RESEARCH ARTICLE

Molecular Detection and Sequencing of Peste Des Petits Ruminants Virus of Saurashtra Region of Gujarat, India

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Abstract

The present study was undertaken with the objective of molecular detection and sequencing of the Saurashtra region's Peste Des Petits Ruminants virus. A total of 119 clinical samples (40 nasal swabs, 20 conjunctiva swabs, 16 oral swabs, and 19 tissues from goats along with 22 nasal swabs and 2 tissues of sheep lung and intestine) were tested by N gene & F gene-based primers. Out of 119 samples, 35 samples were positive for N gene primer, and 32 positives for F gene primer. The N gene-based sequence analysis of three PPRV obtained from Bhavnagar, Amreli, and Rajkot belonged to lineage IV (Indian strains), and all three PPRV sequences showed 100 % genetic identity with each other and 98-99 % genetic identity with PPRV strain of Mehsana (North Gujarat), Bhopal (Madhya Pradesh), Pune (Maharashtra), Jhansi (Uttar Pradesh), and Sungri (vaccine strain)

Keywords: Molecular detection, Peste Des Petits Ruminants virus, (PPR virus), Sequencing, Saurashtra region, Gujarat

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Introduction

Peste des petits ruminants (PPR) caused by PPR virus (PPRV) is a highly contagious, acute, febrile viral disease of sheep and goats, which has emerged as a real threat in India, especially after eradication of Rinderpest. After its first report in India, there has been a pan-Indian increase of incidence including Gujarat (Patel *et al.,* 2017). The PPRV is a RNA virus belonging to the genus *Morbillivirus* and the family *Paramyxoviridae*. The virus is closely related to the rinderpest virus, measles, and canine distemper. The genome of PPRV is negative-sense single-stranded RNA and contains six transcriptional units in the order of '3 N-P-M-F-H-L 5' with N gene getting expressed to a very high level (Diallo, 1990). The PPRV is usually transmitted by the aerosol way but may spread through direct contact, contaminated water or feed, and produce 10–100% morbidity. Among the various techniques developed for the detection of PPRV, polymerase chain reaction (PCR) technique targeting F gene and N gene primers have been the most popular tool so far for diagnosis as well as molecular epidemiological studies (Forsyth and Barrett, 1995; Sakhare, 2019; Chukwudi *et al.,* 2020). Positive RT-PCR results were still obtained with poorly preserved samples, where agar gel immuno-diffusion test and virus isolation failed to detect the virus. RT-PCR also offers the possibility of analyzing the relationship between the different PPRV strains for molecular epidemiological studies (Altan *et al*., 2018). This study was aimed to detect and make sequence analysis of PPRV from goat and sheep samples by RT-PCR.

Materials and Methods

Molecular Detection By RT-PCR

A total of 119 clinical samples (40 nasal swabs, 20 conjunctival swabs, 16 oral swabs, and 9 lung, 4 trachea, 3 spleen, 3 intestine tissues from goat along with 22 nasal swabs and 1 lung, 1 intestine tissues of sheep) and reference vaccine virus were processed for RNA extraction using QIAmp mini viral kit. The study used the lyophilized freeze-dried live PPR vaccine (Sungri 95 strain) as the reference virus. The quantitation and quality assessment of RNA extracted from the samples was carried out by spectrophotometry.

The extracted RNA samples were subjected to PCR amplification using a one-step RT-PCR kit (Qiagen, France)

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by employing the primer pair of NP3 & NP4 and F1 & F2. PCR amplified products and 100 bp DNA molecular weight marker were electrophoresed on 1.5 % agarose gel and were visualized using a gel documentation system (Vilberlourmet, France). The size of the PCR amplicons was analyzed by comparing them with that of the 100 bp DNA molecular weight marker using GeneTools computer software. Two pairs of primers (synthesized by Eurofins, Bangaluru) were used for PCR amplification (Table 1).

The PCR protocol was carried out as followed in Table 2. (George, 2002)

Sequence Analysis and Phylogenetic Study

Purification of PCR Product

N gene amplified products were run in 1.5% AGE in TAE buffer with 50 bp marker (Cat. No. SM0371, Thermo Scientific, USA) and the amplicons were cut precisely for the gel purification. Gel purification was carried out by QIAquick® gel extraction kit (Cat. No. 28704).

Sequencing and Phylogenetic Analysis of the N Gene

Three samples positive for Nucleocapsid gene were obtained from three different locations and were used for sequencing and further phylogenetic analysis. The gel eluted products were commercially sequenced from the Eurofins Genomics Pvt. Ltd. Bengaluru, India, using ABI 3730XL DNA analyzer.

The raw data obtained from the commercial sequencing for N gene was first analyzed for the base call analysis and trimmed by the Chromas software (version 2.5.1). Homology search of the obtained sequences was made using BLASTn interface of GenBank online (http://blast.ncbi. nlm.nih. gov/). Further mismatched sequences were reanalyzed with Chromas software for analysis of base cells. The reverse-oriented sequences were reverse complemented using the Chromas. The contigue were generated by using the online contig software, and consensus sequences were saved for further analysis. The N gene sequences of the present study (PPRV_IND/ Bhavnagar/ GUJ/2020 (ppr g36), PPRV_IND/ Rajkot/ GUJ/2020 (ppr g37), PPRV_IND/ Amreli/ GUJ/2020 (ppr g38) along with N gene sequences spanning genomic position 1232 to 1583 bp to the genomic context of N gene were taken for the analysis from all the available sequences from NCBI belonging to lineage I and Indian isolate of 12 different states of PPRV lineage IV along with vaccine virus Sungri (AY560591) (Fig. 4).

The sequences were analyzed pairwise, and multiple alignments were done using the clustal X. The output file was further extrapolated in the MEGA 7.0 software, and the alignment builder was used to create an input file. Further alignment was carried out using the Clustal W program embedded in MEGA 7.0. The evolutionary history was inferred using MEGA 7.0 software employing the Maximum Likelihood method based on the Kimura 2-parameter model.

Results and Discussion

Detection of Viral Genome in Clinical Samples by RT- PCR

A total of 119 clinical samples of goats, sheep, and reference vaccine virus were processed by N gene-based primers NP3/ NP4, which yielded the expected amplicons of approximately 351 bp in 35 (29.4%) samples. When tested by F gene-based primers F1/F2, the same samples yielded an expected amplicon of approximately 372 bp in 32 samples (26.9 %) (Fig. 1 and 2).

Out of 119 clinical samples, 35 (29.41%) samples and 32 (26.89%) samples were found positive by RT-PCR using N primers and F primers, respectively (Table 3 and 4). Compared to N gene-based RT-PCR, the sensitivity of F gene-based RT-PCR was 91.43 %.

Table 1: Primers used for amplification of N and F gene of PPRV

Primer	*Sequence (5'-3')*	*Position*	*Size*	*References*
NP3 NP4	F- TCTCGGAAATCGCCTCACAGACTG R- CCTCCTCCTGGTCCTCCAGAATCT	1232-1255 1560-1582	351 bp	Couacy-Hymann *et al., (*2002)
F1 F2	F- ATCACAGTGTTAAAGCCTGTAGAGG R- GAGACTGAGTTTGTGACCTACAAGC	777-801 1124-1148	372 bp	Forsyth and Barett (1995)

Table 2: Thermal profile for N gene and F gene amplification

N gene	*F gene*		*Conditions*
50°C for 30 minutes	50°C for 30 minutes	1 cycle	Initial reverse transcription
95°C for 15 minutes	95°C for 5 minutes	1 cycle	Inactivates RT and PCR activation
94°C for 30 seconds	94°C for 30 seconds	denaturation	
58°C for 30 seconds	50°C for 60 seconds	annealing	35 cycles
72°C for 1 minute	72°C for 2 minute	extension	
72°C for 5 minute	72°C for 10 minute	1 cycle	Final extension
4°C (indefinite)	4°C (indefinite)	–	–

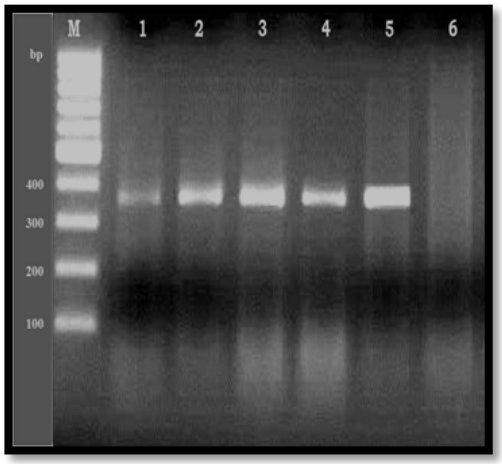

Fig. 1: Agar-gel electrophoresis showing N (351 bp) gene specific product for confirmation of PPRV (1 to 4- Positive sample, 5- Positive control, 6- Negative control)

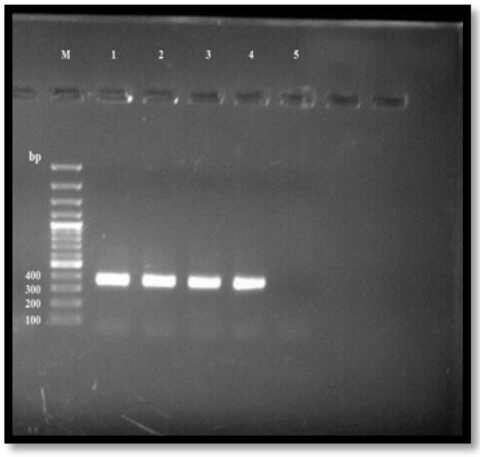

Fig. 2: Agar-gel electrophoresis showing F (372 bp) gene specific product for confirmation of PPRV (1- Positive control 2,3,4- Positive sample 5- Negative control)

Table 3: Detection of PPRV viral genome in clinical samples by RT-PCR using N-primers

	Species of animals					
	Goat		*Sheep*		*Total*	
Type of sample	*Tested*	*Positive (%)*	*Tested*	*Positive (%)*	*Tested*	*Positive (%)*
Nasal swab	40	7 (17.50)	22	5 (22.72)	62	12 (19.35)
Conjunctival swab	20	3 (15.00)	0	0	20	3 (15.00)
Oral swab	16	6 (37.50)	0	0	16	6 (37.50)
Tissue	19	12 (63.16)	2	2 (100)	21	14 (66.67)
Total	95	28 (29.47)	24	7 (29.17)	119	35 (29.41)

Table 4: Detection of PPRV viral genome in clinical samples by RT-PCR using F-primers.

	Species of animals					
	Goat		*Sheep*		*Total*	
Type of sample	*Tested*	*Positive (%)*	*Tested*	*Positive (%)*	*Tested*	*Positive (%)*
Nasal swab	40	6 (15.00)	22	4 (18.18)	62	10 (16.13)
Conjunctival swab	20	2 (10.00)	0	0	20	2 (10.00)
Oral swab	16	6 (37.50)	0	0	16	6 (37.50)
Tissue	19	12 (63.16)	2	2 (100)	21	14 (66.67)
Total	95	26 (27.37))	24	6 (25.00)	119	32 (26.89)

Figures in parentheses indicate the percentage.

Our results align with Cattaneo *et al*. (1987), who suggested that the N gene was more abundant in clinical samples. Couacy-Hymann *et al*. (2002) also used the N gene to detect PPRV in suspected samples. Brindha *et al*. (2001) processed oculo-nasal swabs, and tissue samples collected from PPR suspected sheep and goats for PPRV or its RNA by virus isolation, and RT-PCR yielded 31.8 and 40.9 % samples positive, respectively.

Our results match with the finding of Kgotlele *et al.* (2014), who found PPRV in 21 out of 71 (29.5%) tested animals using primers targeting N gene from Northern and Eastern Tanzania. Our reports are lower (27.37% vs 51.52%) as compared to that of Luka *et al.* (2011) but at the same time higher (27.37% vs 9.7%) as compared to that of Sunderpandian (2014) using primers targeting F gene.

The attempt by targeting the PPRV N gene for PCR gave very promising results. N gene codes for an internal structural protein, and mRNAs of N gene are the most abundant transcripts of the virus, making it an attractive target for developing a highly sensitive and specific diagnostic assay for PPRV (George, 2002). Based on clinical signs and the detection of PPRV using RT-PCR, it could be concluded

that PPRV caused the disease outbreaks in the current study areas.

Sequence and Phylogenetic Analysis

The study was carried out to partial N gene sequencing and phylogenetic analysis, which gives an idea about the genetic correlation of circulating viruses in the region to the global PPRV lineages. The PPR virus (PPRV) was grouped into four distinct lineages covering the globe. However, it has only one serotype, but there are different strains distribution putting the epidemiological investigations always at stake to control the disease. The uncontrolled movement of the animals within states and the porous border between states and countries let the PPRV disease outbreaks spread rampantly. Since the last decade, we have seen a surge in the PPRV outbreaks in the Saurashtra region. Therefore in the present study, an epidemiological dynamic through partial N gene sequencing was carried out. The good quality partial N gene sequence for the three different isolates varied for viral strains (1): PPR/BHV/Guj/Ind 36/2020, (2): PPR/RJT/Guj/Ind 37/2020, and (3): PPR/AMR/Guj/Ind 38/2020, 349 bp each. All three sequences aligned with Indian PPRV isolates, especially with maximum homology (approximately 99%) with PPRV

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ppr_g36 TCGGAAATCGCCTCACAGACTGGGGACGAAAGAACCGCCAGAGGGACTGGGCCTCGACAG 60
ppr_g37 TCGGAAATCGCCTCACAGACTGGGGACGAAAGAACCGCCAGAGGGACTGGGCCTCGACAG 60
ppr_g38 TCGGAAATCGCCTCACAGACTGGGGACGAAAGAACCGCCAGAGGGACTGGGCCTCGACAG 60
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ppr_g36 GCGCAGGTCTCCTTCCTCCAGCACAAAACAGGAGAGGGAGAGTCGTCCGCACCAGCGACCC 120
ppr_g37 GCGCAGGTCTCCTTCCTCCAGCACAAAACAGGAGAGGGAGAGTCGTCCGCACCAGCGACCC 120
ppr_g38 GCGCAGGTCTCCTTCCTCCAGCACAAAACAGGAGAGGGAGAGTCGTCCGCACCAGCGACCC 120
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ppr_g36 AGAGAAGGGGTCAGGGCTGCGATCCCAAACGGATCTGAAGAGAGGGACAGAAAGCAAACA 180
ppr_g37 AGAGAAGGGGTCAGGGCTGCGATCCCAAACGGATCTGAAGAGAGGGACAGAAAGCAAACA 180
ppr_g38 AGAGAAGGGGTCAGGGCTGCGATCCCAAACGGATCTGAAGAGAGGGACAGAAAGCAAACA 180
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ppr_g36 CGCCCAGGAAGGCCCAAAGGAGAGAGACCCCCGGCCAACTGCTCCCGGAAATCATGCCAGAG 240
ppr_g37 CGCCCAGGAAGGCCCAAAGGAGAGAGACCCCCGGCCAACTGCTCCCGGAAATCATGCCAGAG 240
ppr_g38 CGCCCAGGAAGGCCCAAAGGAGAGAGACCCCCGGCCAACTGCTCCCGGAAATCATGCCAGAG 240
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ppr_g36 GACGAGGTCCCGCGAGAGTCTGGCCAAAACCCTCGTGAGGCCCAGAGATCGGCCGAGGCA 300
ppr_g37 GACGAGGTCCCGCGAGAGTCTGGCCAAAACCCTCGTGAGGCCCAGAGATCGGCCGAGGCA 300
ppr_g38 GACGAGGTCCCGCGAGAGTCTGGCCAAAACCCTCGTGAGGCCCAGAGATCGGCCGAGGCA 300
************************************************************

ppr_g36 CTCTTCAGGCTGCAGGCCATGGCCAAGATTCTGGAGGACCAGGAGGAGG 349
ppr_g37 CTCTTCAGGCTGCAGGCCATGGCCAAGATTCTGGAGGACCAGGAGGAGG 349
ppr_g38 CTCTTCAGGCTGCAGGCCATGGCCAAGATTCTGGAGGACCAGGAGGAGG 349
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Fig. 3: Comparison of three isolate of Saurashtra region (ppr g 36, ppr g37, ppr g38) based on partial N gene sequence by Clustal mega software (Online software)

Mehsana, PPRV Gujarat, PPRV Bhopal, PPRV Jhansi, and PPRV Sungri. Among the field isolates of the present study close genetic relationship (100% identity) was observed between the isolates ppr g36 (BHV/Guj/20), ppr g37 (RAJ/Guj/20) and ppr g38 (AMR/Guj/20) (Fig. 3, 4). The other isolates of Gujarat were found closely related to each other with 98-99 percent homology (Nagraj, 2006). The BLASTn homology search analysis confirmed the etiological agent for these outbreaks as PPRV.

All three PPRV sequences showed 99 % genetic identity with PPRV strain of Mehsana (North Gujarat), Bhopal (Madhaya Pradesh), Jhansi (Uttarpradesh), Sungri (vaccine strain), and 98% genetic identity with Pune (Maharashtra) based on partial N gene sequence analysis (Table 5).

The phylogenetic analysis tree constructed using the Maximum Likelihood method revealed that all 59 sequences from the Indian isolates and sequences from different lineages were phylogenetically distributed into four. In the present study, the inclusion of different lineage sequences along with available N gene sequences from India across 12 states and Vaccine viruses Sungri was purposefully done as there is the interstate movement of the goats & sheep for trading as well as for goat farming, or meat purpose, and vaccination mostly with Sungri virus is being carried out (Fig 4). All the Indian isolates from different states were placed in the two different clades I & II under lineage IV. The PPRV strains from the present study were clustered into clade I in lineage IV.

Our findings substantiated the findings reported by Nagraj (2006), Shelke (2017) and Pharande (2018). In India, a comprehensive 'multiple gene assessment of lineage IV isolates of PPRV utilizing sequence data for the study of partial N, F, and H gene revealed effective grouping of PPRV isolates. Also, it indicated that the variable part N gene nucleotides (1253 to 1470) at C terminal give the best phylogenetic assessment of PPRV general isolates clustering

Table 5: Sequence producing significant alignment PPR/BHV/GUJ/IND 36/2020, PPR/RJT/ GUJ/IND 37/2020 and PPR/AMR/GUJ/IND 38/2020

Description	*Max score*	*Total score*	*Query cover*	*E value*	*Identity*	*Accession*
Peste-des-petits-ruminants virus isolate Mehsana/Guj/05 nucleoprotein (N) mRNA, partial cds	634	634	100%	1e-177	99%	DQ267190
Peste-des-petits-ruminants virus isolate Guj/2007 nucleocapsid protein (N) gene, complete cds	628	628	100%	5e-176	99%	JN632530
Peste-des-petits-ruminants virus strain Bhopal-2003 nucleocapsid protein (N) gene, complete cds	628	628	100%	5e-176	99%	FJ750560
Peste-des-petits-ruminants virus strain Sungri-96 nucleocapsid protein (N) gene, complete cds	634	634	100 %	1e-177	99%	AY560591
Peste-des-petits-ruminants virus strain Jhansi-2003 nucleocapsid protein (N) gene, complete cds	628	628	100 %	5e-176	99%	U014571
Peste-des-petits-ruminants virus strain PPR/MH/Pune/ IND/2004 nucleocapsid protein mRNA, partial cds	590	590	99 %	1e-172	98%	KX860076
Peste-des-petits-ruminants virus isolate PPRV-TRIP-AGAR-57/2010 nucleocapsid protein (N) gene, partial cds	584	584	100 %	1e-162	98%	KX860048

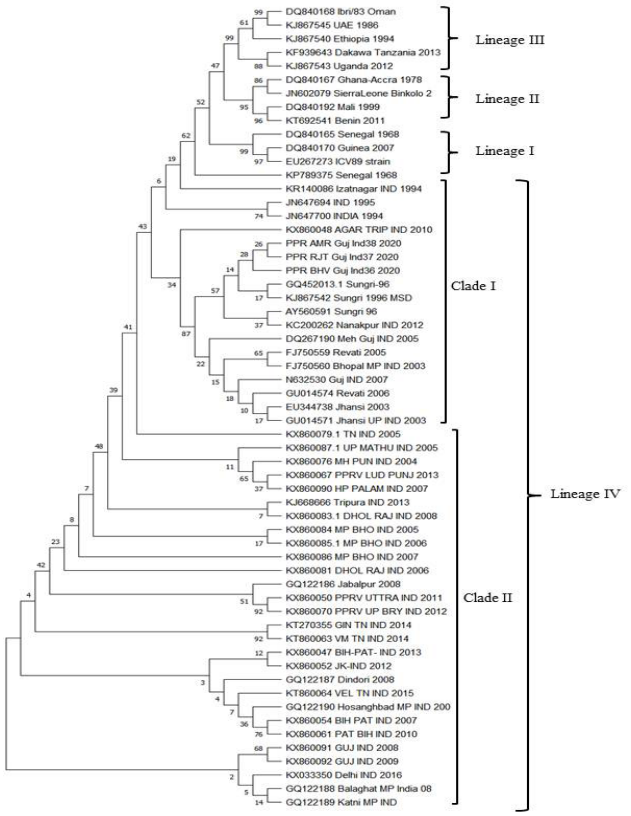

Fig. 4: Molecular phylogenetic analysis based on partial N gene of PPRV of Saurashtra region with 56 partial N gene sequence of all lineage using mega 7 software. (Offline software)

according to geographical isolation (Senthilkumar *et al*., 2014). It was observed that lineage I was limited to Western Africa, lineage II was found in Western and Central Africa, linage III was restricted to East Africa and Middle East (Libeau *et al*., 2014), all four lineages have been detected in Africa, while only lineage IV is circulating across Asia (Banyard *et al*., 2010). From the analysis of the partial N gene, it was observed that only lineage IV was found in India. Thus, phylogenetic analysis was in agreement with other researchers mentioned above.

Conclusion

From the sequence analysis and phylogenetic analysis, it was observed that PPRV isolates of the Saurashtra region were closely associated with the isolates from the Mehsana (North Gujarat), Bhopal (MP), Jhansi(UP), and Sungri (HP), India. It might be due to the wrong way of trading the animals. So, there is a need for control over the trade of animals and regulatory bodies and animal health departments' interventions to prevent the transmission of disease from district to district and state to state for eradication of PPRV like Rinderpest.

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