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Identification and Comparative Phylogeny of Sheep and Goat Pox Isolates from Jammu, India

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ABSTRACT

Background: In view of the limited documentation of sheep and goat pox disease in animals of Jammu region, it was felt necessary to conduct a blanket screening of the small ruminant populations in areas where they are largely reared.

Methods: The investigation was conducted to study the occurrence of capripox infection amongst small ruminants through clinical survey and confirmatory laboratory diagnosis. PCR amplification of a part of the P32 core protein gene successfully confirmed *Capripoxvirus* (CaPV) in clinical scab samples from both sheep and goats and from inoculated CAM. Positive amplified samples yielded a predicted 192 bp product.

Result: Sequencing of the partial *P32* gene of one isolate each from sheep (*Sheeppoxvirus*, SPPV) and goat origin (*Goatpoxvirus*, GTPV) revealed that each isolate were distinct and showed 97.8% identity with each other. The SPPV clustered with respective sheeppox cluster with 100% homology to with other SPPV strains reported within India and abroad and also to vaccine strains Srinagar and Rumanian-Fanar (RF). The GTPV was closely identical (~98-99%) with other strains from India and abroad with some unique residues of its own. Both the SPPV and GTPV were divergent from Lumpy Skin Disease Virus (LSDV) strains. The sequence of SPPV strain being similar to circulating strains and prevalent vaccines in the country favours a common vaccination strategy. However, specific GTPV vaccine is not available in the country. The circulating CaPV isolates were found to be host specific. The possibility of vaccination failures or disease caused by vaccines itself, or cross transmission between hosts cannot be ruled out and in such instances it becomes difficult to distinguish with natural disease.

Key words: *Capripoxvirus* (CaPV), *Goatpoxvirus* (GTPV), *P32* gene, Pathology, Phylogeny, *Sheeppoxvirus* (SPPV).

INTRODUCTION

Capripoxvirus (CaPV) of small ruminants are most severe of the pox diseases of domestic animals and they have a very important role in agricultural economy. They are included in the list of notifiable diseases of Office International des Epizooties (OIE) (Mirzaie *et al.*, 2015). Sheep pox and goat pox are highly contagious viral diseases of sheep and goat which are caused by the epitheliotropic DNA pox viruses of genus *Capripoxvirus,* subfamily *Chordopoxvirinae* and the family *Poxviridae*.

Sheep pox is also known as ovine pox or Laccavalle, caused by virus namely *Sheeppoxvirus* (SPPV) (Buller *et al.*, 2005), while the other related members of the genus include goat pox virus (GTPV) and lumpy skin disease virus (LSDV) (Van Regenmortel *et al.*, 2000). The causative agents are considered to be very host-specific. Even then, cross-transmission of infection has been reported. Phylogenetic analysis exhibit that members of the *Capripoxvirus* could be delineated into three distinct clusters of SPPV, GTPV and LSDV based on the *P32* genomic sequence (Hosamani *et al.*, 2004).

Capripoxviruses are considered to be host-specific and cause outbreaks in a preferred host. However, occasionally some CaPV strains infect both sheep and goats. Instances were recorded when SPPV were found to affect goats in Makhdoom, India (Bhanuprakash *et al.*, 2010) or when GTPV

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were found to affect both goats and sheep in Samba district of Jammu and Kashmir, India (Ramakrishnan *et al.*, 2016). In view of the limited documentation of sheep and goat pox disease in animals of Jammu region, it was felt necessary to conduct a blanket screening of the small ruminant populations in areas where they are largely reared. Preliminary diagnoses of the disease were to be confirmed by employing molecular techniques with the isolation and characterization of the virus for the circulating strains within the region. The present study therefore was proposed to survey, confirm and characterize the circulating *Capripoxvirus* strains in Jammu region.

MATERIALS AND METHODS

Virus isolates

A live attenuated vaccine virus (Raksha-SP) manufactured by Indian Immunologicals (Batch no.01SPPV00317) was used as a reference virus. Viruses obtained from infected tissue or scabs of clinically infected animals were designated putatively as *Sheeppoxvirus* (SPPV) for isolates obtained from affected sheep and *Goatpoxvirus* (GTPV) for isolates obtained from affected goats.

Collection of clinical samples

A survey was carried out for investigating the occurrence of capripox infection in small ruminants from different parts of Jammu, Samba, Kathua, Udhampur, Reasi, Doda, Kishtwar, Rajouri and Poonch districts of Jammu Division (except Ramban district) from June 2017 to November 2018. A total of 47 localities were visited and sheep and goats were investigated for occurrence of the disease. Clinical materials included scabs, skin lesions, or occasional incisional biopsies and lesions from skin from Capripox suspected live or dead animals. Laboratory work was carried out in Veterinary Faculty, Jammu, India.

Isolation of putative CaPV (SPPV, GTPV) from clinical samples

Clinical scab/skin samples of affected animals were processed for virus isolation. A 10-20% solution of the scab samples in PBS was prepared by mechanical homogenization using a sterilized mortar and pestle. Homogenized samples were centrifuged at 3,000 × g for 15 min. The supernatant was then passed through a 0.45 µm syringe filter (Millipore) and incubated with streptomycin, penicillin antibiotic solution (@ 200 IU of penicillin and 200 µg of streptomycin per ml suspension for 1 hr at 37°C. Processed samples were used for infecting CAM for virus isolation. Inoculation in the artificial air sac route was employed as per Cunningham (1973).

Virus isolation in chicken embryonated eggs (CEE)

Approximately 10-12 day old embryonated chicken eggs from Government Poultry Hatchery Unit, Belicharana, under Animal Husbandry Department, Jammu were procured for virus inoculation.

Detection of CaPV nucleic acid

For extraction of viral DNA, commercially available DNeasy Blood and Tissue Kit (Qiagen USA, #69504) was used as per manufacturer's instructions. The *P32* gene encoding a core protein of the virus was targeted using primer sequences (*P32* F 5′-TTTCCTGATTTTTCTTACTAT-3′; *P32* R 5′-AAATTATATACGTAAATAAC-3′) and conditions as previously described by Ireland and Binepal (1998). PCR amplification was performed using 5 µl 10x PCR Buffer, 3 µl $MgCl_2$ (25 mMol), 0.2 µl dNTPs (10 mMol), 0.3 µl *Taq* Polymerase (5 U/ µl), 0.5 µl each of primers (10 pmol), 2 µl DNA template and Nuclease free water up to 25 µl in 0.2 ml PCR tubes (Eppendorf, Germany). Cycling conditions were set at 94°C for 5 min for initial denaturation; followed by 34 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 30 sec and extension at 72°C for 1 min; and final extension at 72°C for 5 min and held at 4°C. The reaction products were separated on a 1.5% w/v agarose gel containing ethidium bromide and visualized in a gel documentation system (BioDoc Analyze, Biometra, Germany).

Partial gene sequencing

Sequencing of PCR prodects were done commercially for SPPV and GTPV at Sci Genome Lab. Pvt. Ltd. (Kerala, India). Nucleotide sequence corresponding to *P32* gene (partial regions) of the Capripox genome was aligned with published sequences obtained from GenBank [using open source BLAST program (National Center for Biotechnology Information, Bethesda MD, http://blast.ncbi.nlm.nih. gov/ Blast.cgi for sequence comparison]. For comparison, different sequences comprising all the notified genogroups, isolates from India, neighbouring countries as well as from other countries as well as vaccine strain were used to draw phylogenetic tree. Phylogenetic relationships were determined based on these alignments using clustalW program of MEGA version 5.0 (Tamura *et al.*, 2011) by neighbour joining method.

RESULTS AND DISCUSSION

Molecular diagnosis and *Capripoxvirus* confirmation

Suspected field samples consisting of skin biopsies and scabs were processed for DNA isolation and subjected to PCR analysis (Table 1). Amplification of *Capripoxvirus P32* gene encoding was carried out for- Standard virus (Sheeppox vaccine virus); Tissue lysates prepared from cutaneous scabs containing putative *Sheeppoxvirus* (SPPV); Tissue lysates prepared from cutaneous scabs containing putative *Goatpoxvirus* (GTPV); Lysates prepared from putative GTPV inoculated on chicken embryonated egg CAM. Positive samples amplified an expected amplicon size of 192 bp PCR product (Fig 1A, B).

Molecular characterization of different *Capripoxviruses*

After purification of the PCR products, one product from sheep origin (*Sheeppoxvirus* isolated in Surinsar area of

Table 1: Detection of P32 core protein gene by PCR amplification in different *Capripoxvirus* suspected samples.

Sample	Source	Putative species	Diagnosis
Sheep pox vaccine	Vaccine	*Sheep pox virus*	+
Sheep cutaneous scab	Surinsar	*Sheep pox virus*	+
Sheep cutaneous scab	Surinsar	*Sheep pox virus*	+
Sheep cutaneous scab	Surinsar	*Sheep pox virus*	+
Sheep cutaneous scab	Surinsar	*Sheep pox virus*	+
Goat cutaneous scab	Akhnoor	*Goat pox virus*	+
Goat cutaneous scab	Akhnoor	*Goat pox virus*	+
Goat pox isolate inoculated CAM	Akhnoor	*Goat pox virus*	+
Goat pox isolate inoculated CAM	Akhnoor	*Goat pox virus*	+

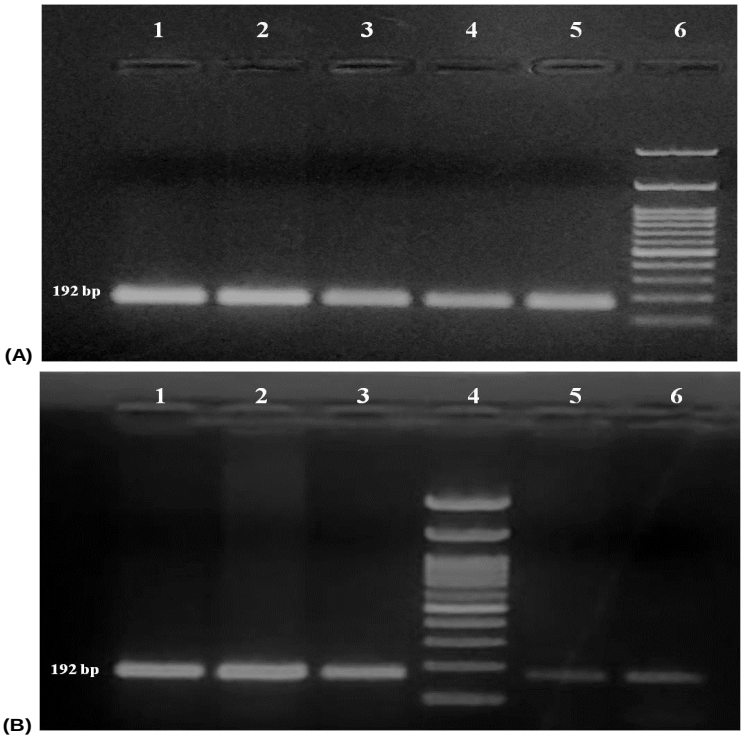

Fig 1: PCR amplification of *P32* gene of *Capripoxvirus* from sheep and goat isolates.

(A) PCR amplification of sheep isolates. Lane 1: Commercial Sheeppox (SPPV) vaccine virus; Lane 2-5: Skin sample from sheep; Lane 6: 100 bp DNA Ladder. **(B)** PCR amplification of goat isolates. Lane 1: Commercial Sheeppox (SPPV) vaccine virus; Lane 2-3: Skin sample from Goat; Lane 4: 100 bp DNA Ladder; Lane 5-6: Chorioallantoic Membrane (CAM) of chicken embryonated egg inoculated with Goatpox isolate.

Jammu district) and another of goat origin (*Goatpoxvirus* isolated in Akhnoor area of Jammu district) were commercially sequenced, annotated and submitted to Genbank, NCBI with published accession numbers (MK358827 and MK358828 respectively).

Phylogenetic analysis of different *Capripoxviruses*

Sequence comparison showed that *Capripoxvirus* isolated from sheep origin grouped under Sheeppox cluster and those isolated from goats grouped under Goatpox cluster (Fig 2). The SPPV strain isolated in the present study was 100% identical with other SPPV strains within India reported from Pune, Tamil Nadu, Bareilly, Akola and even from foreign countries (Tunisia). Interestingly, the SPPV strain was also 100% identical to vaccine strains Srinagar and Rumanian-Fanar (RF). The GTPV closely identical (~98-99%) with other strains from India like Sambalpur and Mizoram and even foreign strains from China, but has some unique signatures of its own. Both the SPPV and GTPV were divergent from Lumpy Skin Disease (LSD) strains. The divergence and relatedness analysis of SPPV and GTPV isolates from Jammu in the present study showed 97.8% identity between the two isolates (Fig 3).

The capripoxviruses have a very large genome and there can be many potential gene targets for diagnosis. The usual choice for using the P32 protein encoding gene is

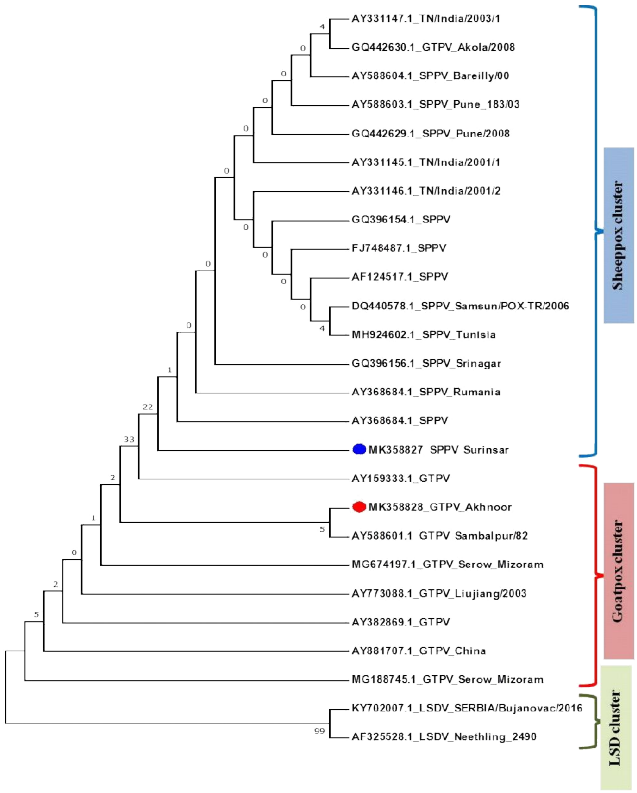

Fig 2: Phylogenetic tree of representative members of *Sheeppoxvirus* and *Goatpoxvirus* using the Neighbour-joining method using clustalW program of MEGA7.*LSD- Lumpy Skin Disease.*

because of its highly conserved nature across all CaPVs and its ability to detect both SPPV and GTPV. The enveloped structural protein contains major immunogenic determinants and therefore has been a preferred target for developing molecular diagnostic tools for detection and differentiation of Capripoxviruses by many workers (Ireland and Binepal, 1998; Heine *et al.*, 1999; Hosamani *et al.*, 2004; Parthiban *et al.*, 2005; Bhanuprakash *et al.*, 2010). It is analogous to P35 protein of Vaccinia virus expressed on the envelope of the mature intracellular virion and may play a role in virus attachment, virulence and virus assembly (Venkatesan *et al.*, 2012). Partial or whole *P32* gene can be targeted with specific primers. Even when all CaPVs can be detected, differentiation with GTPV and SPPV can be possible with additional assays, like RFLP analysis (Hosamani *et al.* 2004; Yan *et al.*, 2010) or by sequence alignment of a part or whole genome of CaPV.

The present results show that there was a high and accurate degree of confirmatory diagnosis based on clinical signs and gross lesions of the disease in both sheep and goats and substantiated by PCR. The application of this method for diagnosing directly from clinical samples is fairly easy and effective.

Although the clinical samples and virus isolates in the present study were segregated according to the host species of animal affected, yet a putative designation of GTPV and SPPV was only confirmed after a sequence alignment and phylogenetic construct of the amplified nucleotides. Although earlier reports from some of the areas (Samba district) showed mixed infection in sheep and goats during an

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	
1		97.8	43.5	97.8	97.6	97.6	94.4	97.8	97.8	97.8	99.3	97.8	97.8	98.6	98.6	95.4	97.8	97.8	97.8	97.8	97.8	43.5	98.6	98.6	97.8	97.8	1
2	2.2		42.9	98.2	100.0	100.0	96.8	99.8	99.8	98.0	98.8	100.0	100.0	98.2	98.2	98.2	100.0	100.0	100.0	100.0	100.0	42.9	98.1	98.1	100.0	100.0	2
3	105.6	107.7		41.7	45.7	45.7	44.2	42.9	42.9	42.3	42.6	42.6	42.6	42.3	42.3	43.1	42.9	42.6	42.6	42.6	42.6	100.0	42.3	42.3	42.9	42.6	3
4	2.2	1.8	113.6		99.2	99.2	96.0	98.0	98.0	99.4	98.8	98.8	98.8	99.6	99.6	97.2	98.2	98.8	98.8	98.8	98.8	41.7	99.5	99.5	98.2	98.8	4
5	2.4	0.0	96.8	0.8		100.0	96.9	100.0	100.0	97.6	98.4	100.0	100.0	98.4	98.4	98.1	100.0	100.0	100.0	100.0	100.0	45.7	98.4	98.4	100.0	100.0	5
6	2.4	0.0	96.8	0.8	0.0		96.9	100.0	100.0	97.6	98.4	100.0	100.0	98.4	98.4	98.1	100.0	100.0	100.0	100.0	100.0	45.7	98.4	98.4	100.0	100.0	6
7	5.8	3.2	102.5	4.1	3.2	3.2		96.8	96.8	94.4	95.2	96.8	96.8	95.2	95.2	95.2	96.8	96.8	96.8	96.8	96.8	44.2	95.2	95.2	96.8	96.8	7
8	2.2	0.2	107.7	2.0	0.0	0.0	3.2		100.0	97.8	98.8	100.0	100.0	98.0	98.0	98.2	99.8	100.0	100.0	100.0	100.0	42.9	97.9	97.9	99.8	100.0	8
9	2.2	0.2	107.7	2.0	0.0	0.0	3.2	0.0		97.8	98.8	100.0	100.0	98.0	98.0	98.2	99.8	100.0	100.0	100.0	100.0	42.9	97.9	97.9	99.8	100.0	9
10	2.2	2.0	110.5	0.6	2.4	2.4	5.8	2.2	2.2		98.8	97.7	97.7	99.6	99.6	96.3	98.0	97.7	97.7	97.7	97.7	42.3	99.5	99.5	98.0	97.6	10
11	0.7	1.2	109.2	1.2	1.6	1.6	4.9	1.2	1.2	1.2		98.8	98.8	99.4	99.4	96.3	98.8	98.8	98.8	98.8	98.8	42.6	99.4	99.4	98.8	98.8	11
12	2.2	0.0	109.2	1.2	0.0	0.0	3.2	0.0	0.0	2.4	1.2		100.0	98.3	98.3	98.2	100.0	100.0	100.0	100.0	100.0	42.6	98.3	98.3	100.0	100.0	12
13	2.2	0.0	109.2	1.2	0.0	0.0	3.2	0.0	0.0	2.4	1.2	0.0		98.3	98.3	98.2	100.0	100.0	100.0	100.0	100.0	42.6	98.3	98.3	100.0	100.0	13
14	1.5	1.8	110.5	0.4	1.6	1.6	4.9	2.0	2.0	0.4	0.6	1.8	1.8		99.8	96.3	98.2	98.3	98.3	98.3	98.3	42.3	99.7	99.7	98.2	98.2	14
15	1.5	1.8	110.5	0.4	1.6	1.6	4.9	2.0	2.0	0.4	0.6	1.8	1.8	0.2		96.3	98.2	98.3	98.3	98.3	98.3	42.3	99.7	99.7	98.2	98.2	15
16	4.7	1.9	107.1	2.8	1.9	1.9	4.9	1.9	1.9	3.8	3.8	1.9	1.9	3.8	3.8		98.2	98.2	98.2	98.2	98.2	43.1	96.3	96.3	98.2	98.2	16
17	2.2	0.0	107.7	1.8	0.0	0.0	3.2	0.2	0.2	2.0	1.2	0.0	0.0	1.8	1.8	1.9		100.0	100.0	100.0	100.0	42.9	98.1	98.1	100.0	100.0	17
18	2.2	0.0	109.2	1.2	0.0	0.0	3.2	0.0	0.0	2.4	1.2	0.0	0.0	1.8	1.8	1.9	0.0		100.0	100.0	100.0	42.6	98.3	98.3	100.0	100.0	18
19	2.2	0.0	109.2	1.2	0.0	0.0	3.2	0.0	0.0	2.4	1.2	0.0	0.0	1.8	1.8	1.9	0.0	0.0		100.0	100.0	42.6	98.3	98.3	100.0	100.0	19
20	2.2	0.0	109.2	1.2	0.0	0.0	3.2	0.0	0.0	2.4	1.2	0.0	0.0	1.8	1.8	1.9	0.0	0.0	0.0		100.0	42.6	98.3	98.3	100.0	100.0	20
21	2.2	0.0	109.2	1.2	0.0	0.0	3.2	0.0	0.0	2.4	1.2	0.0	0.0	1.8	1.8	1.9	0.0	0.0	0.0	0.0		42.6	98.3	98.3	100.0	100.0	21
22	105.6	107.7	0.0	113.6	96.8	96.8	102.5	107.7	107.7	110.5	109.2	109.2	109.2	110.5	110.5	107.1	107.7	109.2	109.2	109.2	109.2		42.3	42.3	42.9	42.6	22
23	1.5	1.9	110.5	0.5	1.6	1.6	4.9	2.1	2.1	0.5	0.6	1.8	1.8	0.3	0.3	3.8	1.9	1.8	1.8	1.8	1.8	110.5		99.8	98.1	98.2	23
24	1.5	1.9	110.5	0.5	1.6	1.6	4.9	2.1	2.1	0.5	0.6	1.8	1.8	0.3	0.3	3.8	1.9	1.8	1.8	1.8	1.8	110.5	0.2		98.1	98.2	24
25	2.2	0.0	107.7	1.8	0.0	0.0	3.2	0.2	0.2	2.0	1.2	0.0	0.0	1.8	1.8	1.9	0.0	0.0	0.0	0.0	0.0	107.7	1.9	1.9		100.0	25
26	2.2	0.0	109.2	1.2	0.0	0.0	3.2	0.0	0.0	2.5	1.2	0.0	0.0	1.8	1.8	1.9	0.0	0.0	0.0	0.0	0.0	109.2	1.8	1.8	0.0		26
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	

1 MK358828 GTPV Akhnoor.seq
2 AF124517.1 SPPV.seq
3 AF325528.1 LSDV Neethling 2490.seq
4 AY159333.1 GTPV.seq
5 AY331145.1 TN-India-2001-1.seq
6 AY331146.1 TN-India-2001-2.seq
7 AY331147.1 TN-India-2003-1.seq
8 AY368684.1 SPPV Rumania.seq
9 AY368684.1 SPPV.seq
10 AY382869.1 GTPV.seq
11 AY588601.1 GTPV Sambalpur 82.seq
12 AY588603.1 SPPV Pune.seq
13 AY588604.1 SPPV Bareilly.seq
14 AY773088.1 GTPV Liujiang 2003.seq
15 AY881707.1 GTPV China.seq
16 DQ440578.1 SPPV Turkey.seq
17 FJ748487.1 SPPV.seq
18 GQ396154.1 SPPV.seq
19 GQ396156.1 SPPV Srinagar.seq
20 GQ442629.1 SPPV Pune 2008.seq
21 GQ442630.1 GTPV Akola 2008.seq
22 KY702007.1 LSDV Serbia.seq
23 MG188745.1 GTPV Serow Mizoram.seq
24 MG674197.1 GTPV Serow Mizoram.seq
25 MH924602.1 SPPV Tunisia.seq
26 MK358827 SPPV Surinsar.seq

Fig 3: Pair-wise sequence comparison of P32 gene nucleotide of *Capripoxvirus* isolates- *Sheeppoxvirus* (SPPV) from Surinsar and *Goatpoxvirus* (GTPV) from Akhnoor using clustal method.

outbreak in January 2013 with a GTPV isolate (Ramakrishnan *et al.*, 2016), the present study however demonstrated host specificity of the CaPVs. This has also been substantiated clinically when only either sheep or goats were affected, even when flocks were co-populated with both animal species. Moreover, sequence alignment showed that *Capripoxvirus* isolated from either animal species clustered under their respective genogroups. However, the possibility of cross transmission cannot be overruled.

Based on the *P32* gene, the SPPV isolate shared nucleotide sequence homology with many Indian isolates and some foreign ones. However, homology with vaccine strains makes differentiation between vaccine and field strains difficult. The SPPV nucleotide sequence was 100% identical with both prevalent vaccine strains [Rumanian-Fanar (SPPV-RF) and Srinagar (SPPV-Srinagar) vaccine strains]. Such similarities are also reported for field SPPV isolates elsewhere in the country, either with SPPV-RF vaccine (Hosamani *et al.*, 2004) or SPPV-Ranipet vaccine strain (Parthiban *et al.*, 2005; Rajamuthu *et al.*, 2009). The Rumanian-Fanar (RF) strain licensed and extensively used in India is attenuated from serial passage in primary lamb testes, while SPPV-Srinagar strain has been developed from a local isolate and attenuated in vero cells (Yogisharadhya *et al.*, 2011) and SPPV-Ranipet used in Tamil Nadu has been attenuated in ovine thyroid cells and lamb testes cells (Bhanuprakash *et al.*, 2004). The homology of the circulating Indian SPPV strains favours devising uniform control strategies throughout the country. Since the live attenuated SPPV have been used in enzootic areas, vaccination failure or vaccine induced diseases also cannot be ruled out.

The GTPV isolate although sharing nucleotide homology with other Indian isolates (~98-99%), yet there could be subtle differences in nucleotide or amino acid sequences. Earlier reports of such changes have been documented (Santhamani *et al.*, 2014; Ramakrishnan *et al.*, 2016), including presence of an additional aspartic acid at 55th position of *P32* of sheep poxvirus that is absent in both goat poxvirus and lumpy skin disease virus (Hosamani *et al.*, 2004). It is envisaged that any genetic changes or mutations in the viral envelope protein genes, such as the *P32* gene which is a major immunogenic protein (Heine *et al.*, 1999), could lead to alterations in the immunogenic properties of the virus. This could lead to vaccination failures when specific GTPV vaccine is not readily available in the country.

CONCLUSION

From the present study it could be concluded that sheep isolates from Jammu are distinct and found to be identical to many circulating strains in the country and abroad, including some widely prevalent vaccine strains currently in use. It is possible that regular vaccination may help combat the disease in sheep. Goat isolates although similar to the sheep isolate and other circulating strains in the country and elsewhere, still there is subtle divergence seen in the *P32* nucleotide sequence and could be unique. Complete *P32* gene sequence of the SPPV and GTPV isolates or whole genome sequence is necessary for estimating the relatedness of the field and vaccine strains. Conversely, vaccine challenge studies are warranted for studying the efficacy of prevalent vaccines.

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