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Identification of interleukin-26 in the dromedary camel (*Camelus dromedarius*): Evidence of alternative splicing and isolation of novel splice variants

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ABSTRACT

Interleukin-26 (IL-26) is a member of the IL-10 family of cytokines. Though conserved across vertebrates, the *IL-26* gene is functionally inactivated in a few mammals like rat, mouse and horse. We report here the identification, isolation and cloning of the cDNA of *IL-26* from the dromedary camel. The camel cDNA contains a 516 bp open reading frame encoding a 171 amino acid precursor protein, including a 21 amino acid signal peptide. Sequence analysis revealed high similarity with other mammalian IL-26 homologs and the conservation of IL-10 cytokine family domain structure including key amino acid residues. We also report the identification and cloning of four novel transcript variants produced by alternative splicing at the Exon 3-Exon 4 regions of the gene. Three of the alternative splice variants had premature termination codons and are predicted to code for truncated proteins. The transcript variant 4 (Tv4) having an insertion of an extra 120 bp nucleotides in the ORF was predicted to encode a full length protein product with 40 extra amino acid residues. The mRNA transcripts of all the variants were identified in lymph node, where as fewer variants were observed in other tissues like blood, liver and kidney. The expression of Tv2 and Tv3 were found to be up regulated in mitogen induced camel peripheral blood mononuclear cells. IL-26-Tv2 expression was also induced in camel fibroblast cells infected with Camel pox virus *in-vitro*. The identification of the transcript variants of IL-26 from the dromedary camel is the first report of alternative splicing for *IL-26* in a species in which the gene has not been inactivated.

1. Introduction

Camelids, the only surviving members of the mammalian group Tylopoda are represented by the New World camelids (Alpaca, Llama, Guanaco and Vicuna) and the Old World camelids (the dromedary and Bactrian camel). The Camelids, believed to have originated in North America during the Eocene period, have exquisitely adapted to inhabit harsh conditions like the hot deserts of Africa and Asia, cold deserts of central Asia and high altitudes of South America (Bravo, 2015). In spite of this diverse distribution and habitats, they seem to share a similar genetic makeup as evidenced by their identical chromosome number (2n = 74) and molecular data emerging from the recent genome sequencing of some of these members (Bravo, 2015). The dromedary camel (*Camelus dromedarius*), one of the major Camelid members is estimated to be domesticated around 3000 BC in the Arabian Peninsula and is an integral part of the life and culture of the region. Although considered a hardy Camelid species, they are also susceptible to many of the diseases of domestic animals (Bravo, 2015). One of the unique features of the camelid humoral immune system is the presence of heavy–chain antibodies (HCAbs) lacking the L-chain, the potential immunological application of HCAbs as nanobodies has evoked considerable interest in antibody-based therapeutics (Muyldermans et al., 2009). Compared to other domestic animals like cattle, pig and horse, investigations into the molecular immunology of dromedary camels have been limited, with only a few cytokines and other immune molecules cloned from the dromedary camels (Nagarajan et al., 2012; Odbileg et al., 2006; Premraj et al., 2013). Recently it has been reported that dromedary camels are infected by zoonotic viruses like MERS Coronavirus (MERS-CoV) and act as intermediate hosts that transmit the virus to human (Adney et al., 2014; Memish et al., 2014). For a better understanding of the dromedary camel immune system during viral infection, the availability of species specific reagents for

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0161-5890/

Table 1
Sequence of oligonucleotide primers used in this study.

Primer	Sequence (5'–3')	Remarks
IL26-IntFP	TGT CAC TCT GTC TCT TGC CAT TGC C	Amplification of the IL-26 fragment
IL26-IntRP	AGT TCA CTG ATG GCT TTG TAG ATT CC	
IL26 3RCF1	ACA GCC TTA GGC AGA AAT TGA GCC GCT G	Primers for 3′ RACE
IL26 3RCF2	TGG TCA ACT CCA GTT ACA AGT TTG CAA GG	
IL26-NF	GAG GGA AAT GTG GGT GAA CTG CAC	Primers for amplification of the full length region
IL26-NR	AAT CCA CTT GGC TTC TTG CAT TAC	
IL26-FF	TGA GTG ACA CGA GCC CAG AGT AGG	
IL26-FR	CTT GCT GTC AGT ACC ATG TAC AC	
IL26XF1	CAA GCT CCA GCA ATC TTC CTT CG	Primers for analyzing the transcript variants
IL26XR1	CTC ACT GAT GGC TTT GTA GAT TCC	

analyzing the major host immune regulatory proteins are essential. This necessitates the identification and isolation of major cytokines of the species which play an important role in the induction of antiviral response.

Interleukin-26 (IL-26) is a member of the IL-10 family of cytokines, which includes other class II cytokines like IL-10, IL-19, IL-20, IL-22 and IL-24. Although the members of this family share low amino acid identity, they share a similar helical structure and functionally target homologous but diverse class II cytokine receptors (Sabat, 2010). Like other members of the IL-10 family, IL-26 also utilizes a hetero-dimeric receptor, IL-26R comprising of IL-20R1 and IL-10R2 receptors. Binding of IL-26 to IL-26R induces signal transduction by phosphorylation and activation of STAT3 and STAT1 (Hör et al., 2004; Sheikh et al., 2004). Initially denoted as AK155, IL-26 was identified in *Herpesvirus saimiri* transformed human T cells and later included as a member of the IL-10 family of cytokines (Knappe et al., 2000). IL-26 is also expressed by activated Th1, Th17, stimulated natural killer (NK) and peripheral mononuclear blood cells (Braum et al., 2013). Of late, IL-26 is gaining significance because of its purported role in many pro-inflammatory diseases and its upstream position in the pro-inflammatory cascade and as the potential drug target for chronic inflammatory disorders (Corvaisier et al., 2012). It has been recently reported that IL-26 is over-expressed in chronically HCV infected patients, enhancing the tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) mediated cytotoxicity and induction of expression of the antiviral cytokines IFN-β and IFN-γ (Miot et al., 2014).

The human *IL-26* gene is mapped to Chromosome 12 (12q15c), where the *IL-22, IL-26* and *IFN-γ* genes are arranged in tandem and transcribed in the same orientation (Donnelly et al., 2010). It is also presumed that these three genes are co-regulated, as IL-26 is reported to be co-transcribed along with IL-22 and IFN-γ (Braum et al., 2013). The human *IL-26* gene consists of five exons, which are interrupted by four introns – three small and one large intron (Knappe et al., 2000). The IL-26 gene is conserved across vertebrates, but interestingly found absent in mouse genome where only short exon fragments of the gene were identified (Braum et al., 2012). Recently, it has been reported that IL-26 is independently inactivated by mutations in exon 2, in several mammals including the members of the Equidae family, African elephant and European hedgehog (Shakhsi-Niaei et al., 2013). There are no reports on the identification and isolation of IL-26 gene from any of the camelids to date.

Alternative splicing, a post transcriptional mechanism for enhancing the diversity of the transcriptome and proteome, has been extensively reported in different cytokines including *IL-1α, IL-2, IL-4, IL-6, IL-7, IL-16* and their receptors in many mammalian species (Sahoo and Im, 2010). It is estimated that around 95% of the human multi exon genes undergo alternative splicing (Pan et al., 2008). As an evolutionary tool, alternative splicing serves as an economical mechanism to produce diversity and specificity at the cellular, tissue or developmental levels and along with non-sense mediated decay, it provides a trial and error mechanism for the evolution of gene structure (Boue et al., 2003). IL-26 has also been reported to undergo alternative splicing in horse, where the gene has been predicted to be inactivated. In this paper we describe the molecular structure of the dromedary camel IL-26 gene and the identification of the novel transcript variants generated by alternative splicing. This is the first report of alternative splicing of IL-26 gene, from a species in which the gene has not been inactivated. The tissue specific distribution of the variants as well as the effect of viral infection on transcription was also studied in cell culture system.

2. Materials and methods

2.1. Dromedary camel tissues

Dromedary camel tissues were collected from three adult female camels that were slaughtered at the abattoir in Al Ain, UAE. Tissue samples (Lymph Node, Liver, and Spleen) were transferred to the laboratory and quickly processed for RNA isolation. Venous blood samples were collected in Vacutainer tubes with anticoagulant and used for RNA isolation.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was isolated from the solid tissues using TRIzol Reagent (Invitrogen) and from blood using TRI Reagent BD (Sigma) following the manufacturer's instructions. RNA pellets were dissolved in nuclease free water and quantified using NanoPhotometer (Implen, Germany). First strand cDNA synthesis was done with oligo dT primers and 1 μg of the total RNA from each tissue using the Reverse Transcription System (Promega) as described previously (Premraj et al., 2013).

2.3. Isolation and identification of dromedary camel IL-26 cDNA

We used a homology based cloning approach to identify the IL-26 sequence of the dromedary camel as no camelid sequences were available. The IL-26 cDNA sequences of human, cattle and whale were aligned to identify areas of high nucleotide conservation. Primers IL26-IntFP and IL26-IntRP (Table 1) were designed at the conserved sites to amplify a fragment of the IL-26 ORF. PCR was performed with these primers using GoTaq Green PCR master Mix (Promega) and cDNA from the dromedary camel as template. Amplicons of the expected size were separated by gel electrophoresis, purified using the Wizard SV gel purification kit (Promega) and cloned into pGEM T Easy vector (Promega). Clones from independent PCR amplicons were sequenced on an Applied Biosystems 3130xl Genetic Analyzer.

We carried out 3′ RACE to determine the missing 3′ end of the dromedary IL-26 ORF. RACE ready cDNA was prepared from total

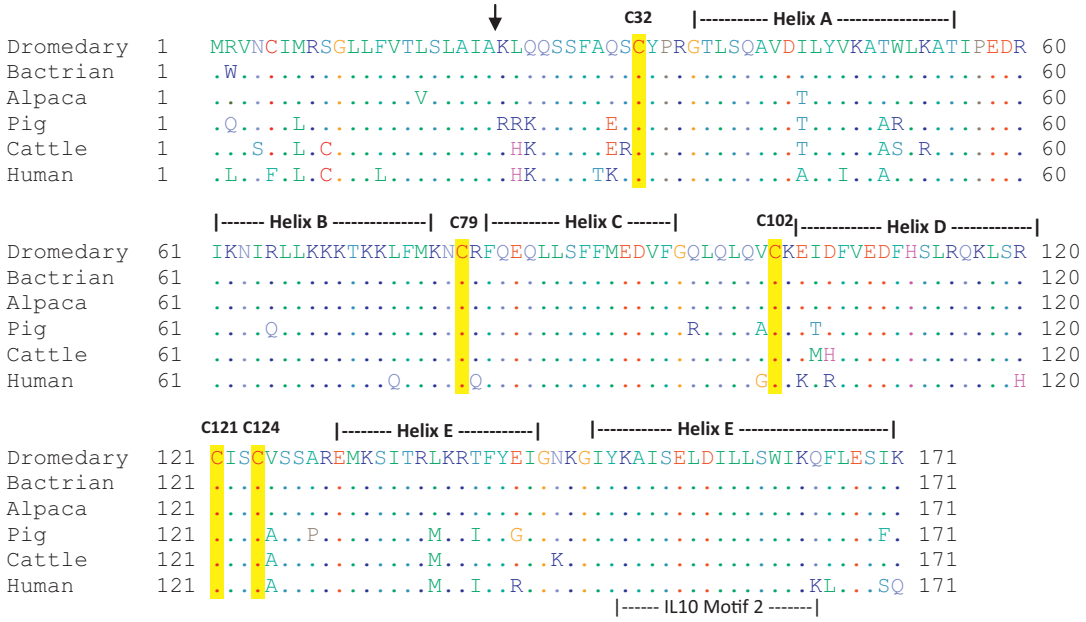


Fig. 1. Alignment of the deduced dromedary IL-26 amino acid sequence with that of other Camelids (predicted from the genome) and mammals. Predicted signal peptide cleavage site is marked by a downward arrow. The five conserved Cysteine residues are marked in highlight and annotated (C32, C79, C102, C121 and C124). The locations of the six alpha helices based on the SOPMA secondary structure prediction are also marked.

camel blood RNA using the SMARTer RACE system (Clontech). 3′ RACE primers 3RCF1 and IL26-3RCF2 (Table 1) were designed based on sequence of the initially cloned partial fragment and 3′ RACE carried out as described previously (Premraj et al., 2013). 3′ RACE amplicons were also cloned and sequenced as described earlier.

2.4. Amplification and cloning of full length splice variants of dromedary IL26

Based on the sequences of the partial IL-26 fragment and the 3′ RACE fragment, new PCR primers were designed to amplify the full length ORF of the dromedary IL-26. Primers IL26-NF and IL26-NR were designed to amplify the exact ORF from the start codon to the stop codon. Another set of primer pair IL-26FF and IL-26FR were designed at the 5′ and 3′ flanking regions of the ORF to amplify additional independent amplicons for verification of Camel IL-26 sequence (Table 1). PCR amplification was carried out with the IL-26FF/FR and IL26-NF/NR primer pairs using cDNA from blood, liver, lymph node and spleen as template. Amplicons for these full lengths IL-26 ORF from various tissues were cloned and sequenced.

2.5. Bioinformatics analysis

Sequence contigs for the clones were assembled and analyzed using Sequencher 4.9 (Gene Codes, Ann Arbor, MI). BioEdit program (Hall, 1999) was used to identify the ORF and the predicted amino acid sequences. Multiple sequence alignments of the nucleotide and protein sequences were carried out using CLUSTAL W (Thompson et al., 1994). Phylogenetic trees were constructed from the dromedary camel and other mammalian IL-26 sequences using the Neighbor Joining method with 1000 bootstrap replications in the MEGA4 program (Tamura et al., 2007).

The whole genome shotgun (WGS) contigs of the *Camelus ferus* released in the NCBI Genbank, was used for the alignment of the cloned cDNA sequences to predict the gene structure. Through BLAST search analysis, we identified one of the contigs (NCBI LOCUS AGVR01041574) that contained the region coding for the IL-26 gene. The gene structure was predicted by aligning the cloned cDNA sequences against the IL-26 gene sequence obtained from the WGS contig using the MGAlignIt program (Lee et al., 2003).

Signal IP server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide coding region of the protein. The protein properties (molecular weight and theoretical pI) were predicted using the ProtParam (http://web.expasy.org/protparam/) program, where as the SMART protein domain identification program (Letunic et al., 2012) was used to search for cytokine family signature domains. The secondary structure prediction and 3D homology modeling of the mature proteins were carried out using the SOPMA (http://npsa-prabi.ibcp.fr/), PHYRE (Kelley and Sternberg, 2009) and SWISS-MODEL (Biasini et al., 2014) servers. Homology models were visualized and images captured using the PyMOL program.

2.6. Analysis of expression of IL-26 splice variants in different tissues, mitogen stimulated PBMC and Camel pox virus infected fibroblasts

To understand the role of the dromedary camel IL-26 expression, we analyzed the expression of IL-26 transcripts in different camel tissues, mitogen stimulated PBMC and camel pox virus infected fibroblasts. For better band size resolution of the small size differences between the transcript variants, we designed a new set of primers (IL26XF1 & IL26XR1) to amplify a shorter fragment of the coding region of the transcripts. These primers spanned the exon 3 and exon 5 region of the mRNA transcript and thus can capture all the variations from the different transcripts within the region. Besides, as there is a large intron between the exon 3 and exon 4 in the predicted IL-26 gene, these primers would not amplify from the genomic DNA.

Camel peripheral blood mononuclear cells (PBMCs) were separated using Histopaque-1077 (Sigma) as per manufacturer's instructions. The isolated PBMCs were washed twice with PBS, followed by one wash with RPMI-1640 medium and resuspended in RPMI-1640 medium with 10% FCS. The PBMCs were simultaneously stimulated *in-vitro* with Poke Weed Mitogen (PWM - 10 μg/ml) or Concanavalin (ConA- 10 μg/ml) for 16 h. Total RNA was isolated from the stimulated cells using TRIZOL reagent. cDNA synthesis

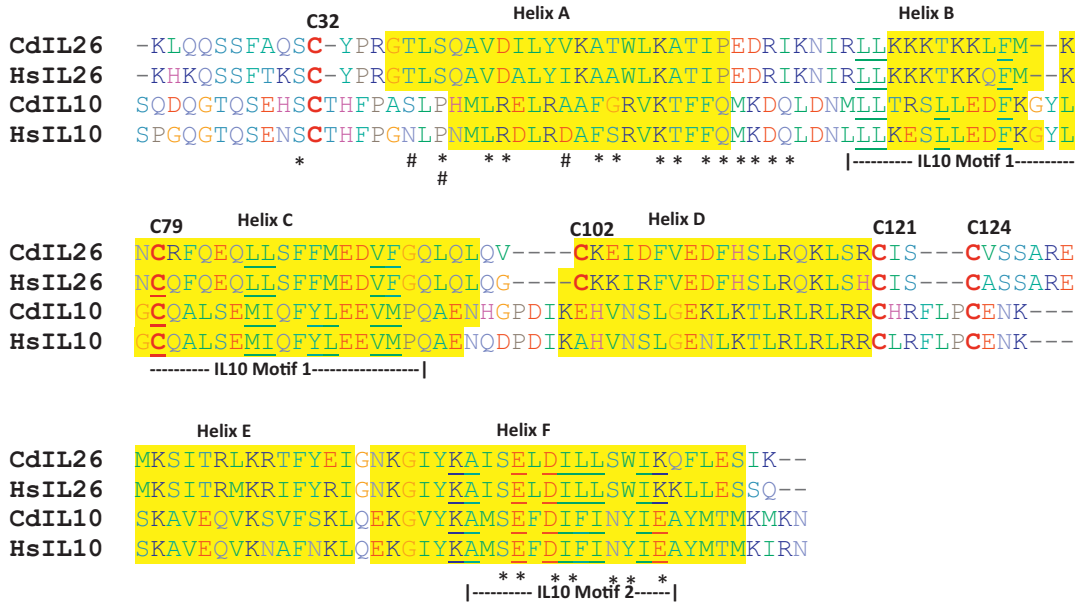


Fig. 2. Alignment of the Camel IL-26 (CdIL26) with Human IL26 (HsIL26), Camel IL10 (CdIL10) & Human IL10 (HsIL10) showing the structural conservation of the IL26 and IL10. Cd IL-26 has 24% amino acid identity and 54% similarity to Hs IL-10. The four cysteine residues and six alpha helices of IL-10 are shared by IL-26 are also marked. IL-26 has an additional Cysteine residue at 102 position, when compared to IL-10. Two signature motifs of IL- 10 family have also been identified and are marked. The residues in the human IL-26 predicted (Donnelly et al., 2010) to be involved in the IL20R1 & IL10R2 are marked by * and #, respectively.

was done with 2 μg of the DNase treated RNA and Random hexamer primers using the Reverse Transcription System (Promega). PCR was carried out using the IL26XF1 & IL26XR1 primers and the products analyzed on an Agilent 2100 BioAnalyzer with DNA 1000 Kit.

Fibroblastic cell line of camel ovary origin maintained in our laboratory was used for virus infection. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal Calf Serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ ml streptomycin and infected with Camel pox virus *in-vitro* (MOI of 0.1). After 48 h of incubation, RNA was isolated from the infected and uninfected controls, and expression of IL-26 variants analyzed as described earlier.

3. Results

3.1. Identification and cloning of IL-26 cDNA from Camelus dromedarius

We obtained the dromedary specific partial cDNA fragment encoding IL-26 by RT-PCR from blood RNA using the primers IL26-IntFP and IL26-IntRP. Using 3′ RACE-PCR we amplified the region coding for the rest of coding sequence and the 3′UTR of the dromedary IL-26 cDNA. Based on the sequences from these two fragments, the full cDNA sequence of dromedary camel IL-26 was assembled. The identified full length camel *IL-26* is 871 bp long with a 34 bp 5′ UTR, 516 bp Open Reading Frame (ORF) and 321 bp 3′ UTR (NCBI GenBank Accession No **KJ862248**).

The entire IL-26 ORF was identified intact without any frameshift mutations, deletions or premature stop codons that cause gene inactivation. Nucleotide sequence analysis by BLAST revealed high level of identity (99%) with two predicted camelid *IL-26* sequences - *C. ferus* (Wild Bactrian camel) and *Vicugna pacos* (Alpaca) with only one and two nucleotide variation between these two sequences, respectively. With the human *IL-26*, the dromedary IL-26 shared 90% nucleotide identity. Three potential mRNA destabilizing ATTTA motifs were also identified in the 3′ UTR.

The 516 bp ORF encodes for a predicted protein of 171 amino acids. A 21 amino acid signal peptide was predicted in the N-terminal of the protein, leaving a 150 amino acid mature protein with an estimated molecular mass of 17.6 kDa. At the amino acid level, the dromedary camel IL-26 shared a high level of identity (99%) with the deduced IL-26 protein from genome sequences of Wild Bactrian camel and Alpaca (Fig. 1). With the *C. ferus* sequence in the NCBI Genbank, there was only one amino acid change (R2W) which was located in the signal peptide. There were two amino acid changes between the dromedary camel and alpaca IL-26; one in the signal peptide (L16 V) and the other in the mature protein (I44D). The five cysteines residues in the mature camelid IL-26 protein corresponded with most mammalian IL-26 (Fig. 1**)**. The mature IL-26 has 27 positively charged amino acid residues (R or K) and seems to be highly cationic with a predicted isoelectric point (pI) of 10.45. The characteristic interleukin-10 cytokine family domain with the two signature motifs was identified inside the mature protein. Alignment of the mature protein sequences of the IL-26 and IL-10 sequences also revealed the positional conservation of cysteine residues which are believed to be involved in the disulphide bond formation (Fig. 2).

Phylogenetic analysis was carried out using the predicted amino acid sequences of dromedary IL-26 and other mammalian sequences. Based on the estimated genetic distances, a phylogenetic tree was constructed using the Neighbor Joining method. The camelid IL-26 clustered together as a distinct clade separate from the Cetacean and Artiodactyl IL-26 (Fig. 3).

3.2. Dromedary IL-26 gene organization

The genomic sequence of the IL-26 was determined by aligning the dromedary camel IL-26 cDNA sequence with a whole genome shotgun sequence contig of the *C. ferus* in the NCBI Genbank. The coding sequence of the camel *IL-26* gene is organized into five exons – Exon1 (171 bp), Exon2 (57 bp), Exon3 (135 bp), Exon4 (66 bp) and Exon 5 (87 bp). The exons are separated by four introns of which intron 1, 2 and 4 are small with sizes 85, 170 and 79 bp respectively.

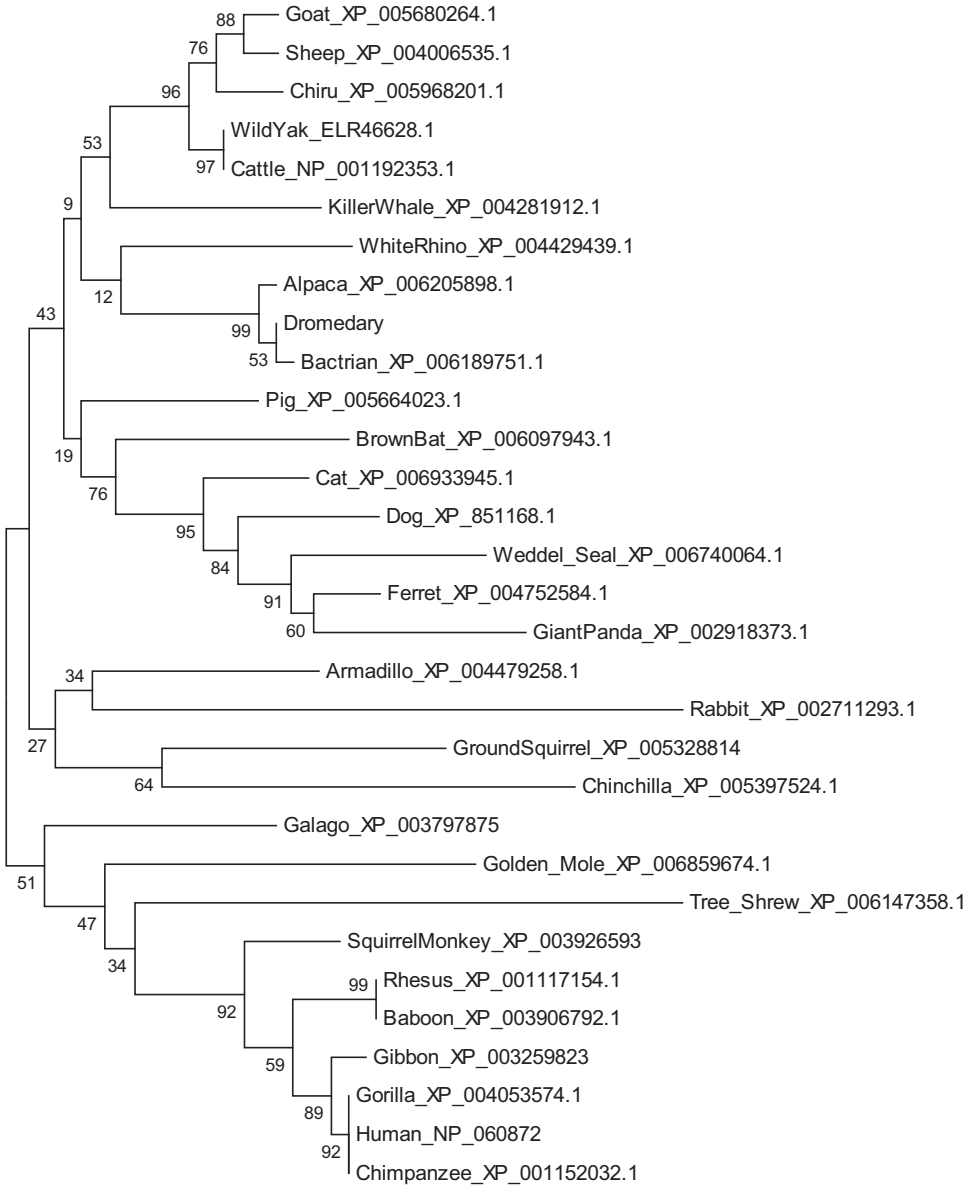


Fig. 3. Unrooted phylogenetic tree of the dromedary camel IL-26 and other mammals. Phylogenetic relationships were reconstructed by the distance Neighbor Joining method using Clustal W multiple alignment, and bootstrapped 1000 times. The scale for the given branch length indicates 0.02 amino acid substitutions per site. XP prefix in the accession number denotes IL-26 sequences in the NCBI GenBank derived through prediction from the genome.

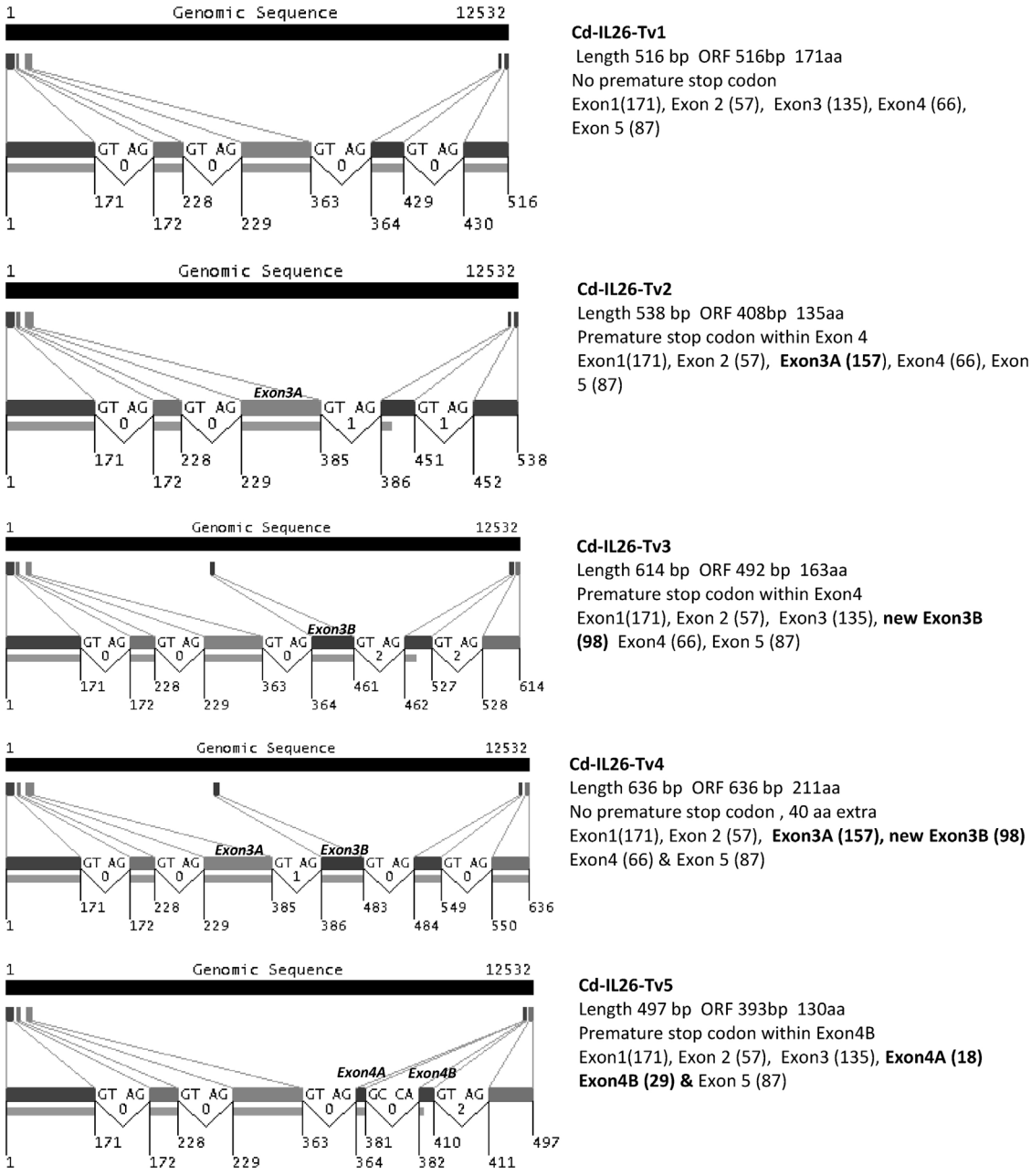
The third intron is the largest with an estimated size of 11.6 kb (Fig. 4).

3.3. Identification of novel alternative splice variants of the dromedary IL-26

Using two sets of primer pairs designed to amplify the full length IL-26 ORF, we obtained multiple bands of different sizes which were cloned and sequenced. From the generated sequences, these were confirmed as novel splice variants. In addition to the normal isoform of the dromedary IL-26, four novel alternative splice variants of *IL-26* were identified in different tissues. The normal camel *IL-26* was designated as Cd-IL-26-Tv1 (NCBI GenBank Accession number **KJ862248**) and the four *IL-26* splice variants as Cd-IL-26-Tv2, Cd-IL-26-Tv3, Cd-IL-26-Tv4 and Cd-IL-26-Tv5 respectively (NCBI GenBank Accession numbers **KJ862249**, **KJ862250**, **KJ862251** and **KJ862252**). These variants are presumed to be originated through alternative splicing at the exon 3–exon 4 regions (Fig. 5) through different mechanisms.

The transcript variant Cd-IL-26-Tv2 is 22 bp longer than the canonical Cd-IL-26-Tv1 isoform. Alignment of the transcript sequence with the predicted *C. ferus* genomic sequence revealed that this variant is the result of the alternative splicing in which the exon 3 is extended by 22 nucleotides (denoted as exon 3A) due to the use of an altered splice site (Fig. 5). This transcript hence has an altered frame of translation resulting in a shorter ORF of 408 bp encoding a truncated protein of 135 amino acids (Fig. 6A). The transcript variant Cd-IL-26-Tv3 has an additional 98 bp sequence at the exon 3 – exon 4 junction when compared to Tv1 (Figs. 4 and 5). In this variant, 98 nucleotides from the intron 3 are alternatively spliced as a new exon – exon 3B (Fig. 4). This transcript also has altered reading frame and results in a shorter ORF (492 bp) encoding a truncated 163 amino acid protein (Fig. 5).

Cd-IL-26-Tv4 is the largest splice variant identified having an additional 120 bp at the exon 3- exon 4 junction of the transcript.



Cd-IL26-Tv1
Length 516 bp ORF 516bp 171aa
No premature stop codon
Exon1(171), Exon 2 (57), Exon3 (135), Exon4 (66), Exon 5 (87)

Cd-IL26-Tv2
Length 538 bp ORF 408bp 135aa
Premature stop codon within Exon 4
Exon1(171), Exon 2 (57), **Exon3A (157)**, Exon4 (66), Exon 5 (87)

Cd-IL26-Tv3
Length 614 bp ORF 492 bp 163aa
Premature stop codon within Exon4
Exon1(171), Exon 2 (57), Exon3 (135), **new Exon3B (98)** Exon4 (66), Exon 5 (87)

Cd-IL26-Tv4
Length 636 bp ORF 636 bp 211aa
No premature stop codon , 40 aa extra
Exon1(171), Exon 2 (57), **Exon3A (157), new Exon3B (98)**
Exon4 (66) & Exon 5 (87)

Cd-IL26-Tv5
Length 497 bp ORF 393bp 130aa
Premature stop codon within Exon4B
Exon1(171), Exon 2 (57), Exon3 (135), **Exon4A (18) Exon4B (29) &** Exon 5 (87)

Fig. 4. Predicted exon intron usage of the dromedary IL-26 transcript variants. The cDNA sequence (start codon to stop codon) of the dromedary camel transcripts was aligned with the *Camelus ferus* shotgun sequence at the NCBI Genbank (LOCUS AGVR01041574) containing the IL-26 encoding region using the MGAlignIt program. Exonic regions are represented by solid boxes and intronic regions by slanting lines. The start and end positions of adjacent splicing exons are marked by numbers. The splice donor and acceptor sites are marked on all exon-intron boundaries. The thin bar below the mRNA sequence indicates the open reading frame.

This additional 120 bp nucleotide sequence corresponds to the 22 bp sequence of variant Cd-IL-26-Tv2 followed by the 98 bp of Cd-IL-26-Tv3 (Fig. 5). Alignment with the genomic sequence revealed that Tv4 transcript variant is the result of two different splicing mechanisms, namely the alternative splicing to extend the length of the Exon 3 from 135 to 157 (exon 3A) as in Tv2 and the introduction of an additional 98 bp – the exon 3B as in Tv3 (Fig. 4). Unlike the Tv2 and Tv3 splice variants, the addition of the 120 bp in the Tv4 transcript is in the same frame of translation and without any interrupting termination codons. The 636 bp ORF of this splice variant thus codes for a longer IL-26 isoform with 211 amino acids. The predicted protein encoded by this variant has 40 additional amino acids when compared to the normal IL-26-Tv1 (Fig. 6A).

We also identified and cloned a shorter 497 bp length transcript variant (Cd-IL-26-Tv5) from liver and lymph node. Unlike the other variants, this was 19 bp shorter than the canonical Cd-IL-26-Tv1 (Fig. 5). The Cd-IL-26-Tv5 is the result of a different splicing mechanism in which the exon 4 (66 bp) is disrupted into two smaller exons (designated as exon 4A (18 bp) and exon 4B (29 bp) by the introduction of a 19 bp intron (intron 4A). It may be noted that this 19 bp intron sequence 'GCTAGAGAGATGAAATCCA' was part of exon 4 in all other variants (Fig. 4). The IL-26 Tv5 also has an altered reading frame resulting in a truncated 393 bp ORF, which encodes for a protein of 130 amino acids (Fig. 6A).

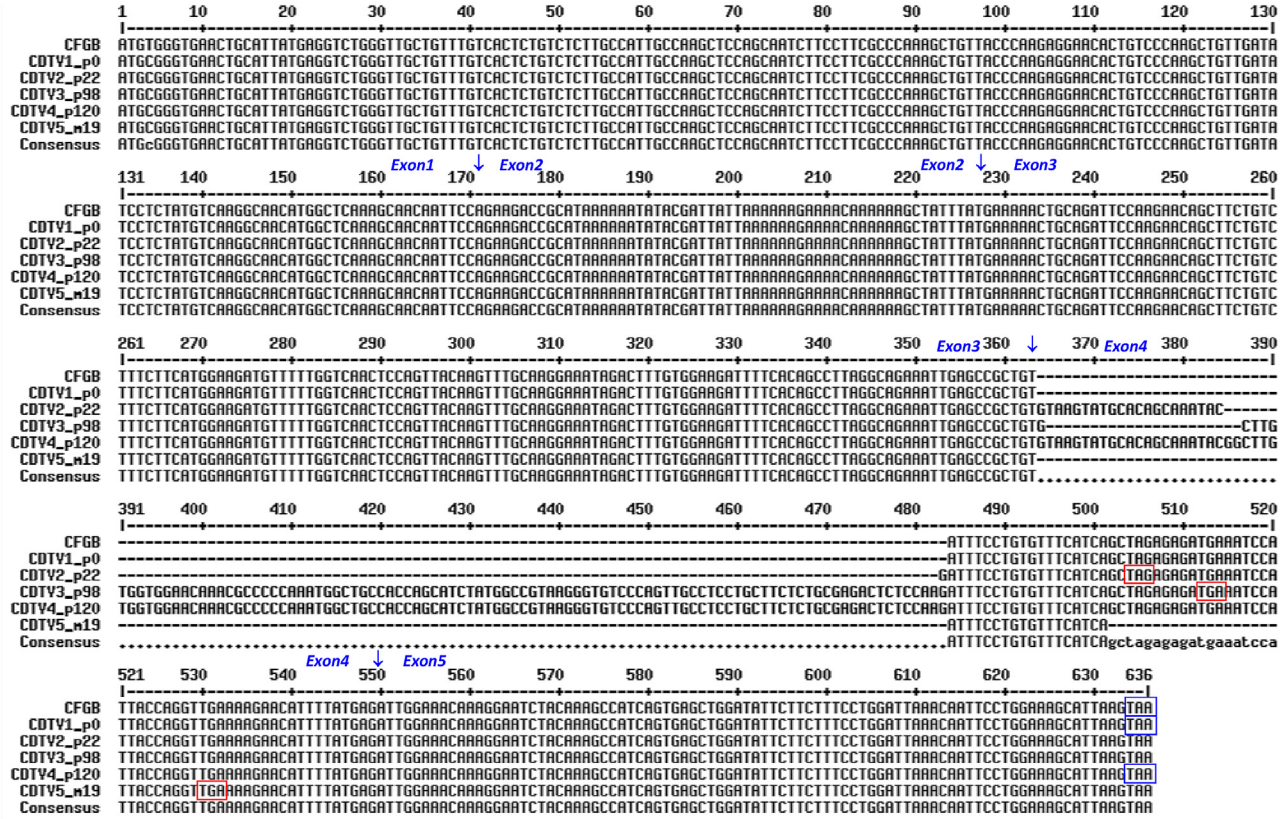


Fig. 5. Multiple Alignment of the nucleotide sequence of the ORF of all the five IL-26 transcript variants in comparison with the predicted Camelus ferus IL-26 sequence (CFGB) in the NCBI Genbank. The predicted splice junctions in the transcript (corresponding to Tv1) are marked by ↓. The termination codons in the protein coding sequence of each transcript are boxed with the premature termination codons (PTC) in the dromedary camel transcript variants Tv2, Tv3 and Tv5 marked in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Structural features of the predicted proteins of the dromedary IL-26 and the splice variants

The predicted protein of the canonical IL-26 isoform (Tv1) has 171 amino acids. Compared to this isoform, the protein encoded by Tv2, Tv3 and Tv5 are shorter comprising of 135, 163 and 130 amino acids, respectively. Only the protein encoded by the Tv4 is longer than the canonical IL-26 isoform, having 40 additional amino acids residues (Fig. 6A). Among the five cysteines in the mature IL-26, four are retained in all the five predicted protein variants (Cysteine at positions 32, 79, 102 and 121). Cysteine at the 124th residue is retained only in Tv1 and Tv5. The 5th cysteine occurs at the 132 aa position in Tv2 and 123 aa position in Tv3. In case of Tv4 protein, after the fourth cysteine (Cys-121), there are 40 additional amino acids compared to the Tv1. However the Cysteine corresponding to Cys-124 (Cys-164) is retained in Tv4 protein. It may be noted that there is an additional Cysteine (Cys-154) among the extra 40 amino acids in the Tv4 protein, making the total number of Cysteines to six in this isoform (Fig. 6A).

We constructed 3-D homology models of the predicted mature proteins of human and all the five camel IL-26 using the PHYRE and SWISSMODEL protein modeling servers (Fig. 6B). The canonical camel IL-26 protein is predicted to contain six alpha helices (Helices A–F) like the human protein (Fig. 6B). The predicted proteins encoded by the transcript variants Tv2, Tv3 and Tv5 had only four alpha helices as they lacked the C terminal region containing Helix E and F. In spite of the extra 40 amino acid residues in the predicted protein of the Tv4, it seemed to maintain the overall six alpha helical structures as per secondary structure analysis and homology modeling (Figs. 6A and B).

3.5. Analysis of pattern of IL-26 transcript variant expression in different tissues, mitogen stimulated PBMC and camel Pox virus infected ovary fibroblasts

To determine the pattern of tissue distribution of the IL-26 transcript variants, we used a new primer pair that amplified a shorter fragment of the IL-26 cDNA. This primer pair produced amplicons of length 333, 355, 431, 453 and 314 bp from the IL-26 transcript variants Tv1, Tv2, Tv3, Tv4 and Tv5, respectively. The amplicons from different tissue cDNAs were analyzed using an Agilent 2100 Bioanalyzer for better size estimation of the transcript variants. All the five transcript variants were detected in lymph node. Four transcript variants (Tv1–Tv4) were detected in blood and spleen. Only Tv1 was detected in kidney and liver (Fig. 7**A**). In the in-vitro stimulation of camel PMBC, it was observed that the expression of IL-26 Tv2 and Tv3 are induced upon Poke Weed Mitogen or ConA stimulation (Fig. 7**B**).

The expression of IL-26 was observed in camel ovary fibroblastic cells maintained at our laboratory. Expression of IL-26 transcript variant Tv2 was induced in these cell lines infected with camel pox virus (Fig. 8).

4. Discussion

We report here the identification and isolation of the orthologue of *IL-26*, a less explored interleukin from a less studied animal, the dromedary camel. Originally identified in Herpesvirus infected cells, IL-26 is also expressed in many other transformed T cell lines, peripheral mononuclear cells, activated NK cells and T cells (Donnelly et al., 2010). The gene organization of the camel *IL-26*

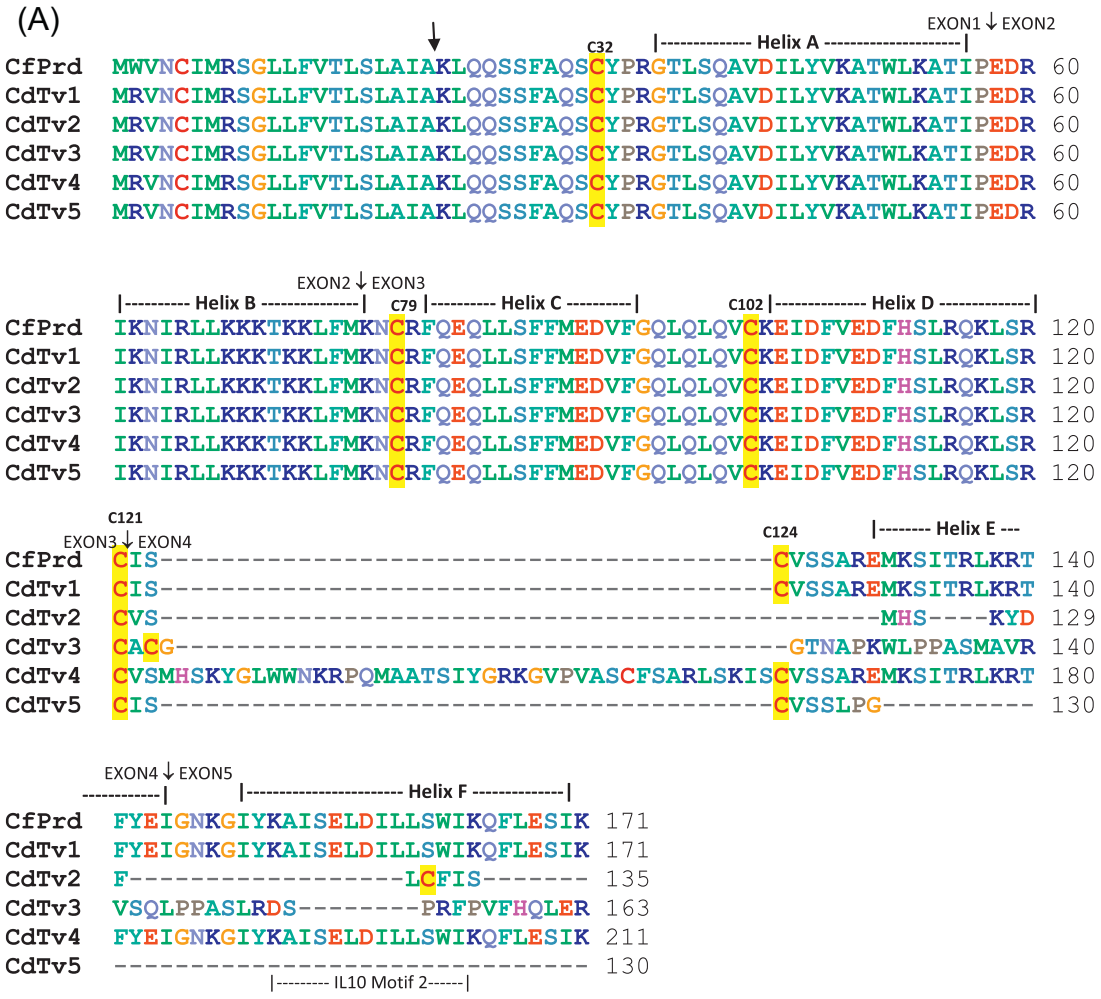


Fig. 6A. Alignment of the predicted amino acid sequences of the five transcript variants of dromedary IL-26. CfPrd refers to the *Camelus ferus* IL26 sequence predicted from the genome (NCBI GenBank Acc No XP 6189751.1). The *IL-26* normal transcript variant, Tv1 has 171 amino acids like the predicted sequence from GenBank. Tv2 Tv3 & Tv5 result in truncated proteins due to premature termination of translation. Transcript variant Tv4 has additional 40 amino acids when compared to the Tv1 protein. IL-26 protein regions corresponding to the exon intron boundaries are marked by downward arrow. The second IL-10 signature motif sequence conserved in most mammalian IL-26 is marked. The truncated proteins encoded by the variants Tv2, Tv3 and Tv5 lack this motif.

was elucidated by aligning the cDNA sequence to a *C. ferus* shot gun scaffold sequence in the NCBI GenBank. We also report for the first time four novel transcript variants of *IL-26* which are generated by alternative splicing at the exon 3- exon 4 regions of the gene.

IL-26 is a member of the IL-10 family of cytokines that includes IL-10, IL-19, IL-20, IL-22 and IL-24. The members of the IL-10 family of cytokines are characterized by a predominantly alpha helical structure composed of five to seven alpha helices. The helices are arranged to form a compact bundle with hydrophobic amino acids constituting an internal hydrophobic core, which is stabilized by one to three disulphide bridges (Zdanov, 2010). The predicted mature protein of the camel *IL-26* also seems to be predominantly alpha helical with the helices constituting around 70.6% of the total structure. Six alpha helical bundles have been predicted for the mature protein encoded by Tv1 (Fig. 1). The crystal structure of IL-26 has not been reported so far and structural models have been predicted based on already identified structures of other IL-10 family cytokines – human IL-10 and IL-19. The structure of IL-26 based on the IL-19 scaffold has been proposed to be a better model than IL-10 as evidenced by the proper alignment of the cysteine residues. Further data suggest both IL-19 and IL-26 can bind to the same receptor IL20R1 (Donnelly et al., 2010). The mature dromedary camel IL-26 features five conserved cysteine residues (at amino acid positions 32, 79, 102, 121 and 124) reported to be conserved in other mammalian IL-26 homologs also (Fig. 1). The IL-26 structure based on the IL-19 scaffold suggests that Cysteine pairs Cys-32/Cys-124 and Cys-79/Cys-121 form disulphide bonds, whereas Cys-102 at the N-terminal end of helix D may be involved in intermolecular disulphide bonds facilitating dimerization (Donnelly et al., 2010).

Two signature motifs belonging to the IL-10 family have been identified and described previously (Ouyang et al., 2014; Pinto et al., 2007) L-[FILMV]-X(3)-[ILV]-X(3)-[FILMV]-X(5)-C-X(5)-[ILMV]-[ILMV]-X(3)-L-X(2)-[IV]-[FILMV] and KA-X(2)-E-X-D-[ILV]-[FLY]-[FILMV]-X(2)-[ILMV]-[EKQZ]. In the camel and human IL-26, the second motif conform to the signature sequence, whereas the first motif is only partially matched (Fig. 2). IL-26 is unique among the members of the IL-10 family due to its highly cationic nature. Human IL-26 has a predicted isoelectric point (pI) of 10.7 which is mainly attributed to the presence of 30 Lys and Arg residues in the mature protein (Braum et al., 2012). The camel IL-26 also has a predicted pI of 10.45 and has 27 Lys and Arg residues in the 150 amino acid mature protein. The Tv2 with the 114 aa mature protein has an estimated pI value of 10.3 with 19 Lys and Arg residues. Similarly Tv3 with the 142 aa mature protein has 23 Lys and Arg residues and a predicted pI of 10.69. IL-26 is reported to have a high heparin binding affinity due to the strong positive charge and the helix A-B region has been suggested as the

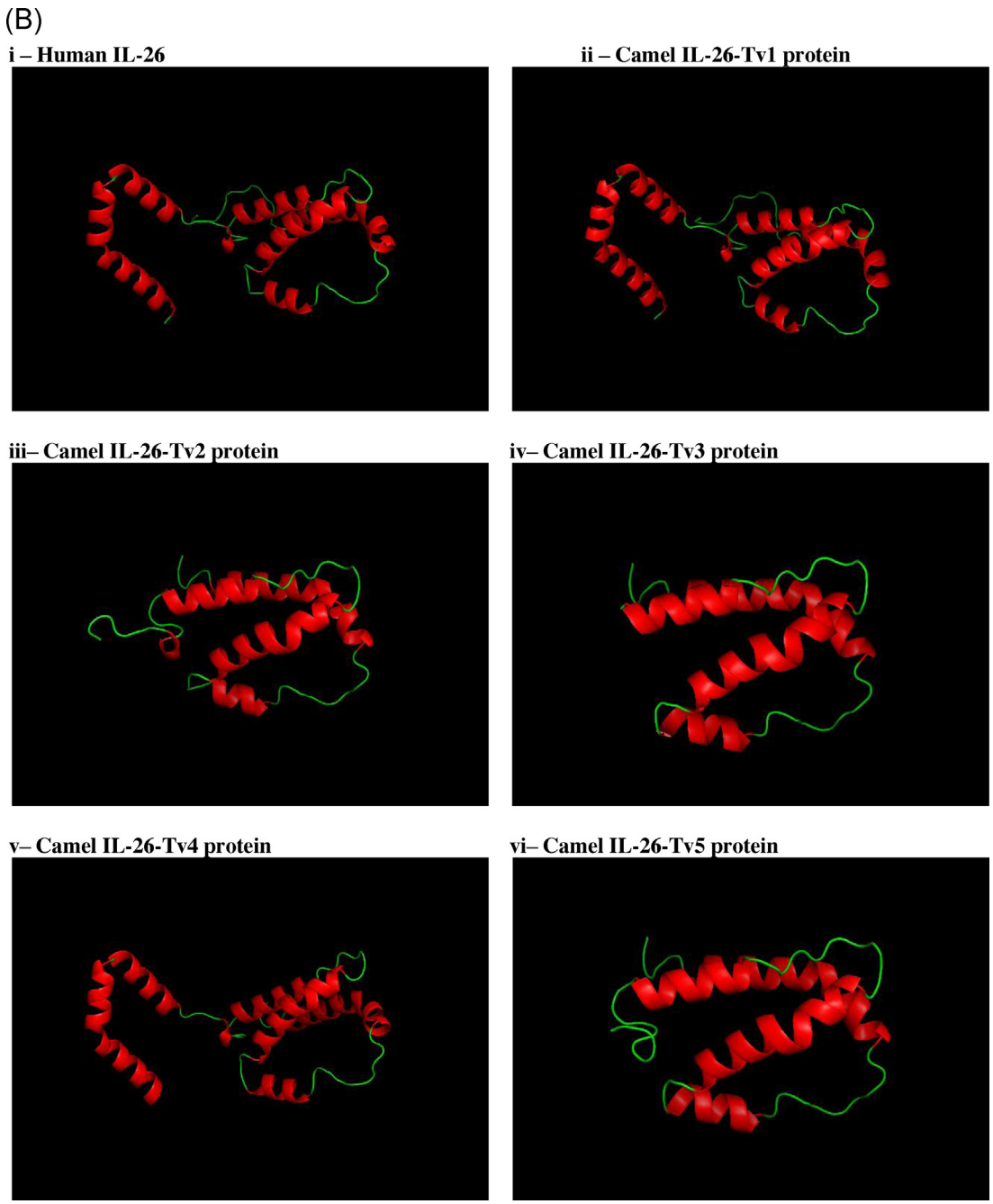


Fig. 6B. Structural comparison of the human IL-26 and the predicted camel IL-26 proteins: Using the PHYRE automatic protein structure modeling server we created 3D homology models for the human IL-26 (i) and the predicted mature proteins of all the five alternative splice variants of camel IL-26-Tv1 to Tv5 (ii to vi)

heparin binding domain for the cytokine (Hör et al., 2004). Due to the affinity of IL-26 to heparin, the secreted IL-26 is presumed to bind immediately to glycosaminoglycan moieties on cell surfaces which leads to local recruitment and enrichment of the IL-26 protein on cell surfaces (Braum et al., 2012).

In the phylogenetic analysis, dromedary IL-26 and other predicted camelid IL-26 sequences (Bactrian camel and alpaca) clustered in one single group (Fig. 3) analogous to the taxonomic relationship among the members of the mammalian superorder Cetartiodactyla. The Tylopods (Camelids), Ruminants (Bovine, Sheep and Goat), Suinae (pig and peccaries) and Cetacodonta (Whales, dolphins & hippopotamus) are the four major groups of this super order. The camelid group stands out as a distinct group in the phylogenetic analysis, which follows a similar pattern with other immune genes of camels reported earlier (Odbileg et al., 2006; Premraj et al., 2013).

In the current study, we also identified four novel splice variants of the dromedary *IL-26*. Based on the alignment of the cDNA sequences with the *C. ferus* genomic sequence, it could be inferred that the *IL-26* isoforms are generated by alternative splicing through different mechanisms. Alternative splicing is a versatile mechanism to increase protein diversity in which diverse transcripts potentially encoding diverse proteins are produced from the same locus of the genome. Recent studies have revealed that many of these alternative splice variants, previously presumed to encode truncated protein variants are actually targeted by a eukaryotic mRNA surveillance mechanism called Nonsense-mediated mRNA decay (NMD) (Lareau et al., 2007). Alternative splicing combined

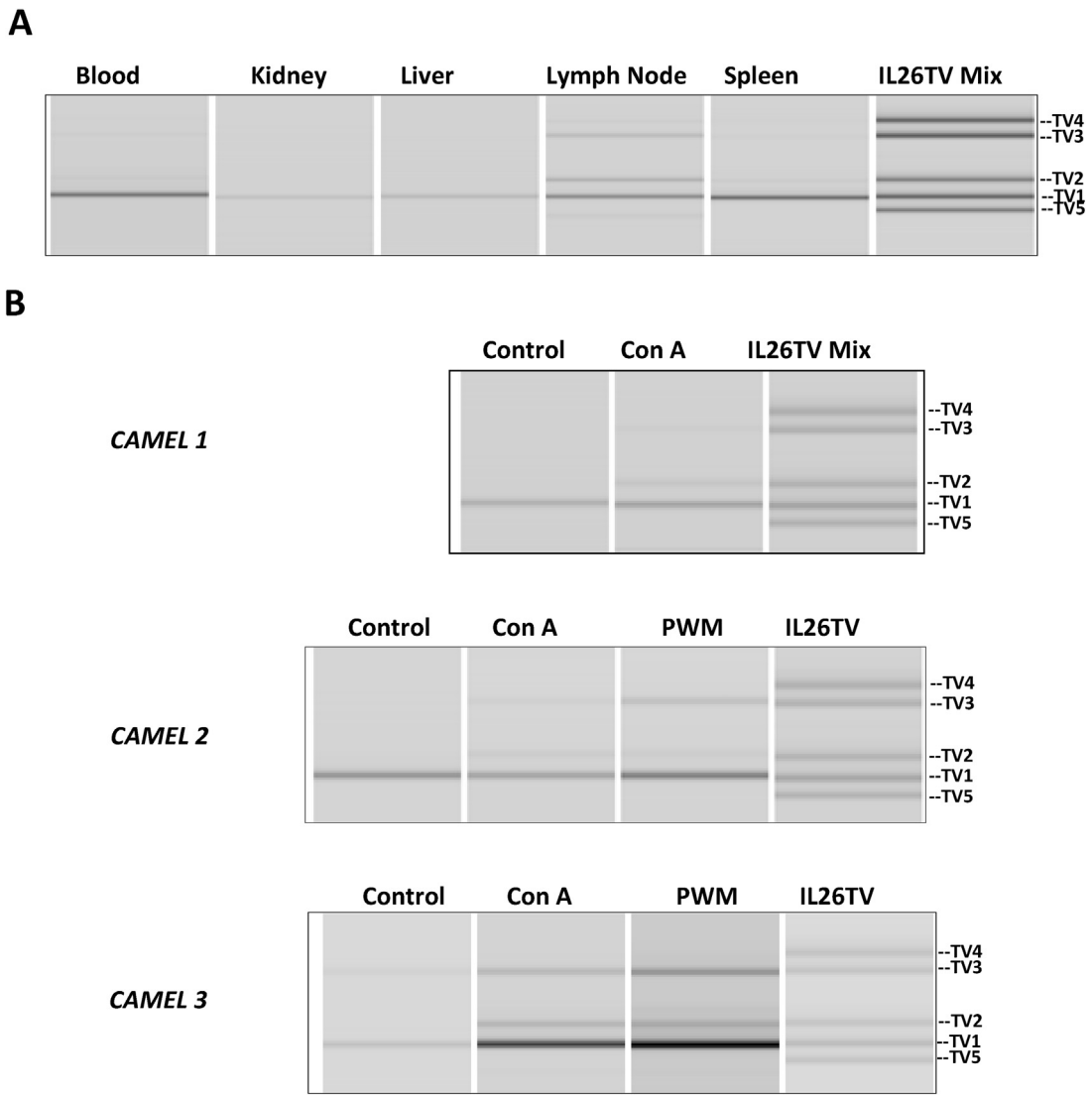


Fig. 7. Expression of IL-26 alternative splice variants in different tissues of dromedary camel tissues and in-vitro mitogen stimulated PBMCs (A) Total mRNA was isolated from camel tissues were reverse transcribed and the cDNA was used as template for amplification with IL26XF1&IL26XR1 primers **(B)** Camel PBMCs were stimulated in vitro with PWM or Con A (10 μg/ml) for 16 hrs. Unstimulated PBMCs under identical culture conditions were also kept as control for 16 h. RNA isolated from stimulated samples and control (3 different camels) was used for cDNA synthesis. IL26 expression profile was analyzed with IL26XF1 and IL26XR1 primers on an Agilent 2100 Bioanalyzer using a DNA 1000 kit. Size standards for IL-26 transcript variants were made by PCR amplification of the previously cloned camel IL-26 Tv1 to Tv5 variants using IL26XF1 and IL26XR1. Equal mixture of these amplicons was used to create the size standards loaded in lane marked IL26Tv Mix.

with NMD serves as an elegant method of regulating gene expression in eukaryotes. It has been proposed that a termination codon located more than 50 bp upstream of the last exon junction is recognized as premature termination codon (PTC) and the transcript is subjected to NMD (Nagy and Maquat, 1998). We analyzed *in-silico* whether the four alternative IL-26 transcript variants could be subjected to NMD based on this rule. For Cd-IL26 Tv2, Cd-IL26 Tv3 and Cd-IL26 Tv5 variants the new stop codons are located only 46, 38 and 20 bp upstream from the last exon junction (Fig. 5) and hence may not be subjected to NMD. The Cd-IL26-Tv4 like the normal Cd-IL-26-Tv1 does not have any interrupting stop codon and hence not subjected to NMD. It appears that all the four transcript variants could be translated normally without NMD and encode IL-26 protein isoforms. However, some of these variant protein isoforms may differ in their activity when compared with the canonical IL-26 protein as the truncated variants lack required regions for receptor binding.

The coding sequences for first four helices of IL-26 protein (Helices A–D) are encoded by the Exon 1–Exon 3. The amino acid sequences of these four helices are identical in all the five transcript variants (Fig. 6A). The second IL-10 signature motif located in the helix F (amino acid residue sequence KAISELDILLSWIK) is completely conserved in human, camel and other mammalian IL-26 (Fig. 1) proteins and contains the amino acids predicted to be involved in binding to the IL20R1 receptor chain (Donnelly et al., 2010). The truncated proteins predicted to be encoded by the variants Tv2, Tv3 and Tv5 lack this motif (Figs. 6A and B) and may exert different biological action compared to the wild type. Among the IL-10 family of cytokines, alternative splicing has been reported in IL-10 (Wu et al., 2005) and IL-24 (Sahoo et al., 2008). In IL-10, the alternative splice variant IL-10 δ3 lacking exon 3 was observed in children with relapsed childhood Acute Lymphoblastic Leukemia (ALL). It was proposed that IL-10 δ3 isoform may be involved in modulating IL-10 mediated biological effects and the expression of IL-10 δ3 isoform was correlated with a better response to chemotherapy in relapsed childhood ALL (Wu et al., 2005). Splice variants lacking exons 3 and 5 of human *IL-24* were identified from normal melanoblasts, and the loss of the expression of this splice

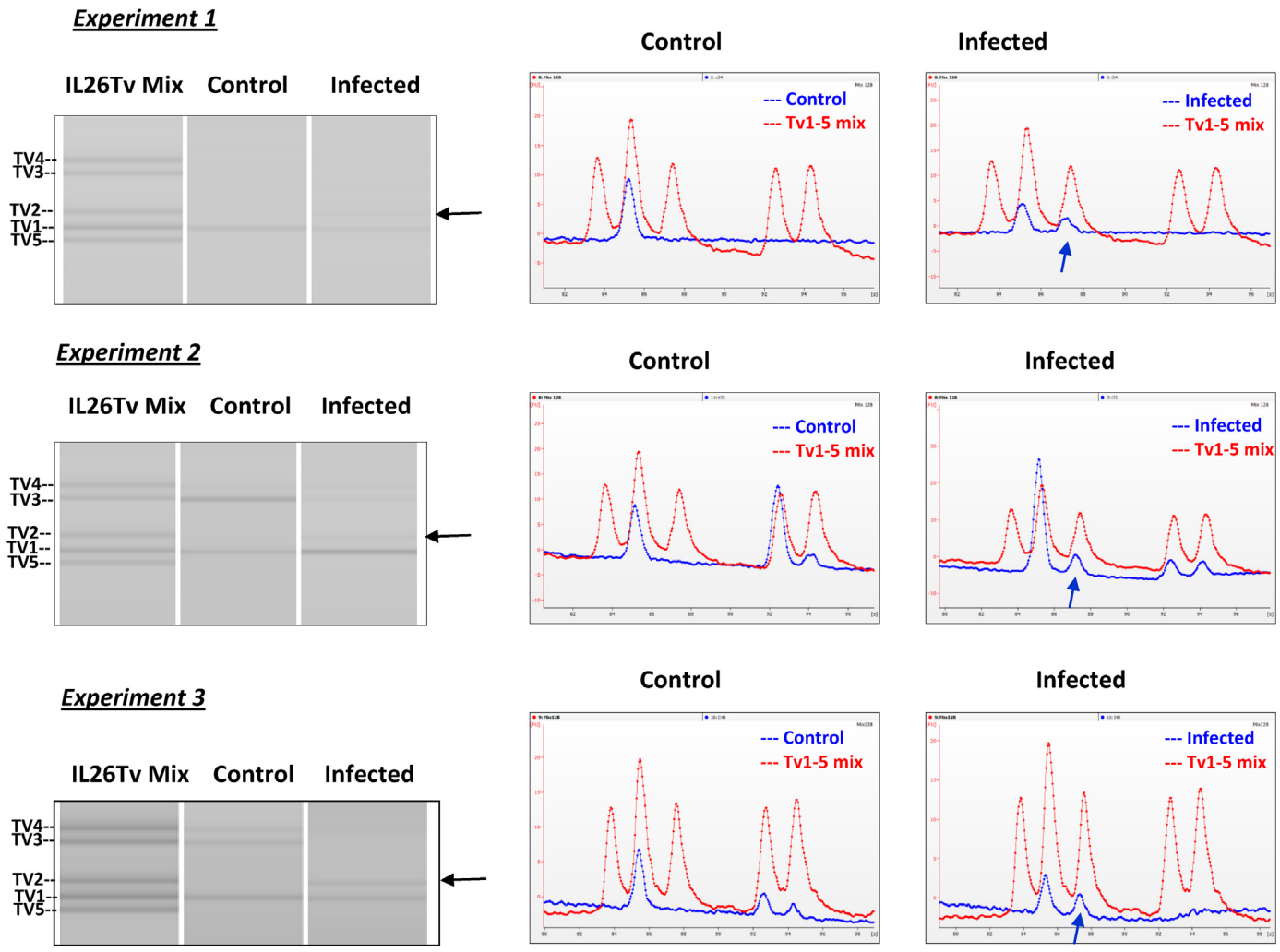


Fig. 8. Analysis of Expression of camel IL-26 alternative splice variants in camel ovary fibroblasts cells infected with Camel pox virus: Camel ovary fibroblasts were infected with Camel Pox virus *in-vitro* and RNA isolated post 48 hrs after infection from virus infected and uninfected cells. IL-26 transcript variants were amplified by RT-PCR using IL26XF1 /IL26XR1 primers and analyzed on Agilent 2100 Bioanalyzer using a DNA 1000 kit. Size standards for IL-26 transcript variants were made by PCR amplification of the cloned camel IL-26 Tv1 to Tv5 variants using IL26XF1 and IL26XR1. Equal mixture of these amplicons was used to create the size standards and analyzed along with the samples. First panel in each set represents the virtual image for the fragments separated on the Agilent 2100 Bioanalyzer. The second and third panel represent the electropherograms of IL-26 transcript variants of control/infected (blue) overlaid with the IL-26Tv standard mix (red) for size comparison. The induction of IL-26 transcript variant 2 in virus infected samples is marked by an arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

variant was associated with metastatic melanoma (Allen et al., 2004). In mouse, a novel splice variant of *IL-24/FISP*, lacking 29 nucleotides from 5′ end of exon 4 was identified and designated as FISP-sp. This variant, unlike the normal IL-24/FISP was not secreted but was retained in the endoplasmic reticulum, dimerised with FISP and inhibited FISP induced apoptosis (Sahoo et al., 2008). Alternative splice variants in many cytokines have been reported to act as functional antagonists of the corresponding wild-type cytokines and suggest that the alternative splicing in response to various stimuli can significantly alter the cellular activity (Sahoo and Im, 2010). The identification of the transcript variants from dromedary camel *IL-26* is the first report of alternative splicing for *IL-26* in species in which the gene has not been inactivated. Based on whole transcriptome data and RT-PCR, alternative transcripts have been identified in horse *IL-26*, but the gene in horse has been inactivated by one base pair deletion in exon2 and this gene inactivating mutation is conserved in related species like the Przewalski's horse and donkey (Shakhsi-Niaei et al., 2013). The protein expression, localization and functional activity of the camel IL-26 isoforms need to be evaluated in future studies.

We also analyzed for the presence of different IL-26 alternative transcripts in various camel tissues and mitogen stimulated PBMC. *IL-26* transcripts were detected from lymph node, liver, blood and spleen (Fig. 7**A**). IL-26 is primarily produced by activated T-cells, but has also been reported to be expressed in peripheral blood mononuclear cells, subsets of NK cells from secondary lymphoid organs (Braum et al., 2013) and certain types of synoviocytes in rheumatoid arthritis (Corvaisier et al., 2012). In liver lesions from HCV infected patients, the IL-26 expression is attributed to the activity of infiltrating lymphocytes and hepatocytes did not seem to express IL-26 (Miot et al., 2014). Among the camel tissues, lymph node seemed to have all the five alternative splice variants, whereas four variants were detected in blood and spleen. Apart from one report of alternate splice variants in horse kidney and liver (Shakhsi-Niaei et al., 2013), there is no other information on tissue distribution of alternate splice variant of IL-26. Splice variants Tv2 and Tv3 seemed to be up regulated in-vitro stimulated camel PBMC (Fig. 7**B**). Infection of camel fibroblasts with camel pox virus seem to induce the expression of IL-26 Tv2 (Fig. 8). IL-26 expression is reported to be induced upon Herpesvirus and Hepatitis C virus infection (Knappe

et al., 2000; Miot et al., 2014). It may be noted that the Tv2 and Tv3 lack the characteristic IL-10 signature motif and residues predicted to be involved in binding to the IL20R1 receptor.

In conclusion, our study reports the identification of a functional IL-26 transcript in the dromedary camel. IL-26 has been reported to be functionally inactivated by independent mechanisms in many diverse groups of mammals like horse, mouse and predicted to be inactivated in elephant and hedgehog. We also demonstrate the presence of unique *IL-26* alternative splice variants; however the functional roles of these variants in camel immune system need to be further elucidated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2015.06.022

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