Cytokine 120 (2019) 15–21

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Poxviral E3L ortholog (Viral Interferon resistance gene) of orf viruses of sheep and goats indicates species-specific clustering with heterogeneity among parapoxviruses

Monu Karki, Amit Kumar, Sargam Arya, M.A. Ramakrishnan, G. Venkatesan*

Division of Virology, ICAR-IVRI, Mukteswar 263 138, Nainital, Uttarakhand, India

ARTICLE INFO

Keywords:
Double-stranded RNA-binding protein
E3L
Orf virus (ORFV)
Parapoxvirus
Viral interferon resistance gene (VIR)
Z-DNA binding domain

ABSTRACT

Orf is a contagious disease posing a serious threat to animal and human health. E3L is one of the evolutionarily acquired immunomodulatory proteins present in orf virus (ORFV) and is responsible for conferring resistance to interferons among poxviruses. Genetic analysis of ORFV isolates of different geographical regions including Indian subcontinent targeting viral interferon resistance (VIR) gene (a homolog of vaccinia virus E3L gene) revealed a high percentage of identity among themselves and other ORFV isolates at both nt and aa levels as compared to low identity among parapoxviruses (PPVs). Phylogenetic analysis showed species-specific clustering among PPVs along with sub-clusters based on host species of origin among ORFVs infecting sheep and goats. Conserved amino acids in N-terminal Z-DNA binding domain and C-terminal ds RNA binding domain of VIR proteins of PPVs corresponding to ORFV VIR positions namely N37, Y41, P57, and W59 (necessary for Z-DNA binding) and E116, F127, F141, and K160 (necessary for dsRNA binding) were found. Further, the predicted protein characteristics and homology model of VIR protein of ORFV showed high structural conservation among poxviruses. This study on E3L genetic analysis of ORFV isolates may provide a better understanding of the molecular epidemiology of circulating strains in India and neighboring countries. Also, E3L deleted or mutated ORFV may be an as vaccine candidate and/or compounds blocking E3L may prove as an effective method for treating broad spectrum poxviral infections, suggesting a wider application in control of poxvirus infections.

1. Introduction

Poxviruses have evolutionary acquired several immunomodulatory proteins like E3L, B8R, B18R, K3L etc to evade host immune response by way of inhibiting host interferons. Among these, E3L protein is conserved among most members of subfamily *Chordopoxvirinae* [7,39]. The E3L protein inhibits activation of double-stranded RNA (ds RNA) activated protein kinase (PKR) [10]. E3L encoded protein contains an amino-terminal Z-DNA-binding (Zα) domain responsible for host range determination [29] and a carboxy-terminal ds RNA-binding domain (dsRBD) [9]. While only the C-terminal domain appears essential for virus replication in cell culture and the N-terminal domain of E3L is dispensable for replication in cell culture [1], both domains are required for pathogenesis in a mouse model [5,29]. Furthermore, VACV with mutated or deleted E3L gene has been a potential replication-competent, attenuated vaccine target against several orthopoxviruses [15,46].

Parapoxviruses (PPVs) include orf virus (ORFV), bovine papular stomatitis virus (BPSV), pseudocowpox virus (PCPV), parapox virus of deer (PVNZ) and sealpox virus (SePPV). Orf or contagious ecthyma, caused by ORFV is a highly contagious disease affecting sheep, goats, wild ruminants and human worldwide. Clinically, the disease is characterized by proliferative lesions on lips, muzzle, eyelids, ears, and around the mouth and nostrils and rarely other body parts in animals [4,44]. Limitations like low mortality, mild clinical presentation of the disease in most of the cases, lack of proper surveillance system and poor reporting of the disease hamper its epidemiological data as well as an effective control strategy. Multi-species involvement and continuous reinfection of the same host further pose difficulty in controlling the disease. Continuous reinfection observed in infection with ORFV is attributed to the subversion of the host immune response by virus-encoded virulence genes. ORFV has a genome size of ~135 kbp encoding ~132 proteins, with slight variations among the species or isolates [14]. ORF020 (homolog of VACV E3L), situated at the left end of ORFV

* Corresponding author.
E-mail address: gnanamvirol@gmail.com (G. Venkatesan).

https://doi.org/10.1016/j.cyto.2019.04.001
Received 31 August 2018; Received in revised form 19 March 2019; Accepted 4 April 2019
Available online 13 April 2019

Table 1
Details of orf virus samples/isolates from India and foreign origin and parapoxviruses used in phylogenetic and multiple sequence alignment studies of viral interferon resistance (VIR) gene.

S. No.	Sample/isolate	Place and Year of isolation	Host species	Gene length (bp)	Accession no.	Reference
1.	ORFV-Mukteswar/05-P51	Uttarakhand, India; 2005	Goat	552	MF414620	This study
2.	ORFV-Mukteswar/05-P6	Uttarakhand, India; 2005	Goat	552	MF414621	This study
3.	ORFV-Meghalaya/03	Meghalaya, India; 2003	Goat	552	MF414625	This study
4.	ORFV-Bangalore/05	Karnataka, India; 2005	Goat	552	MF414622	This study
5.	ORFV-Shahjahanpur/04-P4	Shahjahanpur, Uttar Pradesh, India; 2004	Goat	552	MF414619	This study
6.	ORFV-Jalandhar/07	Punjab, India; 2007	Goat	552	MF414623	This study
7.	ORFV-Assam/LK/14	Assam, India; 2014	Goat	552	MF414624	This study
8.	ORFV-Alwar/08	Alwar, Rajasthan, India; 2008	Goat	552	MF414626	This study
9.	ORFV-Gujarat/06	Gujarat, India; 2006	Goat	552	MF414627	This study
10.	ORFV-Tamil Nadu/08	Tamil Nadu, India; 2008	Sheep	552	MF414618	This study
11.	ORFV-Hyderabad/06	Andhra Pradesh, India; 2006	Sheep	552	MF414628	This study
12.	ORFV-MRCSG/Shuhama/14/1	Jammu and Kashmir, India; 2014	Sheep	552	KU672686	Unpublished
13.	ORFV-SBF/Goabal/15/1	Jammu and Kashmir, India; 2015	Sheep	552	KU672680	Unpublished
14.	ORFV-BSF/Nishat/16/1	Jammu and Kashmir, India; 2016	Sheep	552	KU672684	Unpublished
15.	ORFV-SBF/Dachigam/16/1	Jammu and Kashmir, India; 2016	Sheep	552	KU672681	Unpublished
16.	ORFV-GO/12	Fujian, China; 2012	Goat	552	KP010354	[11]
17.	ORFV-FJ-YT/15	Fuzhou, China; 2015	Goat	552	KU199851	Unpublished
18.	ORFV-SA00	Texas, USA; 2000	Goat	552	AY386264	[14]
19.	ORFV-Gansu/09	Gansu, China; 2009	Sheep	552	JX391976	Unpublished
20.	ORFV-NA1/11	Nongan, China; 2011	Sheep	552	KF234407	[33]
21.	ORFV-SV820/10	Brazil; 2010	Sheep	552	KF927111	[35]
22.	ORFV-D1701/11	Germany; 2011	Sheep	552	HM133903	[36]
23.	ORFV-Iwate/70	Iwate, Japan; 1970	Sheep	552	AB499038	[27]
24.	ORFV-NZ2/06	New Zealand; 2006	Sheep	552	DQ184476	[38]
25.	ORFV-HIS/04	Japan; 2004	Sheep	552	AB522798	[27]
26.	ORFV-IA/82	Iowa, USA; 1982	Sheep	552	AY386263	[14]
27.	ORFV-sCh97	Buenos Aires, Argentina; 1997	Sheep	552	KP244327	[41]
28.	ORFV-B029/96	Germany; 1996	Human	552	KF837136	[17]
29.	ORFV-Takin/01	USA; 2001	Sichuan takin	552	AY424976	[20]
30.	ORFV-IJS081/08	Ishikawa, Japan; 2008	Japanese serows	552	AB492085	[27]
31.	BPSV-AR02/04	USA; 2004	Cattle	588	AY386265	[14]
32.	BPSV-TX09c1/09	USA; 2009	Cattle	597	KM875472	[26]
33.	PPV-Pali/Camel/10	Rajasthan, India; 2010	Camel	552	JQ388235	[40]
34.	PCPV-VR634/63	New Zealand; 1963	Human	543	GQ329670	[23]
35.	PCPV-F00.120R/00	Finland; 2000	Reindeer	549	GQ329669	[23]
36.	PVNZ-HL953/13	Germany; 2013	Red deer	648	KM502564	[16]
37.	SePPV-AFK76s1/15	Poland; 2015	Grey seal	618	KY382358	[19]

genome is a highly conserved gene encoding the viral interferon resistance (VIR) protein involved in inhibition of the antiviral activity of host interferon response [37]. This protein is expressed early in infection and binds the interferon-induced dsRNA dependent kinase (PKR) to make ORFV resistant to both type I and type II interferons [22]. The VIR gene has been a target for molecular diagnosis and genetic characterization of ORFVs in various outbreaks. Keeping this in view, we analyzed Indian and other global ORFVs on the basis of VIR gene along with homology modeling of ORFV VIR protein.

2. Materials and methods

2.1. Virus samples/isolates and DNA extraction

Eleven ORFV samples/isolates belonging to different geographical regions of India maintained at Pox Virus Laboratory, Division of Virology, Indian Veterinary Research Institute (IVRI), Mukteswar, Uttarakhand, India, used in the present study and other ORFVs from other countries included in the analysis are shown (Table 1). Out of these, three ORFV isolates, namely, ORFV-Shahjahanpur/04-P4, ORFV-Mukteswar/05-P51 and ORFV-Mukteswar/05-P6 were recovered in lamb testicle cells (LT) grown in Eagle's minimum essential medium (EMEM) supplemented with 10% bovine calf serum and antibiotics for virus isolation. After incubation for LT cells at 37 °C for 5–6 days and appearance of > 80–90% typical cytopathic effect of ORFV, cells were harvested in 250 µL phosphate buffered saline (pH 7.2) followed by extraction of total genomic DNA using commercial Nucleo-pore DNA-Sure® tissue mini kit [Genetix Biotech Asia Pvt. Ltd., India]. For other samples, crusted scab lesions from infected goats and sheep were homogenized in phosphate-buffered saline with antibiotics followed by freeze-thawing thrice and centrifugation. From supernatant, genomic DNA was extracted as described earlier [44].

2.2. PCR, cloning, and sequencing of VIR gene

The preliminary identity of all samples/isolates was confirmed using DNA polymerase and B2L gene-based PCR as described earlier [3,4]. Full-length VIR gene was amplified from various ORFV isolates by PCR from the extracted DNA using primers designed on the basis of genome sequences of ORFV isolates available at GenBank (fwd: 5′-TTAGAAGCTGATGCCGCAG-3′ and rev: 5′-ACAATGGCCTGCGA GTG-3′). PCR reaction was performed with 2X GoTaq buffer (10 mM dNTPs, 25 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase) [Promega, USA], 10 pmol each primers and 1.5 µL template DNA using optimized cycling parameters: 95 °C for 5 min, 35 cycles of denaturation (95 °C for 30 sec), annealing (58 °C for 1 min) and extension (72 °C for 45 sec); and then final extension at 72 °C for 7 min. The PCR amplicons were purified using SureExtract® PCR Clean-up Gel Extraction kit [Geentix Biotech Asia Pvt. Ltd., India] and cloned into pGEM®-T easy vector [Promega, USA] for sequencing using an automated DNA sequencer [ABI PRISM 3100; Perkin Elmer, Applied Biosystems, USA]. The complete nucleotide sequences of VIR gene of eleven ORFV samples were submitted to GenBank (Table 1).

2.3. Sequence and phylogenetic analysis

The comparative sequence analysis of VIR gene of Indian ORFV isolates was carried out along with other PPV sequences from GenBank. The details of ORFVs and PPVs are presented in Table 1. Nucleotide (nt) and amino acid (aa) identities were compared using ClustalW program of DNASTAR package (Lasergene 6.0, DNASTAR Inc., USA) [43]. Phylogenetic trees based on deduced aa sequences were constructed for PPVs including ORFVs and for other animal poxviruses (Table S1) at 1,000 replicates of the dataset using bootstrap test of phylogeny in the neighbor-joining method in MEGA 7.0 software [31].

2.4. Homology modeling and analysis of functional motifs

Consensus VIR gene sequence of ORFV was analyzed using various protein prediction programs at ExPASy server for secondary structure prediction as well as characterization. A homology model of ORFV VIR was predicted using SWISS-MODEL [2] involving N- and C-terminus regions using the Z-DNA binding domain of Yatapoxvirus E3L [PDB – 1sfu] [21] and interferon-inducible ds RNA-dependent protein kinase activator A [PDB – 2dix], respectively.

3. Results

3.1. Multiple sequence alignment and phylogenetic analysis

PCR amplification of all eleven ORFV samples/isolates under study amplified the product size of ~560 bp with an ORF length of 552 bp (Fig. S1) which was confirmed by sequencing. The sequenced were submitted to GenBank followed by the assignment of accession numbers MF414618 to MF414628. Multiple sequence alignment of ORFV isolates under study demonstrated a significant percentage of identity at nt and aa levels with other foreign isolates as anticipated (Table 2). Indian ORFV isolates including vaccine strain shared 92.4–99.0% and 84.8–99.5% sequence identity among themselves at nt and aa levels, respectively. Indian ORFV-Mukteswar 59/05/Goat/2005(P50) (vaccine strain) shared 95.3% and 92.4% identity with ORFV D1701/USA/Sheep/11 (attenuated strain) at nt and aa levels, respectively and 100% identity, at both nt and aa levels, with its lower passage (P6). With PCPV and BPSV, lower sequence identity was observed at both nt and aa levels (Table 2). Multiple sequence alignment revealed several amino acid substitutions among ORFVs that were demonstrated more towards the N-terminal region (Fig. 1). Two amino acid substitutions, namely, K135E and L140R were found in ORFV isolates of sheep origin, except the later was also found in ORFV-Shahjahanpur/04, an isolate form goat origin. In addition, ORFV-Shahjahanpur/04 showed several substitutions, namely M74D, E78D, T88A, L89R, G91S, D93Y, Q99H, R104A, K106I, N109K, P110R, S112N, H137R, T166S, and S170I. Notably, isolates from the Kashmir region of India exhibited the same substitutions as are seen in ORFVs of foreign origin (Fig. 1). These substitutions were not found in other Indian isolates including isolates under study. Several conserved amino acids were found in all parapoxviruses including those necessary for Z-DNA binding and ds RNA binding (Fig. 1).

Phylogenetic tree constructed based on VIR gene sequence revealed that the members of genus *Parapoxvirus* segregated into three distinct groups namely as ORFV, PCPV, and BPSV (Fig. 2). Indian ORFV isolates clustered only with other ORFV isolates. Within ORFV isolates, two separate sub-clusters were formed on the basis of host species of origin, namely sheep and goats. Attenuated strain ORFV-D1701 diverged separately from all other ORFV isolates. Phylogenetic analysis of various animal poxviruses based on deduced aa sequence of VIR (E3L orthologs) indicated the grouping of respective members of each genus together in clusters of orthopoxviruses, clade II poxviruses (comprising genera *Capripoxvirus*, *Cervidpox virus*, *Suipoxvirus*, *Leporipoxvirus* and *Yatapoxvrius*) and parapoxviruses (Fig. S2).

Table 2
Percentage identities of VIR gene sequences of ORFV isolates with each other and other parapoxviruses.

Virus	ORFV isolates under study	
	nt	aa
ORFV isolates under study	92.4–99.0	84.8–99.5
ORFV Mukteswar 59/05(P50) (Vaccine virus)	95.1–98.9	89.7–97.8
ORFV isolates from Jammu & Kashmir, India	92.9–98.9	85.3–98.4
Worldwide ORFV isolates	92.0–99.1	84.8–97.8
PCPV isolates	74.5–78.8	49.5–75.7
BPSV isolates	65.2–68.3	49.5–58.7

Abbr. - ORFV - Orf virus, PCPV - Pseudocowpox virus, BPSV - Bovine popular stomatitis virus.

3.2. Protein characteristics and homology modeling of VIR protein of ORFV

ORFV VIR protein is 19.9 kDa with 183 amino acids. The protein was predicted to have a DNA binding domain (aa position 3–66) and a double-stranded RNA binding motif (aa position 110–177) (Fig. 3). The sumo-interacting motif (SIM) for sumoylation [L/V/I) × (L/V/I)(L/V/I)] was predicted at aa positions 172–175. Analysis of secondary structure predicted the presence of three alpha helices and two beta sheets in Z-DNA binding domain at N-terminus along with two alpha helices and three beta sheets in ds RNA-binding domain at C-terminus. Homology modeling suggested that ORFV E3L Z-DNA-binding domain consisted of three α-helices (designated α1, α2 and α3) and three β strands (β1, β2 and β3) with α1β1α2α3β2β3 topology, whereas C-terminal ds RNA binding domain consisted two α-helices (designated α1 and α2) and three β strands (β1, β2, and β3) with α-β-β-β-α topology in which the two α-helices are present on the same side of a three-stranded antiparallel β-sheet.

4. Discussion

All poxviruses of vertebrates are known to encode E3L protein, except genera *Avipoxvirus*, *Crocodylidpoxvirus,* and *Molluscipoxvirus* [39]. VACV E3L is most characterized viral interferon resistance (VIR) protein, which is responsible for interferon resistance. Studies on host range genes of PPVs are scarce. Partial [20,30,32,34,41] or full-length VIR gene [27] has been used for characterization of ORFVs due to higher sequence variability than B2L gene. Although high sequence variability in VIR gene has been observed as reported earlier [32,41], the present study showed a higher sequence identity of Indian ORFVs with other ORFV strains than with BPSV and PCPV indicating that VIR gene is relatively conserved among ORFV strains. Among ORFV, N-terminal half of the protein has been shown to be less conserved as compared to C-terminal portion [30]. Among ORFV strains, nucleotide variation resulted in random, individual amino acid changes as shown previously [32,41]. The sequence identity of ORFV-Mukteswar59/05 (P50) vaccine virus and its lower passage (P6) virus indicates that this gene may not be involved in attenuation of virus during cell culture passages. At aa position 135, glutamic acid (E) is found in ORFV isolates of sheep origin whereas lysine (K) is found in ORFV isolates from goats. Also, at position 140, arginine (R) is found in sheep origin ORFV isolates whereas leucine (L) is found in goat origin ORFV isolates except for ORFV-Shahjahanpur/04 which was reported as a mixed infection with PPR. ORFV-Shahjahanpur/04 accumulated several mutations in VIR gene that may be due to selection pressure or virus strategy to evade the host's immune response in concurrent PPR infection in the above infection. Earlier, B2L gene characterization of ORFV-Shahjahanpur/04 isolate has shown slight divergence from other Indian ORFVs [25] and found closely related to Assam and Tripura isolates [45]. Several deletions have been reported in ORFVs as compared to

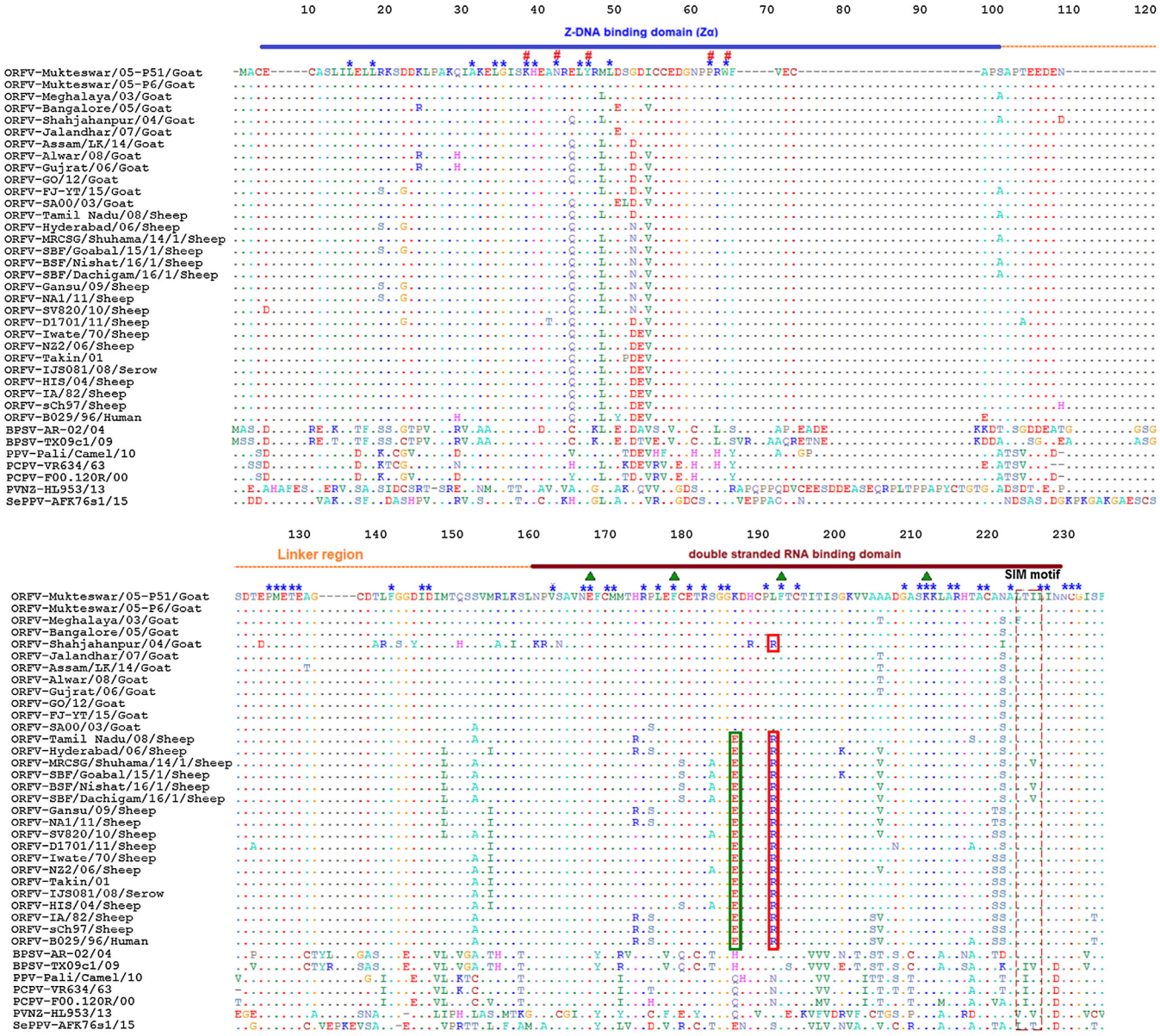

Fig. 1. Alignment of deduced amino acid sequences of VIR gene sequences of orf viruses (ORFVs) along with other parapoxviruses (PPVs) containing Z-DNA binding domain and double-stranded RNA binding (dsRBD) domain. Identity is marked by dots and dashes represent amino acid deletions. Blue asterisks represent conserved amino acids among PPVs. # represents conserved amino acids necessary for Z-DNA binding and green triangles represent conserved amino acids necessary for ds RNA binding. Green and red boxes, respectively indicate conserved E and R amino acids in ORFVs of sheep origin (except ORFV-Shahjahanpur/04). Red dotted box represents SIM (SUMO-interacting motif). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

other PPVs. BPSV E3L has shown sequence variability with 588 bp and 597 bp length in case of BPSV-BV-AR02/04 and BPSV-TX09c1/09, respectively [26]. All ORFV isolates grouped together with PCPV and BPSV being evolutionary distant as previously reported. Phylogenetic grouping pattern independent from geographical origin and time of isolation was seen as some Indian strains grouped with isolates from China and USA. Although, clustering of ORFV isolates on the basis of host species of origin was observed based on full-length VIR suggesting that genetic differences exist between ORFV strains from sheep and goats. Surprisingly, analysis based on partial VIR gene didn't show host-specific clustering [41,34]. Cross-infection of ORFV is possible between sheep and goats but polyclonal antibody raised against sheep ORFV neutralizes sheep ORFV more efficiently than goat ORFV, suggesting that the neutralization epitopes of ORFV of ovine origin might be different than those of caprine ORFV [13]. ORFV isolated from the human (ORFV-B029/96) showed the same substitutions as in ORFV isolates from sheep, indicating the strain might be of sheep origin.

Despite a low 31% aa identity to VACV E3L [37], several conserved amino acids were found in all PPVs at N- and C-terminal domains of VIR protein. Amino acid residues Y48 [28], P63A [29] and W66 [42] in VACV E3L have been shown to be essential for Z-DNA binding. Corresponding residues Y41, P57, and W59 were found to be conserved in ORFV. Several amino acid residues in the C-terminal domain VACV E3L, viz. E124, F135, F148, K167, R168, and K171 form a hydrophilic plane for binding with the minor groove of dsRNA [24]. Out of these, only four amino acids E116, F127, F141, and K160 corresponding to E124, F135, F148, and K167 of VACV E3L i.e. were found to be conserved among ORFV. Implications of the absence of other residues in PPVs need to be carried out. Another important feature of the E3L protein is the presence of SIM-motif (L/V/I) × (L/V/I)(L/V/I) at C-terminus which is responsible for ubiquitination/SUMOylation, and inhibiting PUMA (p53-upregulated modulator of apoptosis) gene

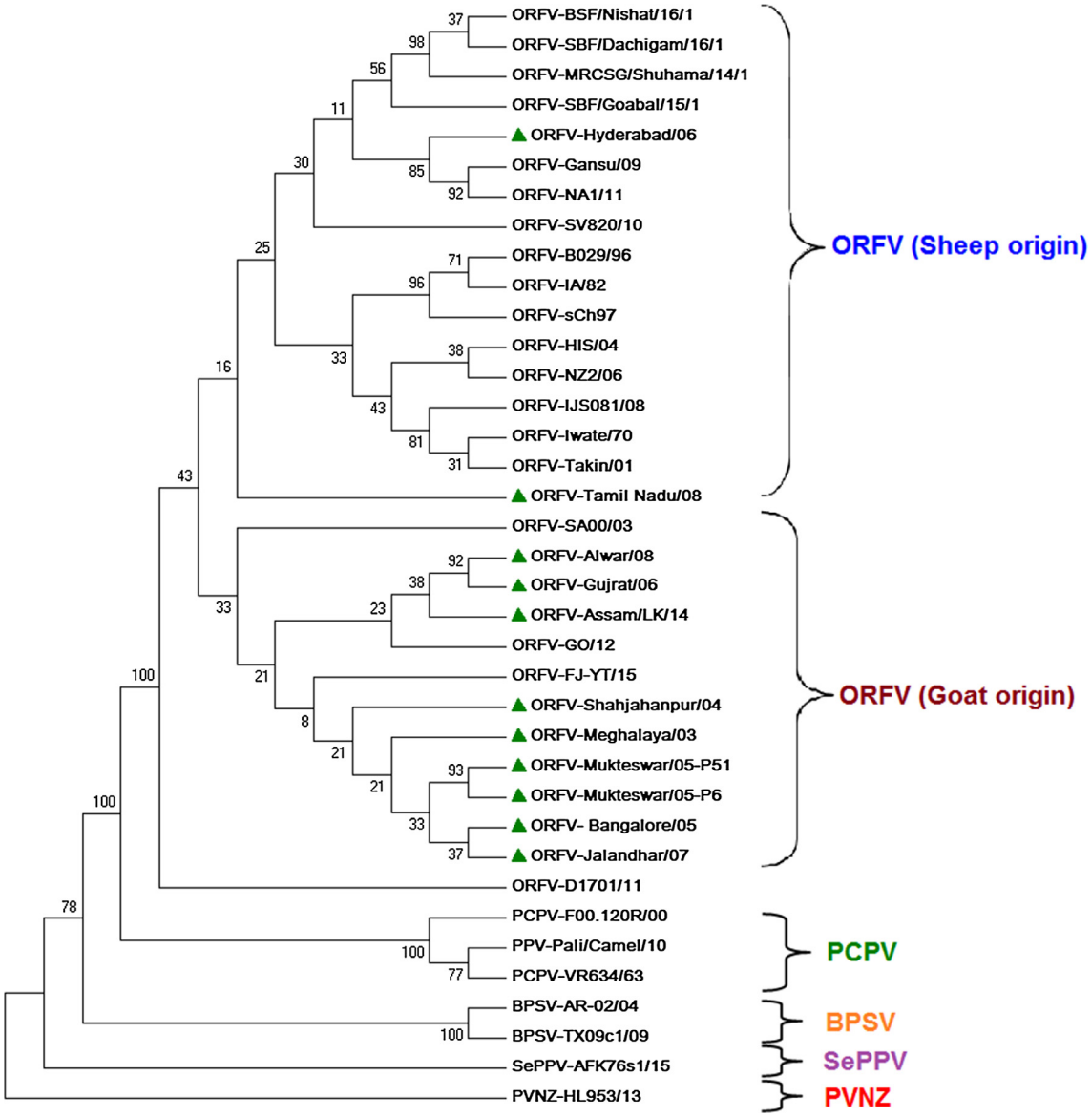

Fig. 2. Phylogenetic tree based on amino acid sequences of VIR genes of Indian ORFV isolates along with other ORFVs and PPV sequences available in GenBank (Refer Table 1 for details) were used. The tree was obtained by the neighbor-joining program of MEGA version 7.0 (bootstrap 1,000). Green triangles indicate ORFV strains under the present study.

involved in apoptosis of the infected cells [18]. Like other Z-DNA binding proteins and E3L of other poxviruses [28,21], ORFV E3L Z-DNA-binding domain consisted of same α1β1α2α3β2β3 topology. C-terminal ds RNA binding domain consisting two α-helices (α1 and α2) and three β strands (β1, β2, and β3) assumed α-β-β-β-α topology in which the two α-helices are present on the same side of a three-stranded anti-parallel β-sheet like other ds RNA binding proteins [8].

Conclusively, VIR gene analysis of ORFVs revealed high sequence variability along with host-specific clustering. Elucidation of the VIR gene of ORFVs may give a better understanding of the molecular epidemiology of Indian ORFV isolates. Future studies are planned to sequence the complete genomes of these ORFV samples/isolates. Elucidation of the localization and dsRNA binding capacity of VIR genes for different isolates under study may shed some light on their virulence. Also, E3L deleted or N-terminal deleted E3L mutant ORFV may be useful as a promising candidate as an improved vaccine candidate [6]. Also, Compounds or siRNA blocking E3L [12] may prove as an effective method for treating broad spectrum poxviral infections, suggesting a wider application in control of poxvirus infections.

Conflict of interest

Authors declare that there is no conflict of interest.

Acknowledgements

The authors thank the Director, ICAR-Indian Veterinary Research Institute for providing necessary facilities to carry out this work and the staff of Pox virus laboratory, IVRI, Mukteswar, for their valuable and timely help in carrying out this work. This study is part of MVSc thesis work of the first author. The financial support provided by DBT, New Delhi, India under North-East Twinning program on DBT-NER on Pox project (BT/385/NE/TBP/2012) is also acknowledged.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.04.001.

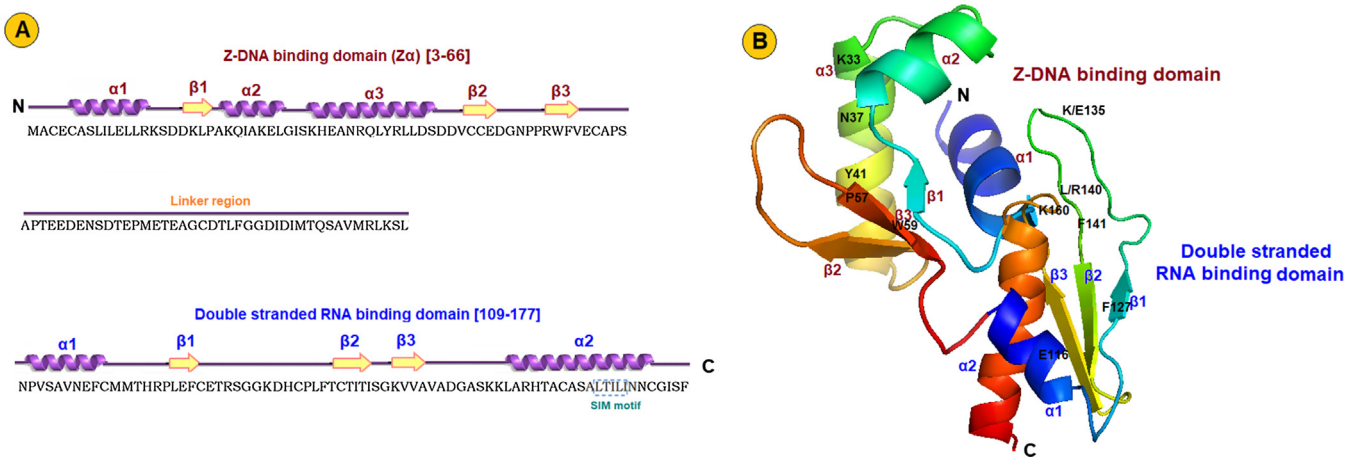

Fig. 3. Secondary structure prediction and homology modelling of ORFV VIR protein. A. Secondary structure of ORFV VIR protein. It consists of Z-DNA binding domain at N-terminus, which possesses three alpha helices (violet, numbered in brown) and three beta sheets (yellow arrows, numbered in brown) connected by loops and double-stranded RNA-binding domain which possesses two alpha helices (violet, numbered in brown) and three beta sheets (yellow arrows, numbered in blue) connected by loops. SIM motif (LTILI) has been denoted as dotted box. B. Ribbon representation of homology model of ORFV VIR protein depicting critical residues generated using automated homology prediction by SWISS-MODEL server by prediction of N- and C-terminus regions using Z-DNA binding domain of Yatapoxvirus E3L [PDB − 1sfu] and interferon-inducible ds RNA-dependent protein kinase activator A [PDB − 2dix], respectively. Alpha helices (numbered in brown) and beta sheets (numbered in blue) connected by loops are depicted in sequential numbers. The abbreviations are as follows: α, alpha helix; β, beta sheet; N – N-terminus; C – C-terminus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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