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Cloning and Sequence Analysis of 163R and 201R Genes of Camelpoxvirus from Indian Dromedaries (Camelus Dromedarius)

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Abstract

In the present study, skin scabs were collected from a total number of 10 severely affected Dromedarian camels (Camelus dromedarius) maintained by the Border Security Force (BSF) of Bikaner, Rajasthan an, India. Two immunomodulatory protein genes, 163R and 201R of camelpoxvirus (CMLV) were amplified by Polymerase Chain Reaction using gene specific primers, followed by cloning and sequencing using the standard experimental protocols. CMLV 163R protein is the orthologue of Vaccinia virus A46R protein possessing the VIPER (viral inhibitory peptide of TLR4) sequence (an 11 amino acid peptide-KYSFKLILAEY) at its α1 helix. The RGD motif necessary for mediating the immunomodulatory mechanism is present in the amino acid sequences of CMLV 201R protein. Sequence analysis of both 163R and 201R genes revealed that CMLV obtained from India shared 100% identity with CMLV-Iran and CMLV-Kazakhstan strains both at DNA and protein level. Based on the nucleotide and amino acid residue sequence identities and phylogenetic analyses of these genes, it is found that CMLV-India is forming a cluster with Kazakhstan and Iranian CMLV isolates.

Keywords: Camelpoxvirus; India; Immunomodulatory protein genes; PCR; Cloning; Sequencing; Phylogeny

Abbreviations: CMLV: Camelpoxvirus; CPXV: Cowpoxvirus; OPV: Orthopoxvirus; RGD: Arginine Glycine Aspartic acid; VACV: Vaccinia Virus; VIPER: Viral Inhibitory Peptide of TLR4

Introduction

Camelpox is one of the most important infectious and contagious diseases of camels in almost every region where the camel is reared with the exception of Australia.

The disease is caused by the camelpoxvirus (CMLV), which belongs to the Orthopoxvirus (OPV) genus of the Poxviridae family. It is closely related to the Variola virus, the etiological agent for smallpox [1-3]. Camelpox inrection is having considerable economic importance due to high morbidity, relatively high mortality in younger animals, loss of condition and reduced milk production in lactating ones [4].

Poxviruses contain many genes that are non-essential for viral replication in cell culture but important for modulating and circumventing the host-response and thus influence the course of poxvirus infection and pathology. Such genes are designated as virulence genes [5]. A portion of these genes has been identified that are important for viral replication in only a subset of tissue culture cells, which were derived from different tissues or animal species and are commonly referred to as host range genes or factors and are thought to be responsible for poxvirus-specific differences in tropism and host range [5,6].

Protein 163R of CMLV is an orthologue of vaccinia virus (VACV) A46R, which targets the host Toll-like–interleukin-1 resistance (TIR) adaptors myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like, TIR domain–containing adaptor inducing IFN- (TRIF), and the TRIF-related adaptor molecule and thereby interferes with downstream activation of mitogen-activated protein kinases and nuclear factor kB [7].

Protein 201R of CMLV contains a signal peptide, an RGD motif and shows amino acid similarity to the C-terminal domain of OPV TNF receptors CrmB and CrmD [8]. RGD motifs mediate the binding of proteins to cell surface integrins: therefore, 201R might be a secreted protein that binds back to infected and/or uninfected cells. A similar protein is encoded by CPV-GRI gene B21R and VVCOP contains a disrupted version of this gene (C13L and C14L) [9,10].

Camelpoxvirus infection of camels can produce severe disease, suggesting CMLV may interfere with the host response to infection. In the context of increasing OPV infections, particular attention should be given to camelpox outbreaks, as well as to the identification of any human infections [1].

Camelpox is considered as emerging public health problem during this decade due to increased reported cases and outbreaks in camels [11]. Additionally, the first conclusive evidence of zoonotic CMLV infection in humans (unvaccinated smallpox individuals) associated with outbreaks in dromedarian camels in the North West region of India has been reported [12]. Further epidemiological studies of camelpox on the endemic regions are necessary to assess the circulation of CMLV, both in camels and humans in order to know its public health significance1. Dahiya and his team recently reviewed camelpox disease with respect to its epidemiology, mode of transmission, virion characteristics and its immune modulation [13].

While several immune-modulations mechanisms have been identified in cowpoxvirus (CPXV) and vaccinia virus (VACV), only few articles reported those of CMLV [14-18]. In order to gain the scientific knowledge in the field of immune escape pathways being used by CMLV, as a preliminary step, the sequence information about the immunomodulatory protein genes encoded by CMLV is essential. Very recently, Bera and his team found out that different strains of CMLVs are circulating in India and the presence of inconsistent mutations within them could play an important role in adaptation of CMLVs in humans [19].

Further, Afonso and his team concluded that major genomic differences between CMLV and other OPVs occur in terminal genomic regions affecting a large number of genes with likely functions involving virulence or host range [20]. The differences clearly distinguish CMLV from other OPVs. CMLV genomic sequence provides a basis from which comparisons with other OPVs may be made, thus contributing to understanding the genetic basis of OPVs virulence and host range. The goal of the present study is to document the sequence information of the two genes viz., 163R and 201R as well as to find out the presence of the mutations within them, if any. The rationale behind in choosing these two genes is that the said two genes present at the terminal region of CMLV are known to play a major role in modulation or evasion of host immune responses as reported earlier [2].

Therefore, on the same line of our previous report pertaining to the phylogenetic analysis of three immunomodulatory protein genes of CMLV obtained from India, the present study was carried out with the objective of sequence analysis of these two immunomodulatory protein genes viz., 163R and 201R as well as comparison of these gene sequences of CMLV isolates obtained from India with that of other orthopoxviruses available in the NCBI database was made using the phylogenetic analysis [21].

Materials and Methods

Scab Materials

An outbreak of camelpox occurred in the Dromedarian camels (Camelus dromedarius) maintained by the Border Security Force (BSF) of Bikaner, Rajasthan in January 2008. The samples of scabs were collected from ten severely infected animals showing clinical signs, symptoms and typical lesions and subjected to PCR.

Polymerase Chain Reaction

Skin scabs were collected from the suspected lesions of camelpox and stored at -20°C until use. DNA was extracted from the skin scabs using GeneiUltrapure™ Mammalian Genomic DNA Purification Kit-Tissues (Bangalore GeNei Pvt. Ltd., India) according to the manufacturer's instructions. Reaction volumes for the PCR of 50 µl were used and contained 5 µl of 10× buffer with 15 mM MgCl2, 10 mM of each dNTPs, 100 pmol of each oligonucleotide primer, 100 ng of DNA sample and 3U Taq DNA polymerase . Both 163R and 201R genes of CMLV obtained from India were amplified from the genomic DNA isolated from the Dromedary camel skin scabs infected with camelpox by PCR using the primers designed based on CMLV gene sequences reported in GenBank Accession No.AF438165 (Table 1). The reaction mixture was subjected to initial denaturation of the template at 94°C for 5 min in a thermal cycler (Eppendorf, Germany). Cycling conditions for PCR were 35 cycles of 60 s at 94°C, 60 s at annealing temperatures depending on the genes to be amplified (Table 1) and 60 s at 72°C, followed by a final extension for 10 min at 72°C. The total genomic DNA isolated from the Dromedary camels infected with contagious ecthyma was included as a negative control in the PCR.

Gene	Primer Sequences (5′-3′)	Predicted size (bp)	Annealing temperature (in Celsius)	GenBank Accession No.
163R	Forward – 5’ cgaaggtaccatggcgtttgatatatcagttaa 3’	723	60°	AF438165
	Reverse- 5’ attggcggccgctacatccgtttccctgtcggttac 3’			
201R	Forward – 5’ cgaaggtaccatgatgatatacggattaatag 3’	570	57°	AF438165
	Reverse – 5’ attggcggccgcaccatcaaccccattactcatcaatc 3’			

Table 1: Primer sequences used to clone 163R and 201R encoding genes of camelpoxvirus.

Cloning and Sequencing of PCR Amplified DNA Fragments

The PCR amplified products amplified from 10 individual scab materials were validated in 1.2% agarose gel. Out of 10 positive amplicons, only three amplicons corresponding to genes encoding 163R and 201R genes of camelpoxvirus (CMLV) were cloned into pGEM-T Easy vector (Promega Corp., Medison, USA). The ligated mixtures for both genes were individually transformed into Escherichia coli DH 5α [22]. The positive clones were confirmed by colony PCR using gene-specific primers and restriction analysis with EcoRI. Three positive clones in each gene were sequenced at the sequencing facility, Delhi University (South Campus), Delhi. Since pGEM-T easy vector was used for the cloning purpose, universal T7 and SP6 primers were used for the sequencing of recombinant clones. The primer sequences used for the sequencing were based on respective promoter sequences. Nucleotide sequences were submitted to Genbank and accession No. obtained (163R gene JQ927476; 201R gene JQ917915). The determined nucleotide sequences and the deduced amino acid residue sequences of 163R and 201R genes of CMLV were analysed with the BLAST program (NCBI) search of GenBank. Nucleotide identity, amino acid identity and comparison of the sequences with published sequences of members of Poxviridae available in the GenBank database were carried out using the computer software computer software CLC Genomics Workbench Version 6.5.2. The multiple alignment of the protein sequences was created using MUSCLE and a phylogenetic tree was constructed based on the amino acid residue sequences of 163R and 201R genes of CMLV, by the Neighbour-joining method using Mega 6 (Molecular Evolutionary genetics Analysis software with bootstrap values calculated for 1000 replicates [23,24]. The open reading frame (ORF) and translation of nucleotide sequences to amino acid residue sequences were predicted by using the computer software Generunner version 3.05 (Hastings Software inc.

Hastings, NY, USA; http://www.generunner.net). In the protein, The domain search was conducted using the Pfam site: http://pfam.xfam.org/.

Results

Cloning and Sequence Analysis of 163R Gene

All the 10 DNA samples isolated from 10 different scab materials were showing the amplification of a fragment of around 723 bp size. Out of 10 PCR positive amplicons, only three representative of 163R encoding gene of camelpoxvirus (CMLV) were transformed into E. coli DH5α. The plasmids containing the gene of interest (recombinant plasmids) were confirmed by colony PCR using gene specific primers and restriction enzyme analysis with EcoRI. Three recombinant plasmids (one for each sample) extracted from individual bacterial colonies grown on LB agar containing 50µg/ml ampicillin were chosen for sequencing. The sequence data obtained using T7 and SP6 primers for all three recombinants were identical and the consensus sequence was confirmed by the use of BLAST program.

The complete amino acid residue sequences of 163R gene of CMLV obtained from India and its comparison to the 163R gene of other orthopoxviruses are shown in Figure 1. The open reading frame of the 163R gene of CMLV obtained from India was 723 bp in length, encoding 240 amino acids. Nucleotide and deduced amino acid residue sequence homologies of 163R gene of CMLV obtained from India and the other orthopoxviruses (complete sequences available) are shown in Table 2. The functional domain found in all the 10 orthopoxviral proteins analysed is Poxvirus (Pfam id: PF06227.7).

S. No.	Virus isolate	Host	Country and year	NCBI Accession No.	% Identity	
					Nucleotide	Amino acid
1	CMLV-India	Camel	India & 2008	JQ927476	-	-
2	CMLV- M-96	Camel	Kazakhstan & 2010	AF438165	100.0	100
3	CMLV- CMS	Camel	Iran	AY009089	100.0	100
4	Cowpox virus	Human	Germany & 1990	HQ420896	98.1	96.6
5	Vaccinia virus	Not available	Canada & 2009	JN654978	97.7	95.0
6	Variola virus -New Delhi	Not available	India & 1953	DQ441428	97.7	97.0
7	Variola virus-Madras	Not available	India & 1953	DQ441427	97.7	97.0
8	Horsepox virus	Horse	Mangolia & 1976	DQ792504	97.6	94.5
9	Rabbitpox virus	Not available	Not available	AY484669	97.6	95.0
10	Ectromelia virus	Not available	Not available	AF012825	97.6	95.0

Table 2: Percent identity of nucleotide and amino acid of 163Rgene of CMLV-India with different orthopoxviruses.

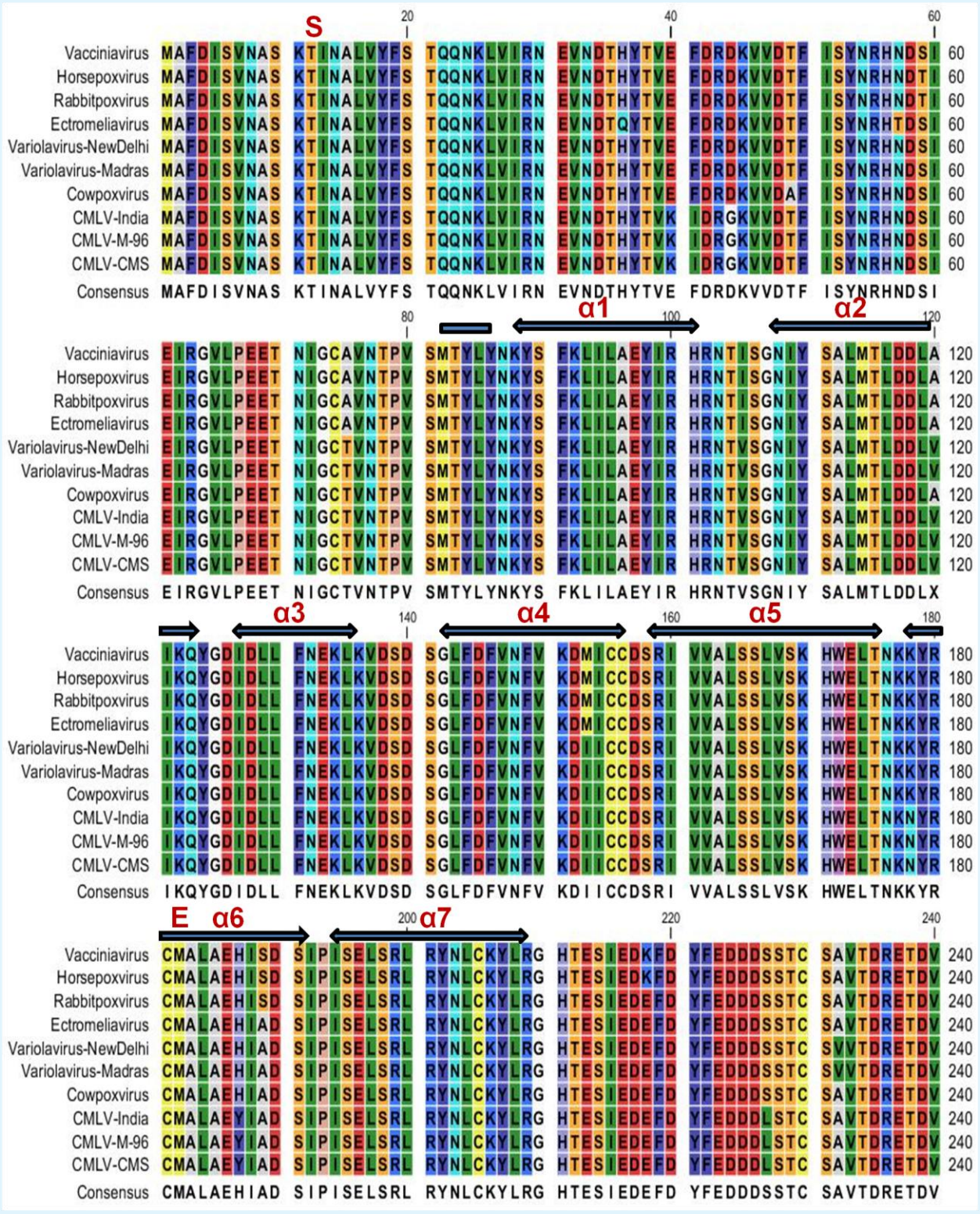

Figure 1: Alignment of amino acid residue sequences of Indian CMLV 163R gene with its orthologues from different orthopoxviruses. S and E denote the start and end of the functional domain Poxvirus (Pfam id: PF06227.7), respectively.

Both the nucleotide and deduced amino acid residue sequences of 163R gene of CMLV obtained from India showed 100% identity with both CMLV-Kazakhstan and CMLV-Iran strains, which was further confirmed by phylogenetic analysis (Figure 2).

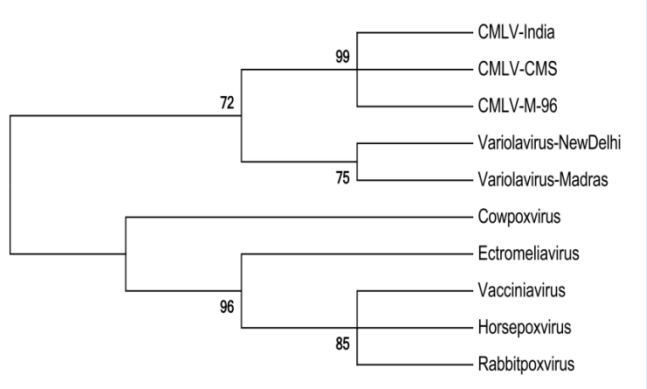

Figure 2: Phylogenetic tree based on amino acid residue sequences of Indian CMLV 163R gene and its orthologues from different orthopoxviruses, constructed by the neighbor-joining method using Mega 6(Molecular Evolutionary genetics Analysis software with bootstrap values calculated for 1,000 replicates. Horizontal distances are proportional to the genetic distances. Vertical distances are arbitrary. The numbers at each branch represent bootstrap values (1000 replicates).

Cloning and Sequence Analysis of 201R Gene

Similar to 163Rgene, in case of 201R gene also, all the 10 genomic DNA samples isolated from 10 different scab materials were showing the amplification of a fragment of around 570 bp size. As discussed above for 163R encoding gene of camelpoxvirus (CMLV), out of 10 PCR positive products representative of 201R encoding gene of CMLV, only three were transformed into E. coli DH5α. The recombinant plasmids were confirmed by standard colony PCR using gene specific primers followed by restriction enzyme analysis with EcoRI. Three recombinant plasmids (one for each sample) extracted from individual bacterial colonies grown on LB agar containing 50 µg/ml ampicillin were chosen for sequencing. The sequence data obtained using T7 and SP6 primers for all three recombinants were identical and the consensus sequence was confirmed by the use of BLAST program.

The complete amino acid residue sequences of 201R gene of CMLV obtained from India and its comparison to the 201R gene of other orthopoxviruses are shown in Figure 3. The open reading frame of the 201R gene of CMLV obtained from India was 570 bp in length, encoding 189 amino acids. Nucleotide and deduced amino acid residue sequence homologies of 201R gene of CMLV obtained from India and the other orthopoxviruses (complete sequences available) are shown in Table 3. Sequence analysis of 201R revealed that Indian CMLV shared 100% sequence identity both at the DNA and protein level with CMLV-Iran as well as with CMLV-Kazakhstan. This was further confirmed by phylogenetic analysis (Figure 4). The functional domain found in all the seven orthopoxviral proteins analysed is DUF 1406 (Pfam id: PF07190.6).

S. No.	Virus isolate	Host	Country and year	NCBI	% Identity	
				Accession No.	Nucleotide	Amino acid
1	CMLV-India	Camel	India & 2008	JQ917915	-	-
2	CMLV- M-96	Camel	Kazakhstan	AF438165	100.0	100%
3	CMLV-CMS	Camel	Iran	AY009089	100.0	100.0
4	Taterapoxvirus	Not available	Not available	DQ437594	97.2	94.7
5	Cowpoxvirus	Callithrix jacchus	Germany & 2002	HQ420898	91.4	89.0
6	Horsepoxvirus	Horse	Mangolia &1976	DQ792504	88.4	83.9
7	Vacciniavirus	Not available	USA	DQ377945	88.4	84.9

Table 3: Percent identity of nucleotide and amino acid of 201R gene of CMLV-India with different orthopoxviruses.

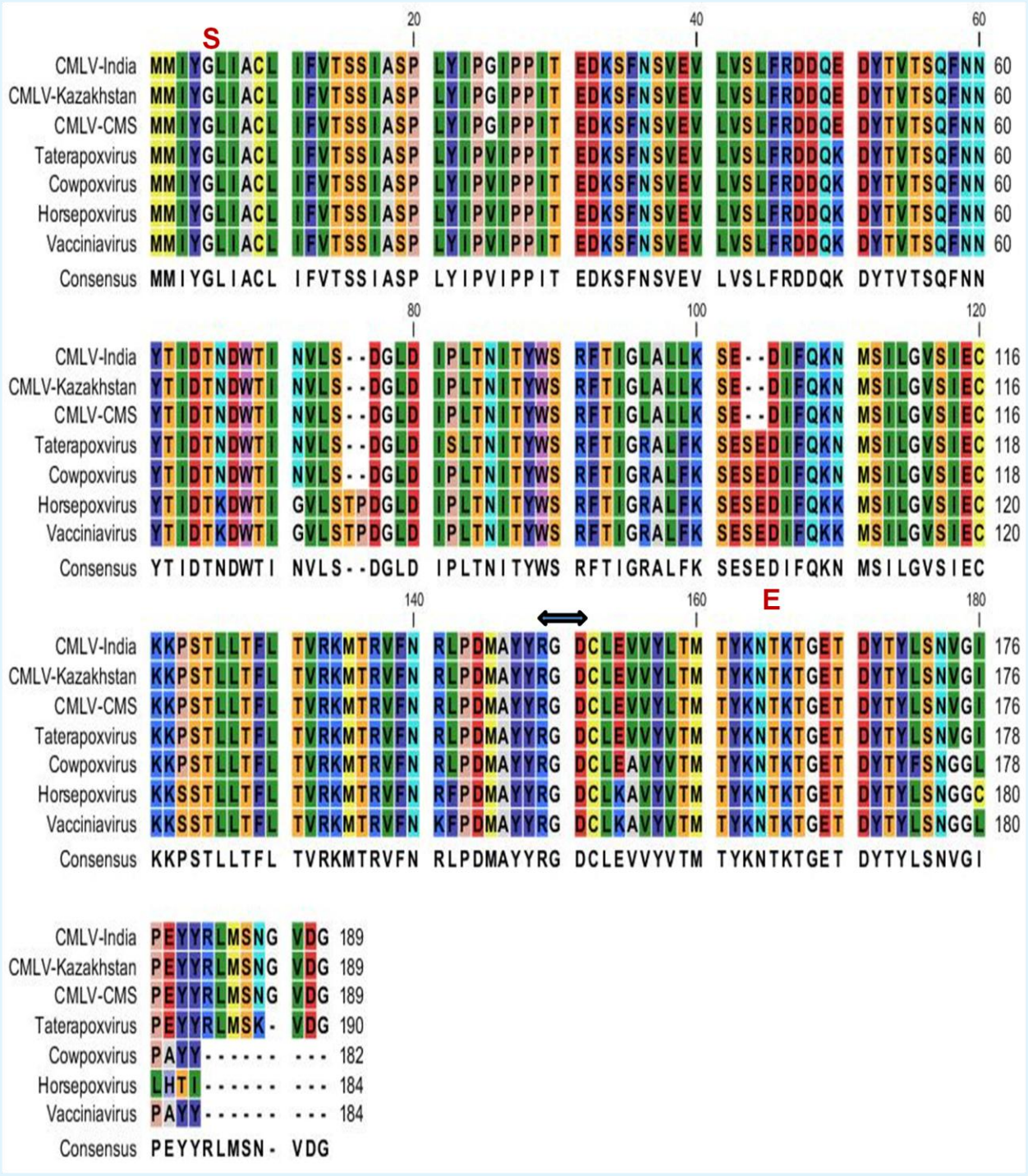

Figure 3: Alignment of amino acid residue sequences of Indian CMLV 201R gene with its orthologues from different orthopoxviruses. S and E denote the start and end of the functional domain, DUF 1406 (Pfam id: PF07190.6), respectively.

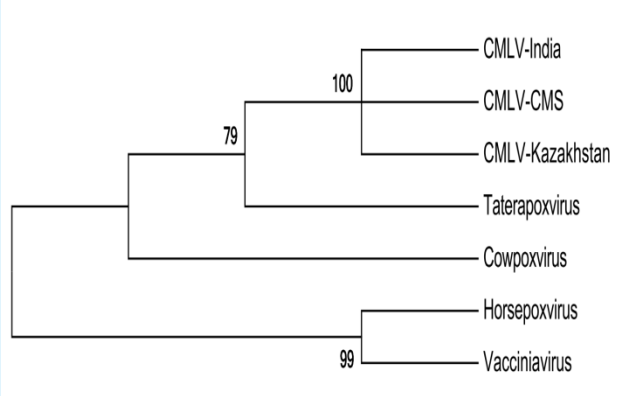

Figure 4: Phylogenetic tree based on amino acid residue sequences of Indian CMLV 201R gene and its orthologues from different orthopoxviruses, constructed by the neighbor-joining method using Mega 6(Molecular Evolutionary genetics Analysis software with bootstrap values calculated for 1,000 replicates. Horizontal distances are proportional to the genetic distances. Vertical distances are arbitrary. The numbers at each branch represent bootstrap values (1000 replicates).

Discussion

In the present study, two immunomodulatory protein genes, 163R and 201R genes of camelpoxvirus (CMLV) obtained from India were cloned and sequenced for the first time. The sequence information pertaining to these two genes would be useful to study the immunomodulation mechanisms being used by CMLV.

Sequence analysis of 163R gene revealed that CMLV obtained from India shared 100% identity with CMLV-Iran and CMLV-Kazakhstan strains both at DNA and protein level (Table 2). A phylogenetic tree constructed based on the amino acid sequences of 163R gene confirmed that the three CMLV isolates from India, Iran and Kazakhstan analysed clustered together and formed a single cluster (Group1). Group 2 was formed by Variola virus where as Group 3 was formed by Vaccinia virus, Horsepoxvirus and Rabbitpox virus (Figure 2).

As Kim and his team proposed the structure of VACV A46R protein, the carboxy terminal domain (CTD) of 163R gene encoding protein of CMLV contained eight a-helices (Figure 1) [25]. Therefore, it is speculated that in 163R gene encoding protein of CMLV, α-helices (α1–α7) without counting the first short α helix (a five amino acid peptide-MTYLY present just ahead of α1 helix in Figure 1) would adopt a typical Bcl- 2-like fold and form a homodimer composed of two subunits in the asymmetric unit with 2-fold non-crystallographic symmetry. Only one amino acid change in α4 (The amino acid at position 153 of VACV is M but in CMLV, it is I) and two amino acid changes in α6 helices (The amino acids at position 178 and 187 of VACV are K and H, respectively but in CMLV, they are N and Y, respectively) were observed between A46R protein of VACV and 163R protein of all the three CMLV isolates. Further, as in the case of A46R protein of VACV, the VIPER (viral inhibitory peptide of TLR4), sequence (an 11 amino acid peptide-KYSFKLILAEY) was located in the a1 helix of 163R encoding protein of CMLV (Figure 1). VIPER motif is believed to be involved in the interaction with MAL protein based on the binding assay [25]. Previous studies have showed that A46-derived peptide named VIPER inhibited both early and late TLR4-mediated responses by interacting with TIR- domain containing adaptor molecules, such as MAL and TRAM, respectively [26]. TLR4 has been implicated in a number of autoimmune and inflammatory diseases, including rheumatoid arthritis, atherosclerosis, and septic shock [27,28]. The functional domain found in all the 10 orthopoxviral proteins analysed is viz., Poxvirus (Pfam id: PF06227.7) is conserved among all the 10 OPVs analysed. The domain viz., Poxvirus covers the amino acid position from 12 to 182 of all the three CMLV 163R proteins (Figure 1).

We also compared the sequences of 201R gene of CMLV obtained from India with the corresponding sequences of other five orthopoxviruses available in the database. Sequence analysis revealed that 201R gene of CMLV obtained from India shared 100% sequence identity both at the DNA and protein level with CMLV-Iran as well as with CMLV- Kazakhstan (Table 3). Overall, CMLV obtained from India grouped with Kazakhstan and Iranian CMLV isolates in all both phylogenetic analyses (Figures 2 and 4).

In the structure of CMLV 201R protein as stated earlier, the RGD motif necessary for mediating the immunomodulatory mechanism is present in all the seven orthopoxviruses including CMLV obtained from India [2]. The RGD motif is actually present at the same position (amino acid 145-147) of all the three CMLV isolates. But, due to the missing of four amino acids in 201R protein, the RGD motif is present at position 149-151 of all the three CMLV isolates (Figure 3). The functional domain viz., DUF 1406 (Pfam id: PF07190.6) is conserved among all the seven OPVs analysed. DUF 1406 covers the amino acid position from 5 to 161 of all the three CMLV 201R proteins. But, due to the missing of four amino acids in 201R protein, DUF 1406 starts from the amino acid position 5 to position 165 of all the three CMLV isolates (Figure 3).

It is proposed that further line of research work needs to be carried out in the development of these two CMLV proteins. Characterization of these two CMLV proteins would be useful to study the pathogenesis, the new strategies for immune modulation and therapeutic intervention in the auto immune as well as inflammatory diseases.

Conclusions

The functional motifs such as VIPER and RGD, needed for modulating the immune responses are conserved in 163R and 201R proteins of CMLV, respectively. The phylogenetic analyses of 163R and 201R genes revealed that CMLV obtained from India is forming a cluster with Kazakhstan and Iranian CMLV isolates.

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