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ORIGINAL ARTICLE

Genetic characterization and phylogenetic analysis of host-range genes of Camelpox virus isolates from India

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Abstract Camelpox virus (CMLV), a close variant of variola virus (VARV) infects camels worldwide. The zoonotic infections reported from India signify the need to study the host-range genes—responsible for host tropism. We report sequence and phylogenetic analysis of five host-range genes: cytokine response modifier B (crmB), chemokine binding protein (ckbp), viral schlafen-like (v-slfn), myxomavirus T4-like (M-T4-like) and b5r of CMLVs isolated from outbreaks in India. Comparative analysis revealed that these genes are conserved among CMLVs and shared 94.5–100 % identity at both nucleotide (nt) and amino acid (aa) levels. All genes showed identity (59.3–98.4 %) with cowpox virus (CPXV) while three genes—crmB, ckbp and b5r showed similarity (92–96.5 %) with VARVs at both nt and aa levels. Interestingly, three consecutive serine residue insertions were observed in CKBP protein of CMLV-Delhi09 isolate which was similar to CPXV-BR and VACVs, besides five point mutations (K53Q, N67I, F84S, A127T and E182G) were also similar to zoonotic OPXVs. Further, few inconsistent point mutation(s) were also observed in other gene(s) among Indian CMLVs. These indicate that different strains of CMLVs are circulating in India and these mutations could play an important role in adaptation of CMLVs in humans. The phylogeny revealed clustering of all CMLVs together except CMLV-Delhi09 which grouped separately due to the presence of specific point mutations. However, the topology of the concatenated phylogeny showed close evolutionary relationship of CMLV with VARV and TATV followed by CPXV-RatGer09/1 from Germany. The availability of this genetic information will be useful in unveiling new strategies to control emerging zoonotic poxvirus infections.

Keywords Camelpox virus · CMLV · Host-range gene · *Orthopoxvirus* · Concatenated phylogenetic tree

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Introduction

Camelpox—a contagious viral disease of camels is manifested with fever and local or generalized pock-like lesions primarily on head, neck, throat, extremities, inguinal region and mucous membrane of mouth [1]. The disease is prevalent throughout the camel rearing regions of the world including India, Pakistan, Afghanistan, the Middle East, north and east Africa, and the United Arab Emirates [2–8]. Frequent outbreaks of the disease have been observed in the North-Central region, the main camel rearing region of the country [7–9]. The causative agent of the disease—Camelpox virus (CMLV), a member of the genus *Orthopoxvirus* of subfamily *Chordopoxvirinae* under the family *Poxviridae* [10] is closely related to Variola virus—causative agent of dreaded smallpox disease [11, 12]. Although, CMLV is highly host—specific and does not infect other animal species, the transmission of CMLV to human has been reported earlier on the basis of clinical symptoms [13–15]. However, the world's first laboratory confirmed human cases of Camelpox infection were reported recently by our group [6], which conclusively

proved the zoonotic nature of the disease. Such change in host-specificity of CMLV could have serious implications from a public health perspective due to the cessation of vaccination against smallpox since 1977 [16]. In this context, the elucidation of host-range genes of CMLV could help in deciphering the role of these genes in host tropism of CMLVs in humans.

CMLV encodes a number of virulence and immunomodulatory genes at both terminal regions of the genome [11, 12] to evade host immune response. Among these proteins: the tumor necrosis factor receptor (vTNFR) named cytokine response modifier B (crmB) encoded by CMLV-ORFs 2L and 205R [12] which inactivates the pro-inflammatory cytokine and certain chemokines [17, 18] for VARV and MPXV [19–21] and CPXV [22, 23] and two CMLV-ORFs-2L and 205R encode secreted 35-kDa vCKBP protein [11, 12] which prevent binding of cellular cognate receptors as in other OPXVs: variola, vaccinia and cowpox viruses [24–26]. Likewise, a 57 kDa cytoplasmic Schlafen-like (v-slfn) protein coding gene encoded by CMLV, CPXV, TATV and MPXV [12, 27]. The ORF176R of CMLV-CMS, Iran isolate [12] and ORF179 of CMV-M96, Kazakhstan isolate [11], encodes v-SLFN protein [12] which inhibits the lymphocyte activity [28]. The genome sequence analysis further revealed that CMLV encodes homologue of myxoma virus encoded M-T4 like protein coding gene -ORF188 in CMLV-M96 isolate [11] and ORF-185 in CMLV-CMS isolate [12] which is involved in inhibition of apoptosis and virus pathogenesis [29] besides down regulating MHC class I molecules [30]. This gene is functional in CPXV, MPXV, and TATV, but it's orthologs are fragmented or absent in other OPXVs. However, the virus envelope protein coding b5r gene-encoded in all OPXVs is known to play a role in formation of extracellular envelope virus (EEV) and subverting the host's immune response [31, 32]. The 178R gene of CMLV encodes this protein similar to other OPXVs [12]. The mutational studies of this gene of VACV strain- WR [32] and RPXV [33] showed decrease in production of EEV, reduced plaque size in vitro and is highly attenuated in vivo compared to the parental strain.

Although, genome sequence analysis have depicted the complete coding sequences of these host-range genes, [11, 12], the detailed genetic and functional studies of these genes of CMLVs circulating worldwide are yet to be deciphered. Further, proven zoonotic nature of the CMLV coupled with close relatedness with variola virus and reduction of cohort immunity of humans against OPXVs, necessitates the genetic and evolutionary studies of these genes. In this context, the current study focuses on characterizing the host-range genes of CMLVs isolated from severe outbreaks (2009) in India to elaborate any mutational changes alongwith phylogenetic evolution.

Materials and methods

Viruses

The CMLVs isolated from outbreaks in camels during 2009 in Delhi (CMLV/camel/Delhi/2009), and Barmer (CMLV/camel/Barmer/2009), Bikaner (CMLV/camel/Bikaner/2009) and Jaisalmer (CMLV/camel/Jaisalmer/2009) districts of Rajasthan state of India, were utilized in the study. The viruses were isolated from scabs collected from infected animals in Vero cell culture as per standard methods.

Amplification, cloning and sequencing

Briefly, Viral DNA was extracted from 200 µl of infected Vero cell culture fluid using the Qiagen DNA Extraction Kit (M/s Qiagen, Valencia, CA, USA) as per the manufacturer's protocol. The DNA was eluted in 50 µl nuclease-free water and an aliquot of 5 µl was used for PCR amplification of targeted genes. For amplification of full-length sequences of crmB, ckbp, v-slfn, M-T4-like and b5r genes, primer sets were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) on the basis of genome sequence of CMLV-M96 isolate (Accession no. AF438165) from Kazakhstan. Primer sequences were: Forward: 5′-AAACAGGAACACGACGA CGA-3′ and Reverse: 5′-TGATTCTACTTCCTTACCGT GAA-3′ for crmB gene; Forward: 5′- TTTCACGGTAAGG AAGTAGAATCAT-3′ and Reverse: 5′-AATCGTTCGA TTAACCCTACTCACC-3′ for ckbp gene; Forward: 5′-AG AGTTAGTTA GTTAAAAAGAGATAACAGT-3′ and Reverse: 5′-AGGAACCCAATTACGATGAGTTTCG-3′ for v-slfn gene; Forward: 5′-TTGCTTGATGATTAGTGA TAGTGGA-3′ and Reverse: 5′-TGTTGGTTTAGT AGCT GTTGAGCAC-3′ for M-T4-like gene and Forward: 5′-ACGGA TTTATATTCACGGCAACAA-3′ and Reverse: 5′-TGTTTCCTTTTATTGCTCGACAGTG-3′ for b5r gene. Although primers were designed based on the CMVL sequence, the specificity of the primers was not restricted to CMLV only as homologue genes are also encoded by other OPXVs. Each PCR reaction mixture contained 5 µl DNA, 15 pmol of each primer, 200 µM dNTPs and 1.0 µl (2.5U) of Qiagen HotStart Hifidelity DNA polymerase (M/s Qiagen, Valencia, CA, USA) in a 50 µl reaction. PCR was performed with the thermal cycling condition of 95 °C for 5 min followed by 35 cycles of 94 °C for 50 s, 51 °C for 1 min, 72 °C for 2 min and final extension at 72 °C for 10 min. The amplified products were purified using Gel purification kit (Zymo Research, Irvine, CA, USA) and cloned into pCRTM-Blunt II-TOPO(R) vector (M/s Invitrogen, Life Technologies, Carlsbad, CA, USA) as per the manufacturer's protocol. The recombinant plasmid DNA

was confirmed by colony PCR, restriction enzyme analysis and sequencing. All clones were sequenced commercially employing automated DNA sequencer (ABI 3730 Genetic Analyzer) from Eurofins Genomics India Pvt. Ltd., Bangalore, India. Three clones for each gene were sequenced to obtain a consensus sequence for analysis.

Sequence and phylogenetic analysis

An open reading frame (ORF) nucleotide as well as deduced amino acid homology search was carried out using NCBI-BLAST server [34]. For comparison, nucleotide (nt) as well as deduced amino acid (aa) sequences were further aligned using Clustal W method of molecular evolutionary genetic analysis (MEGA) version 5 program [35]. Gene sequences of all the members of the OPXV genus were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). The details of these sequences including their accession numbers are mentioned in Table 1. The sequence identity was determined at the nucleotide and amino acid sequence levels.

For construction of phylogenetic tree, aligned individual amino acid sequences of deduced targeted proteins were manually edited and concatenated together. The maximum-likelihood tree was constructed from the artificially concatenated amino acid sequences according to the Jones–Taylor–Thornton model [36] with gamma distribution using MEGA 5 program [35]. Node support was assessed by nonparametric bootstrap analysis using 1000 pseudo-replicates of data set.

Secondary structure prediction

Various online software tools were used for analysis of predicted host-range proteins. Secondary structures of deduced host-range proteins were predicted employing PSIPRED program (http://bioinf.cs.ucl.ac.uk/psipred/).

Results

Homology analysis

The ORFs of targeted host-range genes of all CMLVs comprised of 1050, 768, 1509, 678 and 954 bp nucleotides encoding predicted 349aa, 255aa, 502aa, 225aa, and 317aa residues for CRMB, CKBP, v-SLFN, M-T4—like and B5R proteins, respectively. The nucleotide sequence data was submitted to GenBank, NCBI and assigned accession numbers (Table 1). Comparison of sequences revealed that three genes (ckbp, v-slfn and b5r) showed a similarity of 94.5–100 % with earlier reported CMLVs at both nt and aa levels whereas, crmB and M-T4-like genes shared ~100 % similarity. Homology analysis also revealed that all the targeted genes showed homology with cowpox virus with similarity varying between 59.3 and 98.4 % at both nt and aa levels, whereas only three genes—crmB, ckbp and b5r exhibited identities (92.0–96.5 %) with VARVs. Only two genes (ckbp and b5r) of CMLVs showed homology with VACVs with similarity ranging 46.6–95 %. Furthermore, all the genes showed homology of 46.6–94.1 % with monkeypox virus except in case of M-T4-like gene. The v-slfn gene did not show any homology with VARV and VACV; however it was related to TATV with an identity of 98.6 %. Besides, the CPXV, MPXV and ECTV showed an identity of ~85.5 % with this CMLV protein.

Analysis of amino acid sequence and secondary structure

Amino acid sequences deduced from the targeted host-range genes were compared with CMLV-M96 isolate from Kazakhstan (representative CMLV isolate). Comparative analysis revealed several consensus aa substitutions which have been depicted in Tables 2, 3, 4 and 5 for CRMB, CKBP, v-SLFN, and B5R proteins, respectively. In CMLV-CRMB protein only two aa changes: E247A in Saudi and Iran and V297A in CMLVs from Somalia, Saudi and Iran were found among all CMLVs (Table 2), however, this protein resembles to CPXVs despite fourteen consensus point mutations. In this protein, various secondary structural changes were observed among closely related OPXVs. The major changes include: alteration of strand to coil (at position 51 and 52) in CMLV; coil to strand (at position 96–98) in VARV; at position 250 to 280—one strand and one helix in CMLV, two long helixes in VARV and two small strand structures in CPXV; coil to three small strands (at position 321–343) in CPXV.

The CKBP protein of CMLVs showed six consensus aa changes (Q55K, I/V69N, S86F, T129A, G184E and E226D) compared to other OPXVs viz., CPXV, VARV, VACV and MPXV; whereas the last three changes were absent in VARV (Table 3). The point mutation (I69N) led to major shifting of strand to coil form (at 62–66aa residues) compared to VARV. Interestingly, significant aa substitutions were observed in CKBP protein of CMLV-Delhi09 isolate in which insertion of three consecutive serine residues at position 28, 29 and 30 and six point mutations (K55Q, N69I, F86S, A129T, R150H, and E184G) were found in comparison to other CMLV isolates (Table 3). The insertions of three serine residues were also observed in CPXV-BR strain and VACVs. However, these insertional mutations did not cause any alteration in secondary protein structure. More point mutations were found in this protein encoded by VARVs than CPXV and VACV.

Table 1 GenBank accession nos. of gene sequences reported in the present study and retrieved from the database for analysis

Virus	Strain/isolate	Accession nos. (Gene name)
Camelpox virus	CMLV-M96	AF438165.1
	CMLV-CMS	AY009089.1
	CMLV-DEL09 (This study)	KC841315 (cbp), KC841319 (crmB), KC841311 (B5R), KM234479 (M-T4-like) and KM234483 (v-slfn)
	CMLV-BAR09 (This study)	KC841317 (cbp), KC841321 (crmB), KC841313 (B5R), KM234480 (M-T4-like) and KM234484 (v-slfn)
	CMLV-BIK09 (This study)	KC841316 (cbp), KC841320 (crmB), KC841312 (B5R), KM234481 (M-T4-like) and KM234485 (v-slfn)
	CMLV-JSL09 (This study)	KC841318 (cbp), KC841322 (crmB), KC841314 (B5R), KM234482 (M-T4-like) and KM234486 (v-slfn)
	CMLV-BIK08	JF975616.1 (v-slfn)
	CMLV-HYD06	EF599952.1 (B5R)
	CMLV-BIK97	EF599950.1 (B5R)
	CMLV-SOM78	U87837.1 (crmB)
	CMLV-SAU	AAB94356.1 (crmB)
	CMLV-CP1	AAB94355.1 (crmB)
Variola virus	VARV-IND64	DQ437586.1
	VARV-BSH75	DQ437581.1
	VARV-PAK69	DQ437589.1
Cowpox virus	CPXV-BR	AF482758.2
	CPXV-GRI90	X94355.2
	CPXV-GER09	KC813503.1
Vaccinia virus	VACV-LST	AY678276.1
	VACV-COP	M35027.1
	VACV-WR	AY243312.1
Rabbitpox virus	RPXV-UTR	AY484669.1
Horsepox virus	HSPV-MNR76	DQ792504.1
Taterapox virus	TATV-DAH68	DQ437594.1
Ectromelia virus	ECTV-MOS	AF012825.2
Monkeypox virus	MPXV-ZAR96	NC_003310.1
	MPXV-CON03	DQ011154.1

CMLV Camelpox virus; *M96* Kazakhstan 1996 isolate; *CMS* Iran isolate; *DEL09* Delhi, India 2009 isolate; *BAR09* Barmer, India 2009 isolate; *BIK09* Bikaner, India 2009 isolate; *JSL09* Jaisalmer, India 2009 isolate; *BIK08* Bikaner, India 2008 isolate; *HYD06* Hyderabad, India 2006 isolate; *BIK97* Bikaner 1997 isolate; *SOM78* Somalia-1978 strain; *SAU* Saudi-M3 strain; *CP1* CMLV strain from Iran; *VARV* Variola virus; *IND64* India 1964, ssp. Major; *BSH75* Bangladesh 1975 strain; *PAK69* Pakistan-1969 strain; *CPXV* Cowpox virus; *BR* Brington Red strain; *GRI90* GRI90 strain; *GER09* Germany 2009 RatGer09/1 strain; *VACV* Vaccinia virus; *LST* Lister strain; *COP* Copenhagen strain; *WR* Western Reserve strain; *RPXV* Rabbitpox virus; *UTR* Utrecht isolate; *HSPV* Horsepox virus; *MNR76* Mongalia 1976 isolate; TATV-Taterapox virus; *DAH68* Dahomey 1968 isolate; *ECTV* Ectromelia virus; *MOS* Moscow strain; *MPXV* Monkeypox virus; *ZAR96* Zaire-96-I-16 strain; *CON* Congo 2003 strain

Similarly in the v-SLFN protein, a deletion mutation (204E) was observed in all CMLVs and CPXV-GRI90 isolate compared to other OPXVs expressing functional v-SLFN protein. However, this protein was highly conserved in CMLVs showing ~100 % identity among all CMLVs, and exhibited maximum similarity with TATV depicting six consensus aa changes (I16L, I46V, T150A, S207T, Y303D and V338F) (Table 4). The point mutation (S34P) in v-SLFN proteins of CMLV-Barmer09 and CMLV-Bikaner09 led to alteration in—strand to coil form (at position 33 and 34). However, many secondary structural changes were observed in this protein of TATV, CPXV and MPXV.

There was no mutational change in M-T4-like proteins of CMLVs and showed closest identity to CPXV-GER09 with three point mutations (I123V, L140V and N170D) and TATV with five aa changes (D56G, I123V, L140V, N170D and S179R). The point mutation (V140L) in CMLVs led to change in coil to strand structures at position 134, 135 and 141 compared to CPXV and TATV. Further, substitutions

Table 2 Amino acid substitutions in the predicted CRMB protein sequence compared to CMLV-M96/Kazakhstan isolate

Strain	7	24	25	27	28	30	37	40	41	42	75	80	85	95	121	129	130	132	134	146	149	159	164	169	176	177
CMLV-M96	S	V	T	Y	A	S	N	K	R	H	G	R	P	D	I	K	A	V	Q	H	A	L	R	A	P	S
CMLV-DEL09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMLV-BAR09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMLV-BIK09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMLV-JSL09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMLV-CMS	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMLV-SOM78	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMLV-SAU	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMLV-CP1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CPXV-BR	I	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	T	.	H	V	.	.
CPXV-GRI90	.	I	A	H	.	.	.	N	.	.	.	.	.	.	L	.	.	.	.	.	T	.	H	.	.	.
CPXV-GER09	.	.	A	.	.	.	.	N	.	.	.	.	.	.	L	.	.	.	.	.	T	.	H	.	.	.
VARV-IND64	L	A	A	.	T	P	T	.	.	.	.	.	.	N	L	.	.	.	.	.	V	F	H	.	.	N
VARV-PAK69	L	A	A	.	T	P	T	.	.	.	.	.	.	N	L	.	.	.	.	.	V	F	H	.	.	N
VARV-BSH75	L	A	A	.	T	P	T	.	.	.	.	.	.	N	L	.	.	.	.	.	V	F	H	.	.	N
HSPV-MNR76	L	I	A	H	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	T	.	H	.	.	R
MPXV-ZAR96	.	L	A	H	.	.	.	R	S	R	D	H	Q	.	L	R	T	I	K	Y	T	P	H	T	T	.
MPXV-CON03	.	I	A	H	.	.	.	R	S	R	D	H	Q	.	L	R	T	I	K	Y	T	P	H	T	T	.

Strain	188	205	210	221	224	229	230	247	269	274	280	286	287	289	297	315	326	327	333	342	346	351	352	357	360
CMLV-M96	N	I	S	K	D	E	E	E	G	S	A	G	I	P	V	T	H	A	V	D	L	K	–	H	*
CMLV-DEL09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-BAR09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-BIK09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-JSL09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-CMS	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-SOM78	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	*
CMLV-SAU	.	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	*
CMLV-CP1	.	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	*
CPXV-BR	.	L	.	.	.	N	G	Q	D	.	T	S	.	.	.	.	.	V	.	.	.	N	S	.	*
CPXV-GRI90	.	L	.	.	.	D	G	.	D	.	T	.	.	.	.	.	.	V	.	.	.	.	.	.	*
CPXV-GER09	.	L	.	.	.	.	.	.	D	.	T	T	.	.	A	.	.	.	.	.	.	Q	N	.	*
VARV-IND64	T	L	L	T	N	.	.	.	.	F	.	.	M	S	A	A	R	V	D	N	P	.	.	R	*
VARV-PAK69	T	L	L	T	N	.	.	.	.	F	.	.	M	S	A	A	R	V	D	N	P	.	.	R	*

Table 2 continued

Strain	188	205	210	221	224	229	230	247	269	274	280	286	287	289	297	315	326	327	333	342	346	351	352	357	360
VARV-BSH75	T	L	L	T	N	.	.	.	.	F	.	.	M	S	A	A	R	V	D	N	P	.	.	R	*
HSPV-MNR76	.	L	.	.	N	.	.	.	N	.	.	.	.	.	.	.	.	V	.	N	.	.	.	.	*
MPXV-ZAR96	.	L	.	.	.	A	.	.	D	.	T	T	.	.	.	.	.	M	.	.	.	Q	N	.	*
MPXV-CON03	.	L	.	.	.	A	.	.	D	.	T	T	.	.	.	.	.	M	.	.	.	Q	N	.	*

Amino acid numbering was done from the first amino acid of the deduced protein with signal peptide and gaps as aa residues deleted in many viruses; [.] indicates identity; [*] indicates stop codon

(E/D170N and K/T174S) caused alteration in helix to coil at 173L in comparison to CPXV and similar change was observed at 172K and 173L compared to TATV.

In B5R proteins, five consensus aa changes (G115E, V178I, P221S, A276V and D308N) were found in all CMLV isolates, which were absent in other OPXVs, however, only single point mutation (N120K) was observed in two Indian CMLVs (CMVL-Barmer09 and CMLV-Bikaner09). This protein is more similar to CPXV-GER09, CPXV-BR and TATV proteins depicting 6–7 aa changes in these strains (Table 5). Further, group specific point mutations were also observed in this protein among other OPXVs. The aa substitutions in B5R proteins did not cause major alteration in structure in CMLV and TATV, except change of coil to strand form (at positions 139 and 140) which could be due to mutation at position 136 (D–E) in comparison to VARV.

Phylogenetic analysis

The phylogram of concatenated sequences of five host-range genes revealed that Indian CMLVs were clustered together with CMLVs reported worldwide with a high bootstrap value (Fig. 1). However, CMLV-Delhi09 isolate was distinct from all other CMLVs; which clustered separately from other CMLVs. The topology of the phylogenetic tree depicted close grouping of CMLVs with TATV and VARV with bootstrap value of 65. Cowpox viruses did not group together, while CPXV-GER09 was closest to VARV and CMLVs, the CPXV-GRI90 clustered separately with ECTV and MPXV. The VACV clustered distantly from CMLVs and showed grouping with HSPV and RPXV. However, the phylogeny of individual genes revealed variation in grouping of CMLVs with other OPXVs analysed (data not shown).

Discussion

Ever since the first laboratory confirmation of CMLV zoonosis in the recent past [6] and speculation that conversion of CMLV into a VARV-like virus may result in the emergence of a new human pathogen, studies on host-range genes to decipher the host tropism of CMLVs assume enormous significance. In the present study, we ascertained the sequences of five important host-range genes along with their associated mutational changes in deduced proteins of CMLVs isolated from camels during major disease outbreaks (2009) in Delhi and three districts (Barmer, Bikaner and Jaisalmer) of Rajasthan state of India. The Barmer outbreak was associated with human infections which was the first report of laboratory confirmed camelpox zoonosis in the world [6]. The genetic changes in the

Table 3 Amino acid substitutions in the predicted CKBP protein sequence compared to CMLV-M96/Kazakhstan isolate

Strain	28	29	30	41	55	69	74	86	87	88	90	91	92	100	102	126	129	138	150	151	162	178	184	205	226	240	244	253	258	259
CMLV-M96	–	–	–	H	K	N	S	F	T	S	E	D	V	S	I	D	A	S	R	E	V	K	E	T	D	A	A	S	A	*
CMLV-DEL09	S	S	S	.	Q	I	.	S	.	.	.	.	.	.	.	.	T	.	H	.	.	.	G	.	.	.	.	.	.	*
CMLV-BAR09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-BIK09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-JSL09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	A	.	*
CMLV-CMS	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CPXV-BR	S	S	S	.	Q	V	D	S	E	E	V	K	G	T	V	.	T	.	.	.	M	.	G	.	E	.	.	.	.	*
CPXV-GRI90	.	.	.	.	Q	I	.	S	.	.	.	.	.	.	.	.	T	.	H	.	.	.	G	.	E	.	.	.	.	*
CPXV-GER09	.	.	.	.	Q	I	.	S	.	.	.	K	.	.	.	.	T	.	H	.	.	.	G	.	E	.	.	.	.	*
VARV-IND64	.	.	.	Y	Q	I	.	S	.	.	.	.	.	.	.	N	.	P	.	A	L	Q	.	.	.	V	V	.	S	*
VARV-PAK69	.	.	.	Y	Q	I	.	S	.	.	.	.	.	.	.	N	.	P	.	A	L	Q	.	.	.	V	V	.	S	*
VARV-BSH75	.	.	.	Y	Q	I	.	S	.	.	.	.	.	.	.	N	.	P	.	A	L	Q	.	.	.	V	V	.	S	*
VACV-LST	S	S	S	.	Q	I	.	S	.	.	.	.	.	.	.	.	T	.	.	.	.	.	G	.	E	.	.	.	.	*
VACV-COP	S	S	S	.	Q	I	.	S	.	.	.	.	.	.	.	.	T	.	.	.	.	.	G	.	E	.	.	.	.	*
VACV-WR	S	S	S	.	Q	I	.	S	.	.	.	.	.		.	.	T	.	H	.		.	G	.	E	.	.	.	.	*
RPXV-UTR	S	S	S	.	Q	I	.	S	.	.	.	.	.	.	.	.	T	.	H	.	.	.	G	.	E	.	.	.	.	*
HSPV-MNR76	.	.	.	.	Q	V	D	S	E	E	V	K	R	.	V	.	T	.	.	.	.	.	G	.	E	.	.	.	.	*
ECTV-MOS	A	S	.	.	Q	V	D	S	E	E	V	K	G	T	V	.	T	.	H	.	.	.	G	I	E	V	.	.	.	*
MPXV-ZAR96	.	.	.	.	Q	V	D	S	E	E	V	K	G	T	V	.	T	.	.	.	M	.	G	.	E	.	.	.	.	*
MPXV-CON03	.	.	.	.	Q	V	D	S	E	E	V	K	G	N	V	.	T	.	.	.	M	.	G	.	E	.	.	.	.	*

Amino acid numbering was done from the first amino acid of the deduced protein with signal peptide and gaps as aa residues deleted in many viruses; [.] indicates identity; [*] indicates stop codon

Table 4 Amino acid substitutions in the predicted v-Schlafen protein sequence compared to CMLV-M96/Kazakhstan isolate

Strain	16	34	46	150	204	207	208	213	230	232	235	236	239	248	251	255	258	264	267	269	275	276	278	284	288
CMLV-M96	I	S	I	T	–	S	E	T	Q	D	N	H	M	M	–	R	I	C	I	L	N	L	P	N	R
CMLV-DEL09	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	D	.
CMLV-BAR09	.	P	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.
CMLV-BIK09	.	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CMLV-JSL09	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.
CMLV-BIK08	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.
CMLV-CMS	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TATV-DAH68	L	.	V	A	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CPXV-BR	L	.	V	S	E	T	V	S	K	E	D	Y	.	L	V	H	V	R	V	M	S	P	L	.	K
CPXV-GRI90	L		L	.	–	T	.	A	.	.	.	.	.	.	V	H	.	.	V	M	.	P	.	.	K
CPXV-GER09	L	.	V	S	E	T	V	A	K	E	D	Y	.	L	V	H	V	R	V	M	S	P	L	.	K
ECTV-MOS	L	.	V	A	.	T	.	A	K	E	D	Y	.	L	V	H	V	R	.	M	S	P	.	.	K
MPXV-ZAR96	L	.	V	A	E	T	I	S	K	E	D	Y	.	L	V	H	V	R	.	M	S	P	L	.	K
MPXV-CON03	L	.	V	A	E	T	I	S	K	E	D	Y	.	L	V	H	V	R	.	M	S	P	L	.	K

Strain	289	291	292	294	298	299	300	303	314	324	330	332	338	342	355	360	364	391	399	415	419	477	506
CMLV-M96	S	T	S	I	P	I	T	Y	Q	D	Q	D	V	F	E	V	N	E	L	C	I	T	*
CMLV-DEL09	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	*
CMLV-BAR09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-BIK09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-JSL09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-BIK08	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-CMS	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
TATV-DAH68	.	.	.	.	.	.	.	D	.	.	.	.	F	.	.	.	.	.	.	.	.	.	*
CPXV-BR	T	G	A	L	Y	M	E	E	R	E	E	A	L	.	N	I	H	D	V	Y	V	A	*
CPXV-GRI90	T	G	.	.	S	M	E	E	R	E	E	T	L	.	K	I	H	.	V	Y	V	A	*
CPXV-GER09	T	G	A	L	S	M	E	E	R	E	E	A	L	.	K	I	S	D	V	Y	.	A	*
ECTV-MOS	T	G	.	L	S	M	E	E	R	E	E	T	L	.	K	I	H	.	V	Y	V	A	*
MPXV-ZAR96	T	G	A	L	S	M	E	E	R	E	K	T	L	.	K	I	H	K	V	Y	V	A	*
MPXV-CON03	T	G	A	L	S	M	E	E	R	E	K	T	L	.	K	I	H	K	V	Y	V	A	*

Amino acid numbering was done from the first amino acid of the deduced protein with signal peptide and gaps as aa residues deleted in many viruses; [.] indicates identity; [*] indicates stop codon

Table 5 Amino acid substitutions in the predicted B5R protein sequence compared to CMLV-M96/Kazakhstan isolate

Strain	40	41	50	53	55	82	87	95	96	97	100	102	103	115	120	132	136	152	170	178	221	238	248	276	304	308	317	318
CMLV-M96	D	K	S	Y	L	V	D	A	T	I	I	K	D	E	N	S	E	H	S	I	S	V	E	V	N	N	P	*
CMLV-DEL09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-BAR09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-BIK09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-JSL09	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-HYD06	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-BIK97	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	*
CMLV-CMS	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*
VARV-IND64	.	.	.	.	.	.	N	.	I	.	.	.	.	G	.	.	D	.	T	V	P	I	.	A	.	D	L	*
VARV-BSH75	.	.	.	.	.	.	N	.	I	.	.	.	.	G	.	.	D	.	T	V	P	I	.	A	.	D	L	*
VARV-PAK69	.	.	.	.	.	.	N	.	I	.	.	.	.	G	.	.	D	.	T	V	P	I	.	A	.	D	L	*
CPXV-BR	.	.	.	.	.	.	.	S	.	.	.	.	.	G	.	P	.	.	.	V	.	.	D	A	.	D	.	*
CPXV-GRI90	.	.	Q	H	.	.	.	S	.	M	S	N	G	G	.	.	.	Y		V	P		D	A	D	D		*
CPXV-GER09	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.		.	.	.	V	P	.	D	A	.	D	.	*
VACV-LST	.	.	Q	H	S	.	N	S	.	M	S	N	G	G	.	P	.	Y	.	V	P	.	D	A	D	D	.	*
VACV-COP	N	N	Q	H	S	I	N	S	.	M	S	N	G	G	.	P	.	Y	.	V	P	.	D	A	D	D	.	*
VACV-WR	.	.	Q	H	S	I	N	S	.	M	S	N	G	G	.	P	.	Y	.	V	P	.	D	A	D	D	.	*
TATV-DAH68	.	.	.	H	.	.	.	.	.	.	.	.	.	G	.	.	.		.	V	P	.	.	A		D	.	*
ECTV-MOS	.	.	.	H	.	.	.	S	.	M	R	N	S	G	.	P	.	Y	.	V	P	.	D	A	D	D	.	*
RPXV-UTR	N	N	Q	H	S	I	N	S	.	M	S	N	G	G	.	P	.	Y	.	V	P	.	D	A	D	D	.	*
HSPV-MNR76	N	N	Q	H	S	I	N	S	.	M	S	N	G	G	.	P	.	Y	.	V	P	.	D	A	D	D	.	*
MPXV-ZAR96	.	.	.	H	.	.	.	S	.	M	S	N	G	G	.	P	.	Y	.	V	P	.	D	A	D	D	.	*
MPXV-CON03	.	.	.	H	.	.	.	S	.	M	S	N	G	G	.	P	.	Y	.	V	P	.	D	A	D	D	.	*

Amino acid numbering was done from the first amino acid of the deduced protein with signal peptide; [.] indicates identity; [*] indicates stop codon

CMLV host-range genes (crmB, ckbp, v-slfn, M-T4-like and b5r) were analyzed vis-a-vis other zoonotic OPXVs. Homology analysis revealed similarity of 94.5–100 % among all targeted genes in CMLVs which indicates their conserved nature. Furthermore, identity was observed among three genes—ckbp, crmB and b5r of CMLVs with VARVs; while all genes showed homology with CPXV and only two genes—ckbp and b5r were similar with VACVs. This suggests the variability in functionality of host-range genes between CMLVs and other OPXVs as has also been reported by other workers [11, 12].

Interesting mutational changes specifically insertion of three serine residues observed in CKBP protein of CMLV-Delhi09 isolate similar to CPXV and VACV suggest the possible change in host-specificity of CMLV. These mutations could have a role in favourable interaction with the chemokines in cellular micro-environment which needs further investigation. The consensus mutations observed in crmB proteins of CMLVs which led to alterations in secondary structures assume significance as few studies highlighted the differential activity of crmB proteins of VARV, MPXV, and CPXV wherein only CrmB-VARV inhibited human TNF activity with high efficiency [37, 38]. This indicates that crmB-CMLV protein could also exhibit different TNF suppression activity with their natural or spill-over hosts. Although, genome sequence analysis showed that CMLV encodes schlafen-like and homologue of Myxomavirus T4-like proteins, detailed mutational and functional analysis of these proteins have not been elucidated. Several mutational changes leading to alteration in secondary structures observed in these proteins of CMLVs needs further investigation to elucidate their role in evolution of the OPXVs. Further, the M-T4-like protein of CMLV was more similar to the recent isolate of CPXV (RatGer09/1) from Germany, where four deletion mutations (at aa position 139 and 175–177) observed in CPXV-GRI90 and MPXV- were not found in 2009 isolate of CPXV-RateGer09/1. Another important protein—B5R which is highly conserved in poxviruses, exhibits group-specific mutations among OPXVs. Five CMLV-specific point mutations were found in all CMLVs reported worldwide compared to other OPXVs and single point mutation (N120K) observed only in CMLV-Barmer09 isolate along with earlier isolate (CMLV-Bikaner97) from India [4]. However, these substitutions did not cause alteration in secondary structure of the protein. The functional role of the CMLV-specific changes needs to be analysed to elucidate the host tropism as this protein is involved in formation of EEM and inhibition of complement mediated host defense [31, 32].

To explore the evolutionary pattern of the host-range genes, we compared the ortholog sequences of five host-range genes of OPXVs. The concatenated tree generated

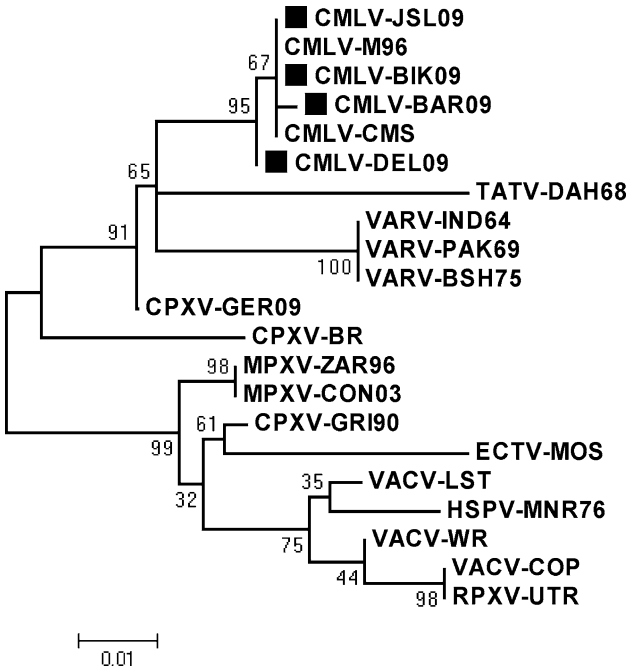

Fig. 1 Maximum-likelihood tree was constructed using concatenated alignment of amino acid sequences of CRMB, CKBP, V-SLFN, M-T4-like and B5R proteins of CMLV isolates reported in the present study and previously reported sequences of OPXVs using MEGA5. The bootstrap scores (1000 replicates) for all nodes are displayed next to the branches. The representative sequences reported in this manuscript are marked by *black boxes*

using protein sequences encoded by these genes represents the species tree more accurately than the single protein based tree. This was supported by previous studies where topologies of phylograms of individual OPXV proteins depicted variable clustering pattern even in closely related species [11, 12, 39]. Phylogeny of the targeted predicted proteins in general showed the clustering of OPXVs into various species-specific groups including CMLVs. CMLV isolates were phylogenetically closest to the variola and taterapox viruses followed by recent CPXV isolate from Germany. Inspite of conserved nature of these proteins in CMLVs, the CMLV-Delhi/09 isolate clustered separately compared to all other CMLVs including Indian isolates. This occurred due to the significant insertional point mutations in this isolate which bearing on evolution of the CMLV in India. The evolution of these host-range genes of CPXVs followed different patterns, however the CPXV-BR and CPXV-GER09 isolates were closet to CMLV, VARV and TATV. The evolution pattern of the targeted host-range genes was similar to the earlier report based on analysis of all OPXV genes [27, 40–42].

To conclude, this is the first report on molecular characterization of important host-range genes of CMLVs isolated from the outbreaks in different geographical locations of the country—one of which was associated with

zoonosis [6]. The sequence and phylogenetic analysis revealed the evolutionarily conserved nature of these host-range genes wherein CMLVs are closely related to VARV and TATV. However, the host tropism of CMLV is different from closely related VARV, as CMLV expresses a unique portfolio of host-range genes, some of which are either fragmented or truncated in VARV. Besides, recent CPXV-RatGer09/1 isolate shared a close relationship with CMLV. The functions of unique point mutations observed in CMLV-Delhi09 isolate similar to zoonotic OPXVs and other specific aa substitutions and deletions found in various genes of CMLVs needs to be investigated to elucidate their role in species tropism in spill-over hosts as well as identifying the underlying cause of CMLV zoonosis.

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