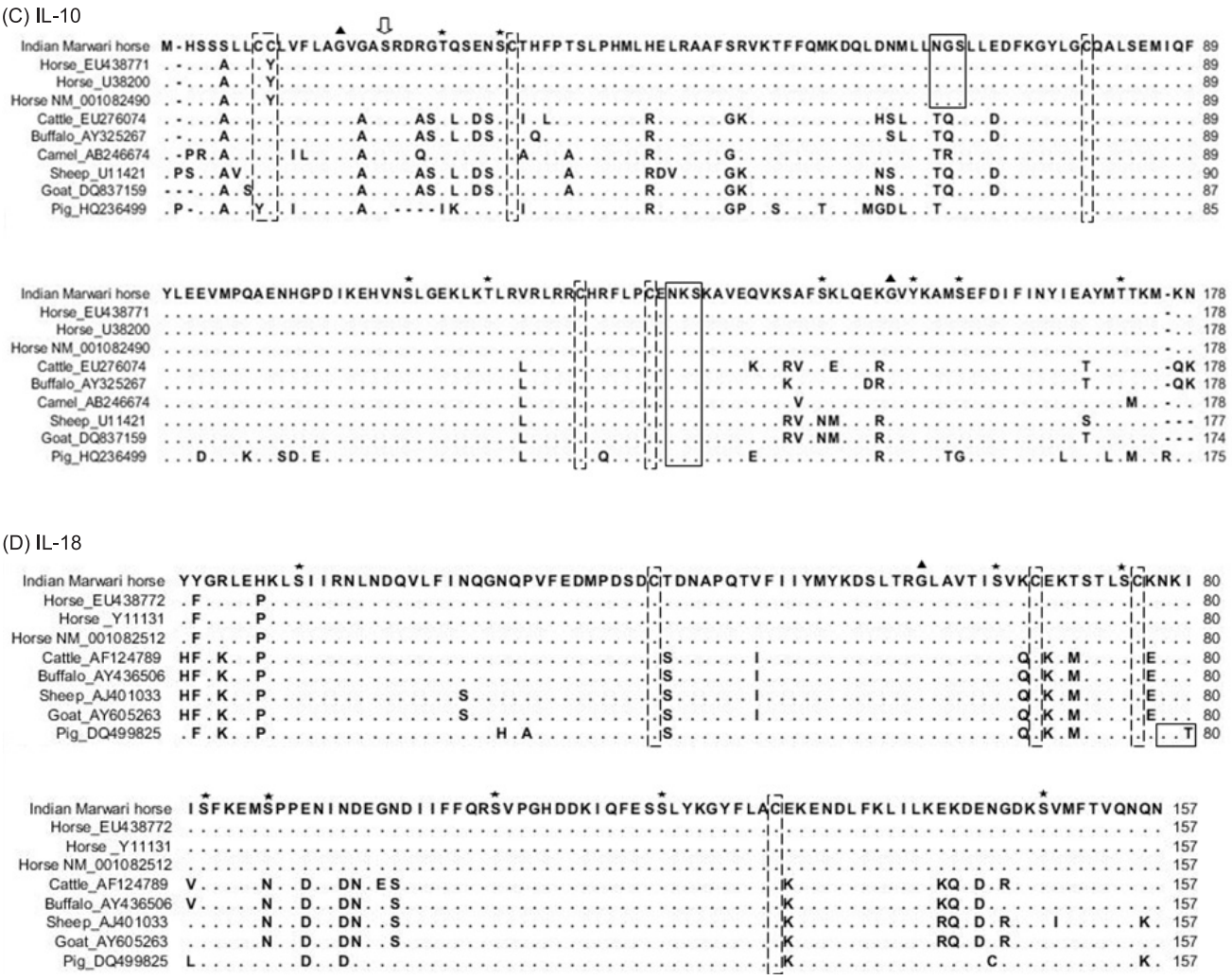


Fig. 2. Alignment of the deduced amino acid sequence of Indian Marwari horse cytokine cDNAs with those of other mammalian species. (A) IL-2, (B) IL-4, (C) IL-10, (D) IL-18. The identical amino acid residues are indicated by dots (.). A dash (-) denotes the absence of amino acids. White arrowheads indicate the amino acid terminus of the mature proteins. The conserved Cys residues or potential *N*-linked glycosylation sites are delineated by dashed boxes and solid boxes, respectively. Potential phosphorylation sites are indicated by a star, while myristoylation sites are indicated by black triangle.

Protein phosphorylation on Ser, Thr and Tyr residues is the most ubiquitous post-translational modification (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).

Horse IL-2 has 33–38 amino acid substitutions and five amino acid deletions at position 91–95. It contains four cystein residues (at position 9, 78, 121, and 141), one potential N-linked glycosylation motif (at position 106–108), seven phosphorylation motif (four Ser-, three Thr- and one Tyr- phosphosites), and two myristoylation motifs (at position 47 and 111) (Fig.2 A). Conservation of cysteine residues in IL-2 among livestock animals and horse suggests conservation of secondary structure of this molecule across the species. Position of N-linked glycosylation site in horse was homologus to pig and camel IL-2 protein. Four phosphorylation sites (Ser-phosphosite at position 26 and 57, Thr-phosphosite at position 61, and Tyr-phosphosite at 122) were conserved in IL-2 sequences in livestock animals including horse. Myristoylation motifs in horse IL-2 were unique.

The horse IL-4 has 25–33 amino acid substitutions. Among the seven cysteine residues in mature IL-4 protein, only four cystein residues showed homologous conservation which indicates horse IL-4 may differs from disulfide bonds position identified in human, murine and ovine (Chaplin *et al*. 2000). Divergence in number and position of possible N-linked glycosylation sites were more prominent in IL-4 among domestic animals. The horse IL-4 contains two potential N-linked glycosylation sites (positions 40–42 and 105–107), cattle and buffalo have single conserved glycosylation site (position 62–64), sheep and goat have two such conserved sites (at positions 62–64 and 96–98), pig has three sites (at positions 62–64, 96–98, and 102–104) and camel has four glycosylation sites (at 62–64, 96–98, 102–104, and 108–110) in IL-4 protein. Non-glycosylated and glycosylated IL-4 reflect a difference in receptor affinity and thus have altered functional properties

Table 2. Percent nucleotide and amino acid similarities of horse cytokines

| IL-2 | Marwari Horse | Horse | Zebra | Donkey | Cattle | Buffalo | Camel | Sheep | Goat | Pig |
|---|---|---|---|---|---|---|---|---|---|---|
| Marwari horse | | 100 (0) | NA | NA | 63.5(38) | 68.3 (33) | 65.4 (36) | 64.4 (37) | 65.4 (36) | 68.3 (33) |
| Horse | 100 | | | | 63.5(38) | 68.3 (33) | 65.4 (36) | 64.4 (37) | 65.4 (36) | 68.3 (33) |
| Cattle | 80.7 | 80.7 | | | | 96.2 (4) | 67.3 (34) | 95.2 (5) | 96.2 (4) | 72.1 (29) |
| Buffalo | 82.9 | 82.9 | | | 98.3 | | 67.3 9 (34) | 97.1 (3) | 98.1 (2) | 73.1(28) |
| Camel | 82.8 | 82.8 | | | 84.3 | 84.6 | | 66.3 (35) | 67.3 (34) | 76.9 (24) |
| Sheep | 81.4 | 81.4 | | | 97.5 | 98.3 | 83.7 | | 99.0 (1) | 73.1 (28) |
| Goat | 82.4 | 82.4 | | | 98.0 | 98.8 | 84.3 | 99.5 | | 74.0 (27) |
| Pig | 82.9 | 82.9 | | | 84.4 | 85.0 | 87.2 | 85.0 | 85.6 | |
| IL-4 | | | | | | | | | | |
| Marwari horse | | 100(0) | 99.1 (1) | 99.1(1) | 73.0(30) | 71.2 (32) | 75.7 (27) | 70.3(33) | 71.2 (32) | 77.5 (25) |
| Horse | 100.0 | | 99.1 (1) | 99.1(1) | 73.0(30) | 71.2 (32) | 75.7 (27) | 70.3(33) | 71.2 (32) | 77.5 (25) |
| Zebra | 98.6 | 98.6 | | 100 (0) | 73.9 (29) | 72.1 (31) | 74.8 (28) | 71.2 (32) | 72.1 (31) | 76.6 (26) |
| Donkey | 98.6 | 98.6 | 98.9 | | 73.9 (29) | 72.1(31) | 74.8 (28) | 71.2 (32) | 72.1(31) | 76.6 (26) |
| Cattle | 80.1 | 80.1 | 79.7 | 80.5 | | 98.2 (2) | 73.9 (29) | 92.8 (8) | 91.0 (10) | 78.4 (24) |
| Buffalo | 79.3 | 79.3 | 79.0 | 79.7 | 98.9 | | 72.1 (31) | 91.0 (10) | 89.2 (12) | 76.6 (26) |
| Camel | 80.8 | 80.8 | 79.7 | 79.5 | 85.0 | 83.6 | | 73.9 (29) | 73.0 (30) | 80.2 (22) |
| Sheep | 78.7 | 78.7 | 79.1 | 79.8 | 95.8 | 94.7 | 85.4 | | 96.4 (4) | 78.4 (24) |
| Goat | 77.9 | 77.9 | 78.3 | 79.1 | 94.1 | 92.9 | 83.3 | 97.2 | | 77.5 (25) |
| Pig | 79.9 | 79.9 | 78.8 | 79.5 | 83.0 | 82.2 | 87.1 | 83.3 | 82.2 | |
| IL-10 | | | | | | | | | | |
| Marwari horse | | 99.2 (2) | NA | NA | 79.8 (26) | 83.7 (21) | 91.5 (11) | 82.9 (22) | 83.7 (21) | 80.6 (25) |
| Horse | 99.6 | | | | 80.6 (25) | 84.5 (20) | 92.2 (10) | 83.7 (21) | 84.5(20) | 81.4(24) |
| Cattle | 89.3 | 89.3 | | | | 92.2 (10) | 80.6 (25) | 90.7(12) | 93.0(9) | 78.3(28) |
| Buffalo | 90.5 | 90.5 | | | 97.1 | | 82.9 (22) | 89.9(13) | 92.2(10) | 77.5(29) |
| Camel | 92.8 | 92.7 | | | 89.3 | 90.5 | | 83.7(21) | 84.5 (20) | 82.2(23) |
| Sheep | 89.3 | 89.3 | | | 95.6 | 95.2 | 89.8 | | 96.9(4) | 78.3(28) |
| Goat | 89.3 | 89.3 | | | 96.3 | 96.3 | 89.8 | 98.2 | | 78.3(28) |
| Pig | 86.4 | 85.5 | | | 85.7 | 85.4 | 86.6 | 85.2 | 85.5 | |
| IL-18 | | | | | | | | | | |
| Marwari horse | | 98.7 (2) | NA | NA | 86.0(22) | 87.3(20) | NA | 85.4(23) | 86.6(21) | 91.1(16) |
| Horse | 99.6 | | | | 87.3(20) | 88.5(18) | | 86.6(21) | 87.9(19) | 91.1(14) |
| Cattle | 90.4 | 90.9 | | | | 98.7(2) | | 96.2(6) | 97.5(4) | 89.2(17) |
| Buffalo | 90.4 | 90.9 | | | 98.3 | | | 96.2(6) | 97.5(4) | 90.4(15) |
| Sheep | 90.7 | 91.2 | | | 97.8 | 97.4 | | | 98.7(2) | 89.2(17) |
| Goat | 90.9 | 91.4 | | | 98.1 | 97.6 | | 99.4 | | 89.2(17) |
| Pig | 92.2 | 92.6 | | | 91.7 | 92.2 | | 92.1 | 92.4 | |

The percent nucleotide and amino acid sequence similarity of the respective cytokines are presented at the lower left and upper right, respectively. The Marwari horse sequence similarity with other animal species is indicated in boldface type. Number in parentheses indicates amino acid substitutions. NA=Not available.

among the glycosylation variants (Thor and Brian 1992). Interestingly structural divergence was also observed in equine IL4 receptor (Solberg *et al*. 2004). Together the structural differences in IL-4 cytokine and its receptor may explain the species-specific activity of this cytokines observed by Mosmann *et al.* (1987). A single conserved myristoylation site was present in cattle, buffalo, sheep, goat, pig and camel IL-4 sequences at position 92, while horse IL-4 contains three myristoylation sites (positions 95, 102, and 106). Four phosphorylation sites (Ser-phosphosite at position 49 and 135, Thr-phosphosite at position 118, and Tyr-phosphosite at 134) in IL-4 sequences were conserved in livestock and horse.

A very high degree of sequence and structural homology was observed between horse IL–10 and companion animals, and was more close to camel IL-10 protein. Deduced amino acid sequences of IL-10 of Marwari horse contains six Cys residues (positions 8, 9, 30, 80, 126, and 132), two N-linked glycosylation sites (positions 67–69 and 134–136), two myrostoylation sites (positions 15 and 153) and nine phosphorylation (five Ser-, three Thr- , and one Tyr-phosphorylation) sites (Fig. 2 C). Interestingly, number and position of Cys residues, N-linked glycosylation sites, and myristoylation sites were well conserved in IL-10 protein of all the animal species including horse. Highly conserved four cysteine residues, glycosylation, myristoylation, and phosphorylation sites in mature IL-10 protein indicate conservation of tertiary structure and functional integrity

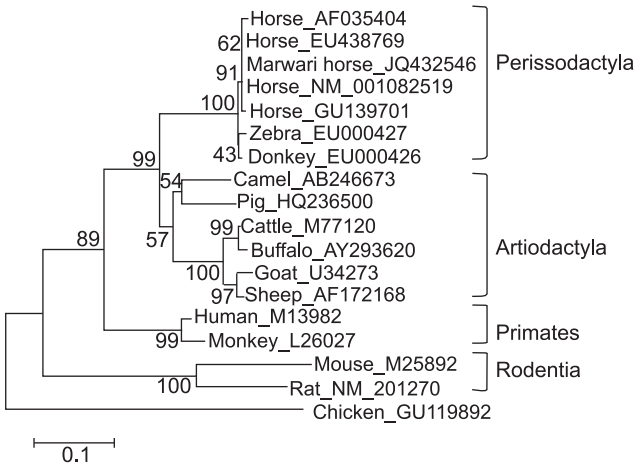


Fig. 3. Phylogenetic tree based on the nucleotide sequences of IL-4 cytokines. The multiple sequence alignments of each cytokines from various species available in GenBank were obtained by CLUSTAL W method and trees were constructed by the neighbor-joining method based on Kimura-2 parameter model using MEGA version 5.05 software packages (www.megasoftware.net). Cytokines obtained from Indian Marwari horse, was indicated by black triangle marker. The tree was rooted using chicken cytokine as an out-group. Horizontal branch lengths are proportional to the genetic distance between species. Bootstrap probabilities of each node were calculated using 1000 replicates.

of this molecule in horse and other animal species as well. Indeed, in a recent experiment conducted by den Hartog *et al*. (2011) demonstrated structural conservation of IL-10 receptor between bovine and human and showed that bovine IL-10 exerts biological activity comparable to human IL-10 on human monocytes and dendritic cells.

Similar to IL-10, IL-18 had high degree of sequence and structural conservation across mammalian species which includes number of amino acids, number and position of cystein residues, serine phosphorylation sites, and myrisylation sites. Sequence and structural identity of horse IL-18 was very similar to pig IL-18 protein (Fig 2 D). Deduced amino acid sequences of mature IL-18 of horse and other mammals contain four Cys residues at position 38, 68, 76 and 127. It was reported that first and fourth Cys residues are necessary for IL-18 to elicit IFN-gamma production from peripheral blood lymphoid mononuclear cells (Pei *et al*. 2005). Conservation of cysteine residues in IL-18 suggests the evolutionary necessity of maintaining the important function of this molecule across the mammalian species. N-linked glycosylation motif was not present in mature IL-18 protein of horse, cattle, buffalo, sheep, goat. However, one glycosylation motif is present in pig IL-18 sequences. The presence of two charged amino acids namely, Glu-42 and Lys–89 (Glu-6 and Lys-53 in mature IL-18 in this paper) are critical for binding to both IL-18 receptor and IL-18 binding protein (Kim *et al*. 2002). Therefore, the homologous conservation of these two charged amino acid residues in horse and other animals underscore the functional similarity of IL-18 across species.

The present work contributes to the understanding of the IL-2, IL-4, IL-10, and IL-18 sequence of Marwari horse in respect of conservation of structural and functional motifs among domestic animals. Interestingly, number of amino acid sequences, structure, and functional motifs of horse IL-10 and IL-18 were highly conserved however, IL-4 and IL-2 showed marked variation in sequences and other vital structure. The availability of biologically active recombinant equine cytokines will be helpful to investigate the physiological roles of this cytokine, as well as its potential efficacy as a therapeutic agent or vaccine adjuvant in horse.

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