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Original research

Genetic characterization and phylogenetic analysis of Peste des Petits Ruminants virus (PPRV) from an outbreak in Puducherry region, India

Nivedha Devanathan[1] ✉, Shashank Chaluva Niranjana[1], Cheruvu Vignatej Reddy[1], Arunkumar N[1], Sambath Kumar[3], Jayalakshmi Vasu[1], Mouttou Vivek Srinivas[2] and Hirak Kumar Mukhopadhyay[1]

[1] Department of Veterinary Microbiology, Rajiv Gandhi Institute of Veterinary Education & Research, Puducherry 605009, India
[2] Centre for Translational Research, Rajiv Gandhi Institute of Veterinary Education & Research, Puducherry 605009, India
[3] Department of Animal Husbandry & Animal Welfare, Puducherry, India

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Correspondence:

Dr. Nivedha Devanathan ✉
nivedhadevanathan28@gmail.com

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ABSTRACT

Peste des petits ruminants (Goat plague) is an acute, highly contagious, and lethal viral disease of goats and sheep. It causes high morbidity and mortality and is considered a significant constraint on the productivity of small ruminants. Four genetic lineages of the PPR virus (Genotype I to IV) have been identified globally. The study was planned to genetically characterize the circulating PPR virus lineages/genotypes in a suspected outbreak in goats from the Puducherry region (Southern India). Fifty-nine nasal/faecal swabs were collected from the goats with the history of profuse diarrhoea, nasal and ocular discharges, stomatitis, and high mortality during the outbreak. A total of 44 out of 59 (74.5%) nasal/faecal swabs were found positive by Reverse Transcriptase PCR targeting the N gene of the PPR virus, with an amplicon size of 359 bp. Based on sequence analysis of three representative samples, the Indian vaccine strain and various global genotypes, forty-one non-synonymous amino acid variations were identified from 110 amino acids. These amino acid variations specific to the study sequences are characteristic of the Genotype IV lineage. Though the vaccine virus (Sungri 96 strain) also had amino acid relatedness with the Genotype IV lineage, nine non-synonymous mutations were repeatedly identified in the field strains, indicating that the virus is under serious positive selection in these regions. In addition, three new mutations were identified at the positions Lys/Gln458→Pro, Thr469→Pro, and Met507→Thr of the N gene. Thus, it could be concluded that the genotype IV lineage of PPRV is circulating in India with three new mutations at the N gene. As the PPRV strains were found to be under immense selection pressure and realizing the constant mutations happening in the field strains, monitoring of circulating genotypes in wider geographical regions of India would be of utmost importance.

Introduction

Peste des petits ruminants, also known as goat plague, is an acute and highly contagious viral disease that results in significant economic losses due to high morbidity (100%) and mortality rates (80%). [1, 2, 3]. This disease primarily affects sheep and goats but can also impact wild small ruminants and camels [4, 5]. India has approximately 74.26 million sheep and 148.88 million goats (20th Livestock Census, 2019; http://dahd.nic.in). This disease threatens the livelihoods of about 70% of landless labourers and small to medium farmers who depend on sheep and goats for their income. Economic losses attributed to PPRV are estimated at around 1,800 million Indian rupees annually, making it one of the most critical threats to the country's small ruminant population [6]. The disease primarily impacts the poorer segments of society, prompting the FAO and OIE to set a target for global eradication by 2030 (http://www.fao.org/ppr/en/).

The PPR virus is an enveloped virus belonging to the family *Paramyxoviridae* of the genus *Morbillivirus* [7]. The genome is approximately 15.9 kb length, non-segmented, negative-sense, and single-stranded RNA.

The genome encodes two non-structural proteins (C and V) and six structural proteins: nucleoprotein (N), RNA-dependent polymerase (L), phosphoprotein co-factor (P), matrix protein (M), fusion protein (F), and hemagglutinin (H) [8]. Although only one serotype of PPRV exists, the virus is classified into four genetic lineages based on variations in the N and F genes, which correspond to its unique geographical distribution pattern [9, 10], which has changed in recent years. Lineage I is found in West Africa and has recently been reported in Central Africa. Lineage II is in Western Africa, Lineage III is in Eastern Africa and certain areas of the Middle East, and Lineage IV is in the Arabian Peninsula, the Middle East, and South Asia [10, 11]. Recent reports indicate the emergence of PPR virus lineage IV in Northeast Africa and North Africa [12]. The distinct geographical dispersal of the virus suggests it existed long before its identification in certain regions. Recent reports of unexpected lineage introductions in areas linked to other lineages indicate a virus flow. These incidents of PPRV in disease-free areas highlight the potential for the virus to spread rapidly and the need for robust surveillance and control measures [13].

In India, PPRV was first reported in 1987 in Tamil Nadu and is now considered endemic [14, 15]. Transmission primarily occurs through close contact between animals. Following an incubation period of 3 to 6 days, symptoms may include high fever, oculonasal discharge, pneumonia, stomatitis, gastrointestinal inflammation, severe diarrhoea, and potential death or recovery [16].

Various DNA-based PCR techniques are employed for routine molecular studies of microbes. However, reverse transcriptase polymerase chain reaction (RT-PCR) is particularly useful for detecting RNA viral material in samples. RT-PCR is being developed for the precise and accurate diagnosis of the PPR virus by targeting different genes, including the F, N, P, M, and H genes. The nucleocapsid protein gene (N gene) is unique and exhibits significant genetic diversity, making it a suitable target for understanding the prevalent PPRV genotypes. Consequently, the N gene is often the preferred choice among researchers for conducting extensive molecular epidemiological studies and is a popular tool for PPR diagnosis [17].

Given these factors, it is essential to identify the exact PPRV genotypes/lineages circulating in the field and to understand their genetic and antigenic relationships with available vaccines and other known PPRV lineages globally. Therefore, this study was designed to identify and genetically characterize the circulating PPR virus lineages/genotypes through sequence analysis of the nucleoprotein (N) gene in an outbreak among suspected goats in the Puducherry State, Southern India.

Materials and methods

Collection of samples and processing

A total of 59 nasal/ rectal swabs were collected from PPR-suspected goats with the clinical features such as profuse diarrhoea, nasal and ocular discharges, stomatitis, and high mortality in outbreak regions of Puducherry State (Southern India). The samples were collected in sterile phosphate-buffered saline (PBS) and processed by centrifugation at 6000 rpm for 10 minutes at 4ºC, and the resulting supernatants were stored at -40°C until further use.

Ethical approval

Ethical approval is not required since this study does not involve animal experimentation. However, samples were collected according to standard procedure without harming or stressing the animals.

Extraction of viral RNA

Two hundred microliters of the nasal or rectal swab samples were subjected to viral RNA extraction using the Magnetic Bead Method with Zybio's Nucleic Acid Extraction Kit (Zybio Inc., Chongqing, China), following the manufacturer's instructions. The extracted RNA was used as a template for reverse transcription. The attenuated live vaccine Raksha PPRV (Sungri96) was used as a positive control, while nuclease free water and nasal swabs from healthy goats were negative controls used in this study.

cDNA synthesis

The RNA templates were subjected to first-strand cDNA synthesis using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit with random hexamer primer, as per the manufacturer's instructions. The reverse transcription reaction was prepared with a total volume of 20µl, including 1µl of random hexamer primer, 2µl of RNA template, 4µl of reaction buffer, 1µl of Ribolock RNase inhibitor, 2µl of 10mM dNTP mix, 1µl of RevertAid M-MuLV RT, and 12µl of nuclease-free water. The reverse transcription was conducted at 25ºC for 5 minutes, followed by 60 minutes at 42ºC in a thermal cycler (Eppendorf, Nexus Gradient). The reaction was terminated by heating at 70ºC for 5 minutes. The final products were stored at -40ºC until further use.

Polymerase Chain Reaction

Following reverse transcription, the synthesized cDNA was screened for the PPR virus through PCR amplification targeting the PPR-N gene, which amplifies a 359 bp fragment using the primer pair listed in Table 2. The reaction mixture (50µl) consisted of 1X Taq DNA polymerase Ampliqon Master Mix Red, 0.2µM of each primer, 2µl template DNA and the rest of the volume is made by adding nuclease-free water. The thermocycling conditions were set as follows: initial denaturation at 95˚C for 5 minutes, followed by 35 cycles of

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denaturation at 95˚C for 30 seconds, annealing at 56˚C for 30 seconds, extension at 72˚C for 1 minute, and a final extension at 72˚C for 10 minutes, with the reaction held at 4˚C afterwards. PCR amplification was performed using an automated thermal cycler (Eppendorf Master Cycler, Germany). The PCR products were resolved by 1.5% agarose gel electrophoresis in 1X Tris-acetate EDTA (TAE) buffer and visualized under UV transilluminator (Syngene).

Sequencing and sequence analysis

Three randomly selected PCR products were gel extracted and custom sequenced in both directions (5'-3' and 3'-5') using the Applied Biosystem 3100 automated sequencer. The sequencing results were evaluated for specificity using the Nucleotide Basic Local Alignment Search Tool (BLAST) to identify the closest known relatives on the NCBI website (http://www.ncbi.nlm.nih.gov). The obtained nucleotide sequences were aligned with corresponding sequences available in GenBank using the Clustal Omega tool in MEGA 11 software [18]. The sequences of the N gene of PPRV obtained from three goats in this study were submitted to GenBank (www.ncbi.nlm.nih.gov/genebank). The deduced amino acid sequences of the N protein from different PPRV genotypes were aligned with multiple PPRV protein sequences from various geographical regions using MEGA 11 software to explore amino acid profiles and potential differences between vaccines and various PPRV genotypes.

Phylogenetic analysis

The PPRV N gene sequences obtained from the three samples were utilized to construct a phylogenetic tree using the neighbor-joining method implemented in MEGA 11 software [18]. The confidence level of branching in the phylogenetic tree was assessed using the bootstrap test based on 1000 resamplings. The phylogenetic tree was constructed with sequences from the vaccine virus, other known Indian PPRVs, and several PPRV genotypes globally (GenBank).

Recombination analysis

GARD analysis (Genetic Algorithms for Recombination Detection) and RDP4 analysis (http://www.datamonkey.org) was performed to identify any breakpoints and recombination events in the N gene of the PPRV study sequences.

Results

In this study, we screened fifty-nine goat samples, of which 44 (74.5%) tested positive using RT-PCR assay targeting the N gene with the expected amplicon size of 359 bp, as demonstrated by the gel electrophoresis of the PCR products (Fig. 1).

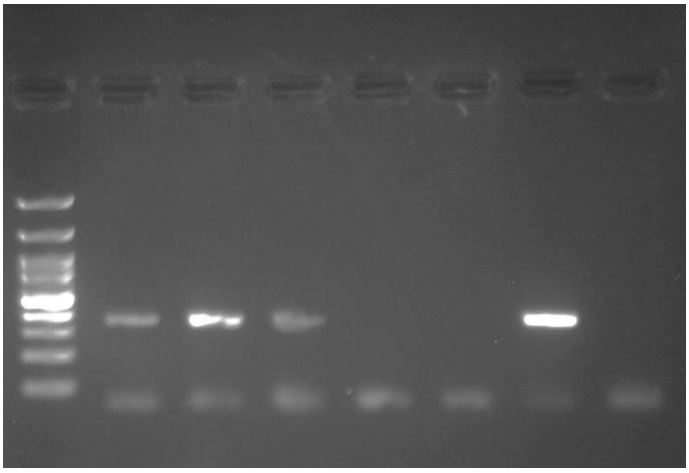

Fig 1. Agarose gel electrophoresis showing the results of PCR amplified product of N gene of PPRV with size 359 bp. Lane 1- 100 bp ladder, Lane 2-6 : Samples from the outbreak (Lane 2-4: Positive for PPRV, Lane 5-6 Negative for PPRV), Lane 7: Positive control (Vaccine) & Lane 8: Negative control (NFW)

Following sequencing, the BLAST analysis of the three study sequences (OQ971954, OQ971955, OQ971956) confirmed that the amplified sequences were highly specific to the PPR virus (PPRV), showing a maximum identity of 94-97% with N gene sequences of other PPR viruses available in GenBank.

During the sequence analysis, we identified 41 non-synonymous mutation sites among the 110 amino acids of the N gene in the PPRV sequences derived from goats in this study (Table 1) compared to other PPRV genotypes. The amino acid variations observed in the three study sequences were characteristic of the Genotype IV lineage. Although the vaccine virus (Sungri96 strain) also showed some amino acid similarity with Genotype IV, nine of the 41 non-synonymous mutations were consistently observed in the field strains, indicating that the virus is undergoing positive selection in these regions. Additionally, three new mutations were identified in the N gene at the following positions: Lys/Gln458→Pro, Thr469→Pro, and Met507→Thr.

Based on N gene phylogeny, the PPRV strains exhibited distinct clustering patterns and formed six clades (Fig. 2). The study sequences clustered with Genotype IV sequences along with the Dubai, Tibet, Chinese, and other Indian genotype IV.

A recombination analysis conducted using RDP4 did not reveal any breakpoints or recombination events in the PPRV sequences in the study (Not shown).

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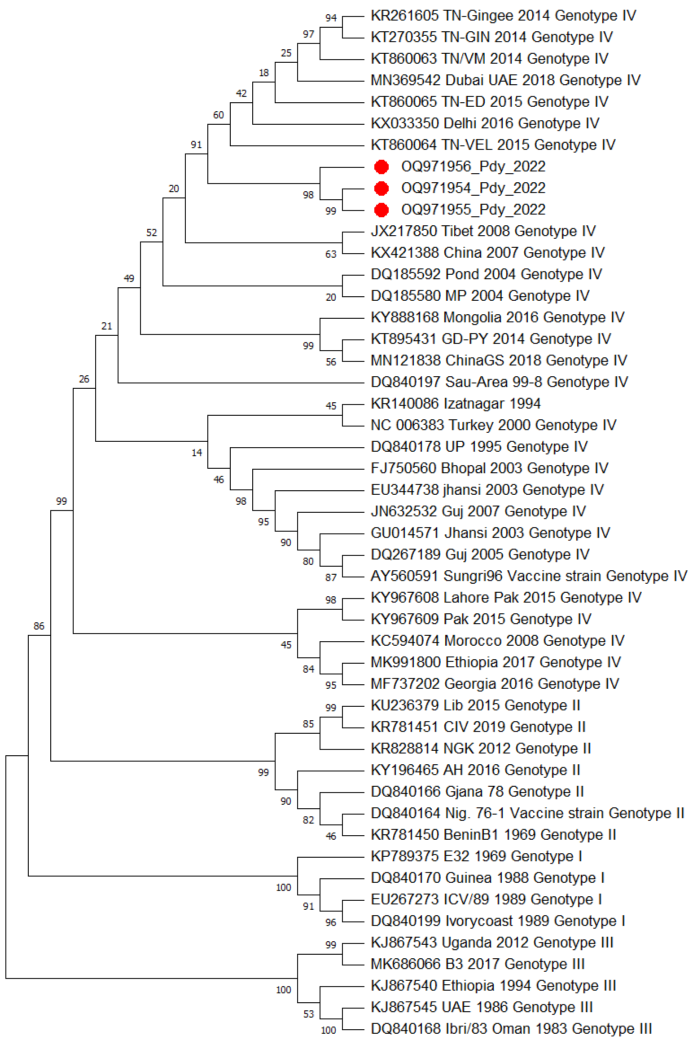

Fig. 2. Neighbour-Joining tree depicting the phylogenetic relationship among PPRV clinical samples. PPRV strains obtained from goat samples sequenced in this study are shown with solid circles.

Table 1. Amino acid residues and their substitutions in the N gene of PPRV study sequences compared with the PPRV lineages circulating globally.

GeneBank accession ID	Place/ Strain	Year	Genotype	Amino acid positions and its substitutions in the N gene of the PPRV																
				436	438	442	458	459	461	465	466	467	468	469	470	472	474	477	481	482
KP789375	E32	1969	I	Thr	Asp	Val	Lys	Thr	Glu	Ser	Ala	Pro	Val	Thr	Arg	Glu	Lys	Ile	Ser	Glu
EU267273	ICV/89	1989	I	Thr	Asp	Val	Lys	Thr	Glu	Ser	Ala	Pro	.	Thr	Arg	Glu	Lys	Ile	Ser	Glu
KU236379	Lib	2015	II	Thr	Glu	Val	Lys	Ile	Glu	Pro	Thr	Pro	Ala	Thr	Lys	Glu	Lys	Ile	Ser	Glu
KR781451	CIV	2019	II	Thr	Glu	Val	Lys	Ile	Glu	Pro	Thr	Pro	Ala	Thr	Lys	Glu	Lys	Ile	Ser	Glu
KJ867540	Ethiopia	1994	III	Thr	Asp	Ala	Lys	Ile	Glu	His	Ala	Ser	Ala	Thr	Arg	Glu	Lys	Thr	Pro	Asp
KJ867543	Uganda	2012	III	Thr	Asp	Ala	Lys	Ile	Glu	His	Ala	Ser	Ala	Thr	Gly	Glu	Lys	Thr	Pro	Asp
KR140086	Izatnagar, India	1994	IV	Ala	Asp	Ala	Lys	Thr	Glu	Ser	Ala	Pro	Ala	Thr	Arg	Gly	Lys	Ile	Ser	Glu
NC 006383	Turkey	2000	IV	Ala	Asp	Ala	Lys	Thr	Glu	Ser	Ala	Pro	Ala	Thr	Arg	Gly	Lys	Ile	Ser	Glu
KX421388	China	2007	IV	Ala	Asp	Ala	Gln	Thr	Gly	Ser	Ala	Pro	Ala	Thr	Arg	Gly	Lys	Ile	Ser	Glu
KT860064	Tamilnadu, India	2015	IV	Ala	Asp	Ala	Gln	Thr	Gly	Ser	Ala	Pro	Ala	Thr	Arg	Gly	Lys	Ile	Ser	Glu
KX033350	Delhi, India	2016	IV	Ala	Asp	Ala	Gln	Thr	Gly	Ser	Ala	Pro	Ala	Thr	Arg	Gly	Lys	Ile	Ser	Glu
AY560591	Sungri96 Indian Vaccine strain		IV	Ala	Asp	Ala	Lys	Thr	Glu	Ser	Ala	Pro	Ala	Thr	Arg	Gly	Arg	Ile	Ser	Glu
OQ971954	Puducherry, India	2022	IV	Ala	Asp	Ala	Pro	Thr	Gly	Ser	Ala	Pro	Ala	Pro	Arg	Gly	Lys	Ile	Ser	Glu
OQ971955	Puducherry, India	2022	IV	Ala	Asp	Ala	Pro	Thr	Gly	Ser	Ala	Pro	Ala	Pro	Arg	Gly	Lys	Ile	Ser	Glu
OQ971956	Puducherry, India	2022	IV	Ala	Asp	Ala	Gln	Thr	Gly	Ser	Ala	Pro	Ala	Thr	Arg	Gly	Lys	Ile	Ser	Glu

GeneBank accession ID	483	484	486	487	488	489	491	493	494	495	497	498	500	502	504	506	507	511	512	513	515	519	542	545
KP789375	Glu	Arg	Lys	Arg	Arg	Thr	Ser	Arg	Pro	Arg	Glu	Thr	Gly	Pro	Leu	Ile	Met	Glu	Val	Ser	Glu	Thr	Gly	Glu
EU267273	Glu	Arg	Lys	Arg	Arg	Thr	Pro	Arg	Ser	Arg	Glu	Ile	Ser	Pro	Leu	Ile	Met	Glu	Ala	Ser	Glu	Thr	Gly	Gly
KU236379	Gly	Arg	Ile	Lys	Arg	Thr	Pro	Lys	Pro	Arg	Glu	Thr	Gly	Leu	Leu	Ile	Met	Gly	Val	Ser	Glu	Asn	Asp	Glu
KR781451	Gly	Arg	Ile	Lys	Arg	Thr	Pro	Lys	Pro	Arg	Glu	Thr	Gly	Leu	Leu	Ile	Met	Gly	Val	Ser	Glu	Asn	Asp	Glu
KJ867540	Glu	Lys	Lys	Lys	Arg	Ala	Ser	Arg	Pro	Arg	Gly	Thr	Asp	Leu	Leu	Ile	Met	Glu	Val	Pro	Gly	Asn	Gly	Glu
KJ867543	Glu	Lys	Lys	Thr	Arg	Ala	Ser	Lys	Pro	Arg	Gly	Thr	Asp	Leu	Leu	Ile	Met	Glu	Val	Pro	Gly	Asn	Gly	Glu
KR140086	Glu	Arg	Arg	Lys	Gln	Thr	Ser	Arg	Pro	Arg	Glu	Thr	Ser	Leu	Leu	Ile	Met	Glu	Val	Pro	Glu	Asn	Asp	Glu
NC 006383	Glu	Arg	Arg	Lys	Gln	Thr	Ser	Arg	Pro	Arg	Glu	Thr	Ser	Leu	Leu	Ile	Met	Glu	Val	Ser	Glu	Asn	Asp	Glu
KX421388	Glu	Arg	Arg	Lys	Gln	Thr	Pro	Arg	Pro	Arg	Glu	Thr	Gly	Leu	Leu	Ile	Met	Glu	Val	Ser	Glu	Asn	Asp	Glu
KT860064	Glu	Arg	Arg	Lys	Gln	Thr	Pro	Arg	Pro	Arg	Glu	Thr	Gly	Leu	Leu	Phe	Met	Glu	Val	Ser	Glu	Asn	Asp	Glu
KX033350	Glu	Arg	Arg	Lys	Gln	Thr	Pro	Arg	Pro	Arg	Glu	Thr	Gly	Leu	Leu	Phe	Met	Glu	Val	Ser	Glu	Asn	Asp	Glu
AY560591	Glu	Arg	Arg	Lys	Gln	Thr	Pro	Arg	Pro	Lys	Glu	Thr	Gly	Leu	Pro	Ile	Met	Glu	Val	Pro	Glu	Asn	Asp	Glu
OQ971954	Glu	Arg	Arg	Lys	Gln	Thr	Pro	Arg	Pro	Arg	Glu	Thr	Gly	Leu	Leu	Phe	Thr	Glu	Val	Ser	Glu	Asn	Asp	Glu
OQ971955	Glu	Arg	Arg	Lys	Gln	Thr	Pro	Arg	Pro	Arg	Glu	Thr	Gly	Leu	Leu	Phe	Thr	Glu	Val	Ser	Glu	Asn	Asp	Glu
OQ971956	Glu	Arg	Arg	Lys	Gln	Thr	Pro	Arg	Pro	Arg	Glu	Thr	Gly	Leu	Leu	Phe	Thr	Glu	Val	Ser	Glu	Asn	Asp	Glu

Discussion

This study confirmed that the Peste des Petits Ruminants Virus (PPRV) is the causative agent of outbreaks among small ruminants, based on observed clinical signs and molecular diagnosis. High morbidity and mortality rates were linked to secondary infections, such as pneumonia and gastroenteritis, which were exacerbated by malnutrition and parasitism [19]. Oculo-nasal swabs were tested using RT-PCR targeting the N gene, yielding a detection rate of 74.5%. The study results significantly exceed the 29.5% detection rate reported in Tanzania [20]. The N gene was selected due to its high abundance and structural significance, making it ideal for sensitive and specific diagnosis [6, 21].

PCR effectively detected viral RNA in nasal swabs, which were optimal for clinical sampling [22, 23]. Although nasal swabs were the most reliable samples, other sample also showed potential for detecting the virus.

Globally, PPRV is classified into four lineages (I–IV), with lineage IV having the broadest distribution across Asia, the Middle East, and Africa [14, 15]. Sequence and phylogenetic analyses confirmed that the identified sequences were characterized as Genotype IV lineage

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PPR virus, which is in consistent with previous reports in India [16, 24].

Sequence and phylogenetic analysis in this study revealed 97.7% to 100% identity within the Asian lineage IV at both the nucleotide and amino acid levels and 93.2% to 98.7% similarity with other PPRV lineages, reinforcing the distinctiveness of Genotype IV lineage.

Unique substitutions in the N protein at the positions Lys/Gln458→Pro, Thr469→Pro, and Met507→Thr may result in new genetic variants. Based on partial N gene phylogeny, the PPRV strains exhibited distinct clustering patterns and formed six clades. The study sequences clustered with the Dubai, Tibet, Chinese, and various Indian genotypes.

These findings underscore the value of N gene-based RT-PCR in diagnosing PPRV and tracking its genetic evolution. The sequence identity among field isolates and vaccine strains confirms the prevalence of Genotype IV lineage in the Indian subcontinent. This research highlights the importance of molecular diagnostics and phylogenetics in understanding PPRV epidemiology and lineage distribution.

Conclusion

This study investigates the prevalence of Peste des Petits Ruminants Virus (PPRV) infection among goats in the Puducherry region of Southern India. Sequence and phylogenetic analyses indicate that the outbreak PPR virus is characterized as Genotype IV lineage. Notable substitutions in the N protein—specifically Lys/Gln458→Pro, Thr469→Pro, and Met507→Thr—indicate that these PPRV strains are under significant selection pressure, which may lead to the emergence of new genetic variants. The study also highlights the importance of N gene-based RT-PCR for diagnosing PPRV and monitoring its genetic evolution. Given the ongoing mutations observed in field strains, it is crucial to identify the specific strains and lineages circulating across different geographical regions of India. This identification will aid in developing effective strategies for the therapeutic prevention and management of PPRV infections.

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Disclosure statement

The author reported no potential conflict of interest.

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