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Genetic diversity of fusion gene (ORF 117), an analogue of vaccinia virus A27L gene of capripox virus isolates

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Abstract The fusion gene (ORF 117) sequences of twelve ($n = 12$) capripox virus isolates namely sheeppox (SPPV) and goatpox (GTPV) viruses from India were demonstrated for their genetic and phylogenetic relationship among them. All the isolates were confirmed for their identity by routine PCR before targeting ORF 117 gene for sequence analysis. The designed primers specifically amplified ORF 117 gene as 447 bp fragment from total genomic DNA extracted from all the isolates. Sequence analysis revealed a significant percentage of identity among GTPV, SPPV and between them at both nucleotide and amino acid levels. The topology of the phylogenetic tree revealed that three distinct clusters corresponding to SPPV, GTPV and lumpy skin disease virus was formed. However, SPPV Pune/08 and SPPV Roumanian Fanar isolates were clustered into GTPV group as these two isolates showed a 100 and 99.3 % identity with GTPV isolates of India at nt and aa levels, respectively. Protein secondary structure and 3D view was predicted and found that it has high antigenic index and surface probability with low hydrophobicity, and it can be targeted for expression and its evaluation to explore its diagnostic potential in epidemiological investigation in future.

Keywords Capripox virus · Fusion gene · PCR · Sequence analysis · Phylogenetic tree

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Sheeppox and goatpox termed as capripox are caused by viruses that are members of the genus capripox virus posing a significant economic threat globally in general and particularly in developing countries like India and are notifiable to Office Internationale des Epizooties (OIE) [1]. Capripox viruses (CaPV) are difficult to be distinguished by serological methods, but possible by molecular tools [2] and they usually have no host preference [3]. CaPV isolates are shown to have differential host preference demonstrated by experimental challenge studies and virus isolation [4] and considered as different entities within the Indian subcontinent [5]. Capripox is enzootic in Southwest and Central Asia, Northern and Central Africa and Indian subcontinent [1] and pose potential economic threat to the small ruminant industry [6].

The present study is focused on (1) genetic analysis based on ORF 117 of capripox virus isolates ($n = 12$) of Indian origin and their relationship with other isolates worldwide in order to identity the variation and (2) prediction of the secondary structure of the protein. This structural protein is a homologue to the vaccinia virus (VACV) A27L protein. This fusion protein, which is present mainly on intra-cellular mature virus (IMV), mediates the virus interaction with cell surface heparan sulphate [7]. The A27L protein was found to be a trimer, highly hydrophilic, and its functional properties were analogous to the hemagglutinin protein of influenza virus [8]. It was also found to be similar to gp41 protein of human immunodeficiency virus, except for the presence of a protein-

anchoring domain instead of a transmembrane domain. There are only a few reports of genetic analysis based on the ORF 117 gene of CaPV virus isolates available [9]. Therefore, the study was taken to analyse Indian CaPV isolates including vaccine strains of sheeppox and goatpox targeting ORF 117 gene sequence, one of the structural protein genes of the genus, and to identity the variation and genetic relatedness between and among SPPV and GTPV isolates.

Primers specific to ORF 117 gene sequence were designed (ORF 117 F: GG**CCATGG**CGATGGACAGAGCGTTAT CAAT and ORF 117 R: GG**AAGCTT**TCATAGTGTT GTACTTCGTCCTG) on the basis of laboratory published sequences of GTPV Ladakh/01/01 and GTPV Sambalpur/82 (GenBank Accession No.: AY588605, AY588606, respectively) to amplify the full-length gene. Live attenuated goatpox virus, strain Uttarkashi (passage 60) and capripox virus (CaPV) isolates namely SPPV Srinagar (P7), SPPV Makdhoom (P3), SPPV Ranipet (P55), SPPV Pune/08 (P2), SPPV Romanian Fanar (P50), SPPV Jaipur (P3), GTPV Akola (P5), GTPV CIRG/16 (P3) and GTPV Ladakh/01 (P3) available at Pox Virus Laboratory, Division of Virology, IVRI, Mukteswar were revived and propagated in Vero cell line using Eagle's minimum essential medium (EMEM) (pH 7.4) containing foetal bovine serum (FBS) as per standard protocols (OIE, 2008). After infection, cells were observed daily for the appearance of cytopathic effects (CPE). At 80 % of CPE, the infected cells were harvested and total genomic DNA (gDNA) was extracted using QIAamp® DNA Mini Kit (M/s Qiagen, USA) as per manufacturer's protocols. In this study, two skin scabs of goat received (Pune/09 and CSWRI/05) for diagnosis were also included, 10 % suspension of which was then used for total gDNA extraction and PCR reaction. All the virus isolates and clinical samples were initially confirmed to be SPPV and GTPV by diagnostic PCR and PCR-RFLP as described elsewhere [10, 11].

After confirmation of the isolates as CaPV, full-length ORF 117 gene was amplified from extracted gDNA using custom synthesized (M/s Metabion International AG, Martinsried, Germany) Capripox-specific primers using standard protocol. In brief, PCR was performed in a 50-μL reaction containing 100 ng of DNA, 10 pmol of each primer, 5 μL of 10× buffer with MgSO4, 0.4 mM each of deoxyribonucleotide triphosphate and IU of Pfu DNA polymerase (MBI Fermentas, USA) in a thermal cycler (BioRad, USA) at a standard cycling conditions with an optimized annealing temperature at 59 °C. The PCR products were checked in 2 % agarose gels stained with Ethidium bromide and they were gel purified using commercial gel extraction kit (MBI Fermentas, USA) as per manufacturer's protocol and sequenced commercially (M/s SciGenome Labs, Kochi, India).

The nucleotide sequences were aligned using Sequencher (Sequencher® version 5.1, Gene Codes Corporation, USA) and the aligned sequences ($n = 12$) were submitted in GenBank (Accession No. *KF153667* to *KF153678*). Comparative sequence and phylogenetic analyses of ORF 117 gene sequences of CaPV isolates were made with published reference sequences from members of CaPV genus (Table 1) using suitable bioinformatic tools. The percent identity among various capripox isolates at nt and aa levels were determined by Clustal W method of MegAlign (Lasergene 6.0, DNASTAR Inc., USA). Phylogenetic tree based on deduced aa sequence of ORF 117 was also constructed at 1,000 replicates using bootstrap test of phylogeny in the neighbour-joining method in MEGA5.02 software [12]. The structure of the translated protein sequence was visualized using Phyre^2, an online free tool for predicting the secondary structure and fold recognition [13].

Among the proteins of pox viruses, A27L, B5R, D8L, H3L and L1R have been shown to induce neutralizing antibodies [14]. A27L, D8L and H3L proteins are found on intracellular mature virus particle (IMV), and B5R and L1R proteins are found on extracellular enveloped viruses (EEV). Therefore, in the present study, ORF 117 gene of CaPV, which is an analogue of A27L protein of VACV, was selected for amplification and sequencing to study the genetic relatedness among CaPV isolates.

The infected cells by different CaPV isolates including vaccine strains had shown a characteristic CPE [15], and the virus isolates were confirmed as CaPV using in-house-developed diagnostic PCR [10]. A product of 447 bp in size could be amplified from all virus isolates. Sequence alignment revealed that the 447-bp amplicon from all the isolates corresponds to the full-length ORF 117 gene of CaPV. Sequence analyses of these virus isolates demonstrated a significant percentage of identity at nt and aa levels with other CaPV isolates as anticipated. It revealed 98.4–100 and 96.6–100 % identity among all GTPV isolates including other isolates worldwide at nt as well as aa levels, respectively, whereas it showed 96.2–100 and 93.2–100 % identity among SPPV isolates at nt and aa levels, respectively. Between SPPV and GTPV isolates, it had shown a percentage of identity of 95.3–100 and 91.2–100 at nt and aa levels, respectively. Among Indian origin, GTPV isolates had shown a percent identity of 98.2–100 and 96.6–99.3, whereas SPPV isolates revealed a 96.4–100 and 93.3–99.3 % of identity at nt and aa levels, respectively. Further, it showed a percentage of identity of 96.4–100 and 93.3–99.3 at nt and aa levels, respectively, between GTPV and SPPV isolates of Indian origin. Among these, SPPV-Pune/08 and SPPV-Roumanian Fanar had shown a percentage of identity of 100 and 99.3 with GTPV isolates.

Table 1 Sequences used for analysis of CaPV isolates based on ORF 117 gene

Sl. no.	Virus/isolate/strain/sample	Species	Country and year of isolation	Accession number
1	GTPV Uttarkashi	Goat	India; 1976	KF153667
2	GTPV Akola/08	Goat	India; 2008	KF153668
3	GTPV CSWRI/05	Goat	India; 1946	KF153669
4	GTPV CIRG/08	Goat	India; 2008	KF153670
5	GTPV Ladakh/01/P3	Goat	India; 2001	KF153671
6	SPPV Srinagar 38/00	Sheep	India; 2000	KF153672
7	SPPV Makhdoom	Sheep	India; 2007	KF153673
8	SPPV Ranipet	Sheep	India; NA	KF153674
9	SPPV Pune/08	Sheep	India; 2008	KF153675
10	SPPV Roumanian Fanar	Sheep	Kenya; NA	KF153676
11	SPPV Jaipur	Sheep	India; NA	KF153677
12	GTPV Pune/09	Goat	India; 2009	KF153678
13	GTPV Ladakh/01	Goat	India; 2001	AY588605
14	GTPV Sambalpur/82	Goat	India; 2001	AY588606
15	GTPV Tirupathi	Goat	India; 2005	FJ716697
16	GTPV G20-LKV	Goat	Kazakhstan; 2000	AY077836
17	GTPV Pellor	Goat	Kazakhstan; 2000	AY077835
18	GTPV AV41(G56-42)	Goat	China; 2007	EU024477
19	SPPV TU	Sheep	Turkey; 1970	AY077832
20	SPPV NISHKI	Sheep	Kazakhstan; 1994	AY077834
21	LSDV Neethling/LW/1959	Cattle	S. Africa; 1959	AF409138
22	LSDV NI-2490	Cattle	Kenya; 1958	AF325528
23	LSDV NW-LW	Cattle	S. Africa; 1999	AF409137

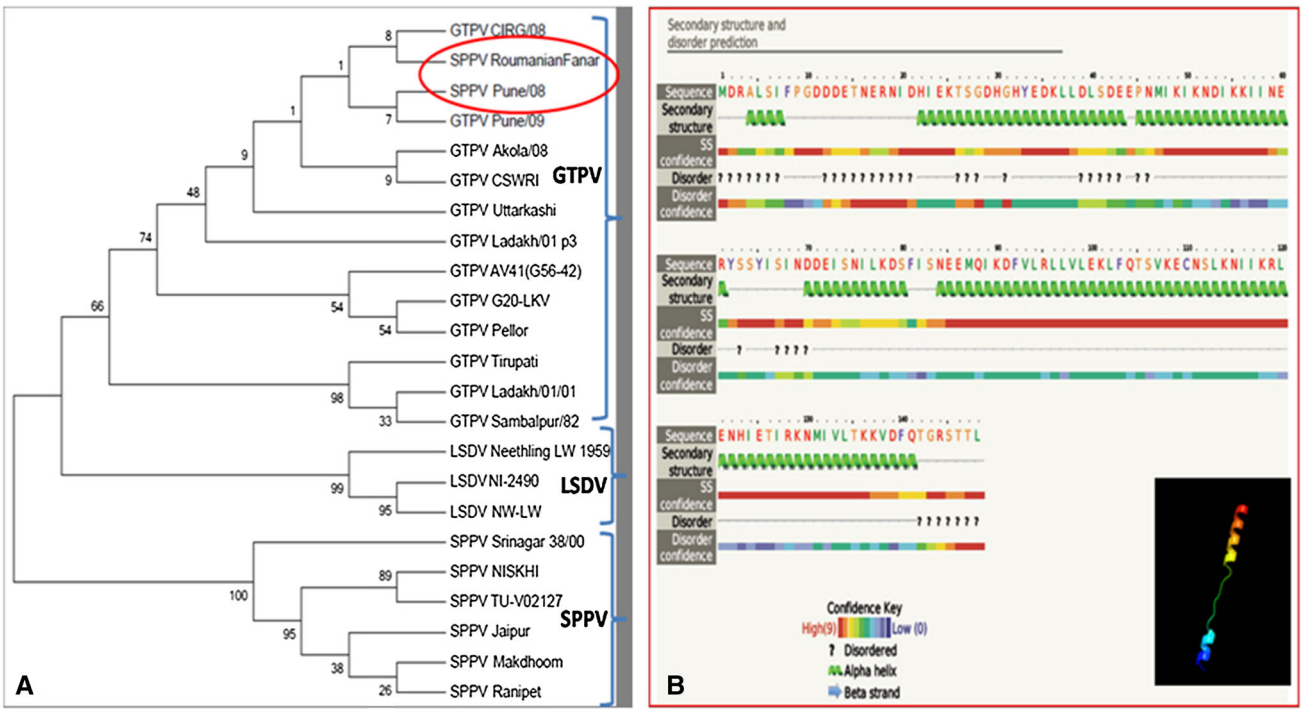

Fig. 1 **a** Phylogenetic tree based on deduced aa sequence of ORF 117 constructed at 1,000 replicates using bootstrap test of phylogeny in the neighbour-joining method in MEGA 5.02 software. Values of the major clusters are indicated in the node or branch of the tree, which represents the bootstrap confidence, tested using 1,000 replicates of the dataset. *Bar* represents the genetic distance (i.e., number of substitutions per site). **b** The secondary structure prediction of translated protein sequence of ORF 117 gene by Phyre[2] online free software (secondary structure and 3D view predicted at 93.5 % confidence value)

The phylogenetic tree resolved CaPV into three different distinct tight clusters, namely GTPV, SPPV and LSDV as anticipated. Interestingly, two SPPV isolates namely Pune/08 and Roumanian Fanar are placed within the GTPV cluster rather than SPPV group (Fig. 1a). Sequence analysis about homology among species infers the conserved nature of the gene among the species but the clustering of two SPPV isolates into GTPV group in phylogenetic tree needs to be investigated further through experimental infection in the homologous hosts to trace its host discrimination [5]. Therefore, the target gene cannot be used for distinguishing the species, and the conserved nature of the sequence may pave way for development of a recombinant antigen-based diagnostic kit for sero-surveillance of capripox viruses in endemic areas. The consensus tree also revealed that GTPV and LSDV are more closely related to each other than to SPPV based on tree topology and also supports the hypothesis that GTPV and LSDV are from a common ancestor close to SPPV as reported earlier [2, 16]. Further, the translated gene sequence of the entire ORF of this gene revealed a good antigenic index with less hydrophobicity when demonstrated using the protean program in the DNA STAR software package. The secondary structure of the protein was predicted to have arrangement of α-helix and β-pleated sheets, and 3D model of the protein had 93.5 % confidence with vaccinia virus A27L protein (Fig. 1b). Because of the conserved nature of the ORF 117 gene among the genus CaPV particularly between SPPV and GTPV and also good antigenic index of the translated protein demonstrated by bioinformatic analysis, it could be selected as a candidate gene for further expression studies which could detect both SPPV- and GTPV-specific antibodies in immuno-assays like ELISA.

It is concluded that the ORF 117, an analogue of vaccinia virus A27L gene, had shown a high homology in sequence of the ORF 117 gene from the isolates of Indian origin as well as from isolates worldwide and also indicated high antigenic index of the protein encoded by this gene. Therefore, it can be targeted for expression in heterologous system and the antigen could be explored for its diagnostic potential to use in ELISA for the sero-surveillance of capripox disease as an alternate to existing immunological assays.

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Conflict of interest All the authors declare that they have no conflict of interest.

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