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Molecular Characterization of Sheep Pox Outbreak in the State of Uttar Pradesh in India

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A B S T R A C T

Sheep pox virus is widespread in India and clinical cases are reported across the country throughout year, particularly during extreme climatic conditions. In India sheep rearing is associated with a particular community and being land less and economically weaker, owners used to migrate one to another place for grazing of their flocks. Such animal husbandry practices make these animals exposed and susceptible to viral diseases. Thus, the thorough investigations of outbreaks are essential to establish linkage and lineages of prevailing virus and also to find out presence of any new strain. The current report investigated an outbreak of sheep pox during April to September, 2024 in Uttar Pradesh. The samples were confirmed through *P32*gene based amplification and subjected to sequencing of 4 representative samples collected from 4 different villages. Phylogenetic analysis of the full-length *P32*gene revealed homology with sheep pox isolates previously reported from various places of India and subsequently with neighboring countries. It suggested high lineage-specificity of Indian sheep pox virus and its correlation with the original host. Thus, continuous and proper vaccination of existing sheep population with homologous vaccine strain is recommended to prevent and control it's incidences as well as outbreaks.

Introduction

As per the 20th annual livestock census India stands forth with a total sheep population of 74.26 million with a 10.1% increase from the previous census [https://dahd.gov.in/sites/default/files/2024-10/Brochure of21stLivestockCensus.pdf]. In India, sheep farming is a conventional agricultural practice associated with its rural community comprised of marginal farmers and landless population residing in villages. The existence of sheep pox endemicity across the country limits the trade of sheep and directly affects the rural economy. There are limited studies on sheep pox particularly in northern India. However, in a study conducted in the state of Maharastra, sheep pox was reported with major impact on the state economy. It revealed 53.5% morbidity and 49.5% mortality rates. The multiple studies have been conducted in state of Karnataka including the recently published study that reported 11 outbreaks of sheep pox (Manjunatha Reddy *et al.,* 2024). The study also confirmed sheep pox in 95 animals through PCR out of 105 animals exhibiting clinical signs resembling to sheep pox. Under such circumstances, the sheep plays crucial role in the socioeconomic status starting from earning of livelihood particularly for economically weaker sections of society surviving on the earnings

from sheep (Bhanuprakash *et al.,* 2011). Being landless or less holding community, shepherds used to graze them in open grass lands and also migrate in search of pastures. This migration may be localized or across the state due to adverse climatic conditions either due to chilling cold or hot summer. The environmental stress and exposure to virus circulating in wild and domestic animals make them prone to infection. The migratory nature of these communities may fail to get their animals regularly vaccinated through the vaccination schemes being run by the Government. In spite of availability of safe and effective vaccine reports of large number of outbreaks (Manjunatha Reddy *et al.,* 2024) enforce the need of continuous monitoring of the disease and its source of infection. Moreover, there is no test is avialble for the differentiation of infected and vaccinated animals. The molecular method particularly P32 gene based PCR has been well accepted for the confirmation of the infection. This is robust method of confirmation without isolation of virus in laboratory. Further, sequencing and alignment of sequences with previously reported sequences support to understand lineage-specificity of Indian sheep pox virus and its correlation with the original host (Reddy *et al.,* 2015; Manjunatha Reddy *et al.,* 2017). The current paper details an outbreak of sheep pox in province of Uttar Pradesh in India and molecular confirmation and characterization of virus involved in the outbreak with the objective to identify the causing agent and also to affirm it's origin and resemblance to previously reported virus to access the effectiveness of current vaccine program.

Materials and Methods

Samples: All the skin scabs amples were collected in virus transport medium (HiMedia, Mumbai) during April to September, 2024 after due approval of IAEC from the clinical cases. A total of 12 samples were collected (three samples from a village from a village). These villages were Kosi Kalan, Barsana and Govardhan of District Mathura and village Dankur of District Gautam Buddh Nagar. Thee geographical location of villages are depicted in image 1. The details are provided in Table 1.

DNA extraction: The scab samples were homogenized in PBS (7.4) and 2 ml aliquot was transferred to sterile tubes and centrifuged for 15 min at 15,000g at 4ºC. The supernatant was discarded, and the pellets were suspended in 2 ml of lysis buffer composed of 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100) and lysozyme in the concentration of 20 ug/ml. Thereafter, the incubation period was increased many folds to 5 hours and further processed by Gene JET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) as per manufacturer's instructions. Extracted DNA samples were stored at −20°C until amplification.

Molecular confirmation of virus: All the samples were subjected to DNA extraction as per the previously described protocols (REF). All the DNA were subjected to PCR based amplification for the P32 gene using previously reported set of primers (F: ACACA GGGGGATATGATTTTACC and R: ATACCGTTTTT CATTTCGTTAGC genus-specific confirmation (Reddy *et al.,* 2015). The DNA of the vaccine strain of sheep pox virus maintained at BioMed, Gaziabad was used as served as a positive control.

Sequencing, phylogenetic analysis and multiple sequence alignment: All the PCR products were purified by the Gene JET Gel Extraction Kit of Thermo Fisher Scientific (Waltham, MA, USA) and outsourced to (Centyle Biotech, New Delhi) for Sanger sequencing. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of P32 gene was generated from forward and reverse sequence data using BioEdit (Hall, 1999). The sequence was used to carry out BLAST with the database of NCBI gen-bank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGAX (Kumar *et al.,* 2018).The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985).

Results and Discussion

Clinical signs and Symptoms: The animals showed all the stages of clinical signs including fever, difficult breathing, purulent to mucoid discharge from nostrils due to secondary bacterial infections, anorexia and non interest in feeding. The animals with later stages have developed scabs and these were collected for confirmation of virus.

Amplification of p32 gene and phylogenetic analysis: The p32 gene of the Capri pox virus was successfully amplified from all field samples using specific primers, producing an amplicon of approximately 237 base pairs (bp), as visualized by agarose gel electrophoresis (Fig.1). The phylogenetic tree (Fig.2) revealed that the sequenced isolates—SPH1, SPH2, SPH3, and SPH4—clustered closely with other known Sheep pox virus (SPV) strains, indicating their strong genetic relatedness. These isolates grouped tightly with Sheep pox virus and showed a close relationship with Indian field strains. The clustering was supported by high bootstrap values, indicating reliable genetic grouping. All four isolates (SPH1 to SPH4) distinctly grouped within the Sheep pox virus clade and clearly separated from Goat pox virus (GTPV) and Lumpy skin disease virus (LSDV) strains. The distant placement of Vaccinia virus (M35027.1) as an out group confirmed the specificity and divergence of Capri pox virus sequences. Further, the identity matrix based on nucleotide sequence similarity supported the phylogenetic analysis (Image 3). The SPH isolates showed >99% sequence identity among themselves, indicating minimal intra-group variation.

Table.1 Details of samples collected during study

S. No.	Sequence ID	Gene	Amplicon Size	Collection Date	City/Country	Lat_Lon	Accession Number
1.	SPH1	P32 gene	237bp	07/04/2024	Kosi kalan, UP, India	27.78769, 77.43679	PV782988
2.	SPH2			05/05/2024	Govardhan, UP, India	27.49395, 77.46781	PV782989
3.	SPH3			21/07/2024	Dankur, UP, India	28.35352, 77.55326	PV782990
4.	SPH4S			08/09/2024	Barsana, UP, India	27.49148, 77.48078	PV782991

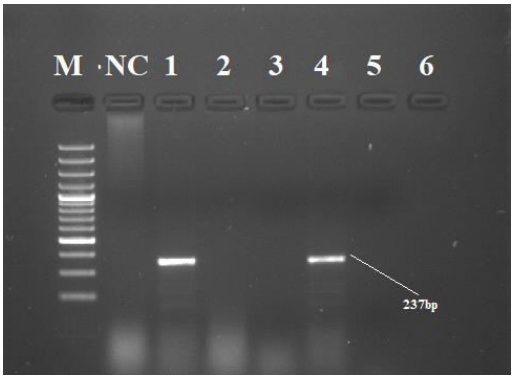

Fig 1. Agarose gel electrophoresis showing amplification of the p32 gene (237 bp) from Sheep pox virus isolates.

When compared with reference Sheep pox virus sequences (e.g., SPV Jaipur, SPV Srinagar), they exhibited 97–99% identity, highlighting their close genetic relatedness to other Indian field isolates. In contrast, identity values between SPH isolates and GTPV or LSDV sequences ranged between 88–92% confirming species-level differentiation. These findings suggest that the circulating field isolates belong to the Sheep pox virus group and show strong genetic conservation with known Indian and international strains. The analysis supports the reliability of the p32 gene as a molecular marker for Capri pox virus classification and differentiation. The values on the matrix (Fig.3). Correspond to percent identity scores calculated from the multiple sequence alignment. This matrix supports the phylogenetic relationships shown in phylogenetic tree.

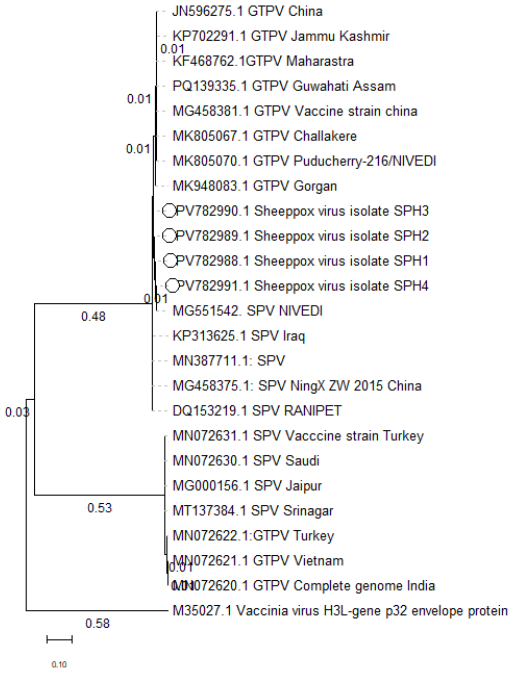

Fig 2. Phylogenetic tree based on the nucleotide sequence of the p32 gene from Capri pox virus isolates. The tree was constructed using the Maximum Likelihood method in MEGA X.

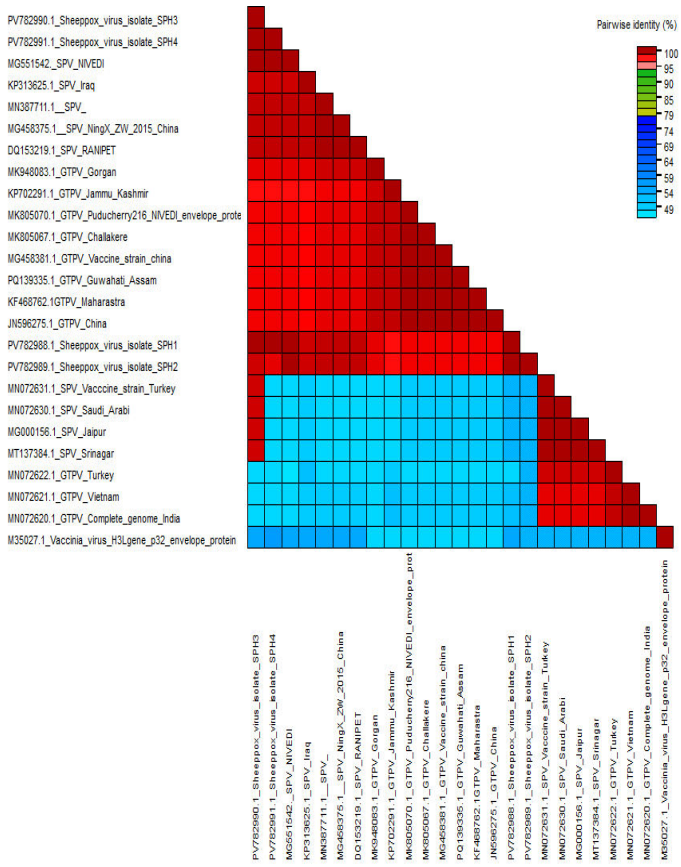

Fig.3 Pair wise sequence identity matrix of p32 gene nucleotide sequences. The values on the matrix correspond to percent identity scores calculated from the multiple sequence alignment. This matrix supports the phylogenetic relationships shown in phylogenetic tree.

Sheep are the animals reared by the most backward classes and economically weaker sections of the country. Any infection may severely affect their socioeconomic status. Thus, proper monitoring, surveillance and vaccination can save such losses to these communities. Moreover, sheep pox is highly contagious and may cause significant losses due to substantial morbidity and mortality (Bhanuprakash *et al.,* 2005). The persistence of sheep pox virus may be attributed to multiple factors. These may be physiological factors like age, sex, pregnancy, lactation, nutritional factors affecting breed immunological status of susceptible population, managemental factors like low or no vaccination, migration of animals and exposure to adverse climatic (Chahota *et al.,* 2022, Manjunatha Reddy *et al.,* 2024).

The current study reports the disease during the months of hot and humid climate as also suggested by previously published reports. The factors like adverse temperatures, lack of nutrition, water scarcity and stresses of migration would have contributed through compromised immunity (Bhanuprakash *et al.,* 2006; Manjunatha Reddy *et al.,* 2017, Chahota *et al.,* 2022, Manjunatha Reddy *et al.,* 2024). The sheep pox virus stability in environment and availability of suitable temperature, humid climate might have supported the survival of the virus on contaminated wool and objects for extended periods exacerbating the level and spread of infection (Rao *et al.,* 2000; OIE, 2012, Hurisa *et al.,* 2018, Hota *et al.,* 2018). The clinical signs of then animals also resembles with the previously reported incidences (Zangana *et al.,* 2013; Al- Shabebi *et al.,* 2014; Manjunatha Reddy *et al.,* 2017; Sumana *et al.,* 2020; Manjunatha Reddy *et al.,* 2024), including fever, respiratory distress, reluctance to feed, characteristic lesion starting from vesicle to scab formation on ear, eye lids, muzzle and udder and confirmed both vesicular and nodular forms of disease in affected animals.

The PCR based molecular detection is well accepted method for the confirmation of Capri pox viruses, including sheep pox due to its specificity, sensitivity and reproducibility (Haegeman *et al.,* 2013, Manjunatha Reddy *et al.,* 2024). The *P32*gene, used for the confirmation of sheep pox infection, is a structural gene coded for a major pathogenic viral determinant. It is the most common gene used for the diagnosis of Capri pox virus infections (Beard *et al.,* 2010; Reddy *et al.,* 2015; Manjunatha Reddy *et al.,* 2017) and similar to previous reports revealed a band of 237 bp (Image 2).

The current study report the outbreak in 4 villages belonging to 2 districts of western Uttar Pradesh (Table-1). These two districts are adjoing districts and likely to get the exposure of virus due to movement of flocks or procurement of exposed/ infected animals. A recently published paper from the Karnataka state of India reported 11 outbreaks in different districts (Manjunatha Reddy *et al.,* 2024). These reports are suggestive of continuous persistence of Sheep pox infection from southern to northern states of India in spite of continuous vaccination programmed. Thus, the sequencing as well as phylogenic studies are also required. The sequence based phylogenetic analysis and BLAST-n alignment confirmed the all four strains belonging to sheep pox virus (SPPV) under investigation revealed all belonging to the sheep pox group and align closely with other known Sheep pox virus (SPV) strains, indicating their strong genetic relatedness. These findings are in concurrence with the recently published report covering

11 outbreaks in Karnataka State (Manjunatha Reddy *et al.,* 2024). Genetic closeness of these viruses involved in disease at multiple places suggests less mutation in migratory viruses State (Sumana, *et al.,* 2020; Manjunatha Reddy *et al.,* 2024). However, the occurrence of these number of out breaks in spite of ongoing vaccination programmes are of concern. Further, analysis revealed their grouping with sheep pox virus NIV and other Indian field strains (Figure-2). Their clustering is supported by high bootstrap values suggestive of reliable genetic grouping (Kimura, 1980). In spite of close genomic relationship of SPPV and the Goat pox virus (GTPV), all four align distinctively in Sheep pox virus clade and clearly separated from Goat pox virus (GTPV) and Lumpy skin disease virus (LSDV) strains (Image 3), However, there are reports showing more than 97% similarity in spite of presence of species-specific genes (Biswas *et al.,* 2020). These species specific genes can be used to differentiate these viruses and there are reports of species specific infections in mixed group of sheep and goats without cross-species infection (Rao *et al.,* 2000; Bhanuprakash *et al.,* 2006). The P32 gene amplicon sequence analysis of 4 strains in this study also underscored species specificity and distinctiveness from GTV and LSDV. The close resemblance to other Indian SPPV isolates, previously reported in different regions of the country suggests the circulation of genetically closely related virus strains in the country. These observations are suggestive of the high lineage-specificity of Indian sheep pox viruses. Thus, use of homologous vaccine may be an effective way to prevent and control of sheep pox viruses. However, continuous reports from the different part of country need elaborative studies or whole genome sequences of field strains and vaccine strain to find out homology in protective proteins to obtain better protection and reduction in the incidences of the disease.

In conclusion, the current study report sheep pox outbreaks and also underlines the probable risk factors associated with the disease in Uttar Pradesh. The genetic analysis of the representative strains revealed close relatedness with other previously reported strain and aligned in the same clade. This close relationship may support one vaccine for all across the country to overcome sheep pox disease in India.

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Author Contributions

Himanshu Agarwal: Investigation, formal analysis, writing—original draft. A. P. Garg: Validation, methodology, writing—reviewing. Surendra Upadhyay:—Formal analysis, writing—review and editing. Amit Kumar: Investigation, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval: IAEC/SVPUAT/2023/144 dated 27/12/2023.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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