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Phosphoprotein gene of peste des petits ruminants virus: unsuitable for lineage determination

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ABSTRACT

The nucleotide sequence of phosphorprotein (P) gene of a recent isolate of Peste des petits ruminants virus (PPRV/INDIA/TN/Apampattu/2014) was determined by RT-PCR amplification and sequencing. The sequence analysis showed that phosphoprotein gene sequence of this isolate has 87-98 percent identity with other Indian PPRV. At amino acid level, the identity was 84-98 percent with recent Indian PPRV isolates and 95 percent with Sungri/96 isolate. Surprisingly, when phylogenetic analysis was conducted with all the available 'P' gene sequences in genbank along with our sequence, it depicted more than four lineages in the phylogenetic tree. This is not in compliance with the PPRV 'F' and 'N' gene based and whole genome sequence based phylogenetic analysis. This concluded that PPRV 'P' gene based analysis is not suitable for lineage detection. Meanwhile, the PPRV/INDIA/TN/Apampattu/2014 isolate was found to group with Indian isolates coming under lineage IV. Therefore, 'P' gene based analysis could be of help to cluster new isolates into close related groups.

Key words: Nucleotide sequence, Peste des petits ruminants virus, Phosphoprotein, Phylogenetic tree.

INTRODUCTION

India being a developing country has vast resources of sheep and goat population which forms the backbone of rural economy. The major threat to sheep and goat industry in India and also, worldwide, is outbreak of infectious diseases. Peste des petits ruminants (PPR) being the leading one, is a highly contagious and economically important viral disease of domestic and wild small ruminants with a widespread distribution across sub-Saharan Africa, the Arabian peninsula and the Indian subcontinent (Nanda *et al*., 1996; Dhar *et al*., 2002 and Yapici *et al*., 2014). In India, the disease is equally considerate and dreadful. It is not heartening to say that the disease is endemic in most of the states of India (Manimaran *et al*., 2016) and require special attention owing to its transboundry and emerging nature. The causative agent of PPR is PPRV (Parida *et al*. 2015 and Baron *et al*. 2016), a non-segmented negative sense RNA genome, classified under *Morbilivirus* genus of family *Paramy- xoviridae* (Gibbs *et al*. 1979; Afonso *et al*. 2016 and ICTV, 2016). The disease in small ruminants is characterized by pyrexia, necrotic stomatitis, pneumonia and enteritis and also immunosupression (Kul *et al*., 2007; Pope *et al*., 2013; Baron *et al*., 2016; Wernike *et al*., 2014). It is estimated to cause global economic losses of US$ 1.5 to 2 billion every year. Therefore targeted for global control and eradication by the year 2030 (OIE and FAO, 2015).

The 'P' gene is reported to be a multifunctional, multiprotein gene of 1650 nucleotides in length with an open reading frame of 1530 nts, extending from 1807 to 3336 in position of whole PPRV genome. It encodes three separate proteins such as P, C and V (Bellini *et al*., 1985; Cattaneo *et al*., 1989 and Mohapatra *et al*., 2003). Phosphoprotein is a direct transcript of the P gene while V and C proteins are produced as a result of alternate open reading frame and mRNA editing of P transcript (Vidal *et al*., 1990 and Hausmann *et al*., 1999). Phosphoprotein (P), phosphorylated protein of PPRV, plays a significant role in viral replication by forming RNP complex in association with 'N' and 'P' protein. Here, we sequenced and analyzed the 'P' gene of PPRV/TN/Apampattu/ 2014 isolate and constructed phylogenetic tree to examine its relevance in identifying the lineages.

MATERIALS AND METHODS

Virus and cell line: PPRV/IND/TN/Apampattu/2014 strain isolated from field outbreak and preserved in lyophilized form at Vero passage 10 (P10) in the Department of Veterinary Microbiology, Madras Veterinary College, Chennai-600007, was revived and used for the present study.

RNA extraction and cDNA synthesis: Total RNA was extracted from infected Vero cell culture fluid using Trizol reagent (Life technologies, USA, Cat # 15596-026) as per the manufacturer's instruction. The concentration of RNA was estimated with spectrophotometer (Nanodrop 2000, Thermo Scientific) using DNase and RNase free water as blank. The RNA at a minimum concentration of 1 µg/20 µl

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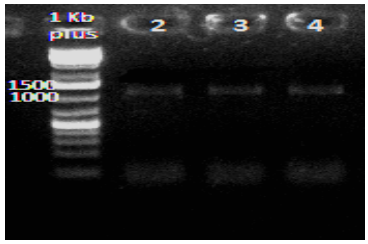
and purity close to 2.0 was used for reverse transcription. Reverse transcription was performed with random hexamers using Verso cDNA synthesis kit**.** The components of RT reaction mixture were having 0.5 µl of reverse transcriptase enzyme, RT enhancer and random hexamer; 1 µl of dNTP; 2 µl of buffer and 5 µl of cDNA template.The reaction condition was standardized for PPR Virus to 42°C for 1 h, 95°C for 2 minutes and 4°C for infinity following the manufacturer's instruction with little modification in temperature and time. The obtained cDNA was then checked for concentration and purity in Nanodrop.

Sequencing and analysis: Whole P gene was amplified in three overlapping fragments F1, F2 and F3.The cDNA was subjected to PCR amplifications for all the three fragments as described in Table 1.PCR products of fragments F1 to F3 were purified from 1 % agarose gel using QIAquick® Gel Extraction Kit (Qiagen, cat # 28704).The gel purified PCR products with a concentration of >50 ng/µl and purity >1.9 at A_{260}/A_{290} were sequenced by Sanger's dideoxy sequencing method using BDT v3.0cycle sequencing kit on ABI 3730x1 Genetic Analyzer (Eurofins India Private Ltd, Karnataka, India).

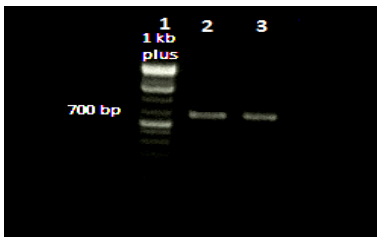
The obtained sequences were checked for homology and percent identity with all the available sequences in Genbank by blastn program. Sequences of all the three fragments of 'P' gene were contiguously aligned using Bioedit software version 7.0.5.3 (Hall, 1999) to construct the complete P gene sequence. Multiple sequence alignment and phylogenetic analysis was done using Mega 7.0 software programme by Maximum Likelihood (MLH) method using bootstrap values of 500. The nucleotide and deduced amino acid sequence were analysed by blastn and blastx program, respectively, to derive percent indentity of 'P' gene the given isolate with other isolates.

RESULTS AND DISCUSSION

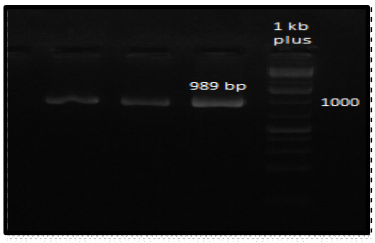
The P gene of above PPRV isolate was amplified as three overlapping fragments yielding 1116, 625 and 989 bp products. The PCR amplicons of these three fragments are depicted in Plate 1. Nucleotide sequences of these three PCR amplicons were aligned using BioEdit 7.1 software tool to obtain a complete P gene sequence (provided in Fig 1).



a. RT- PCR amplified PPRV 'P' gene 1st Fragment



b. RT- PCR amplified PPRV 'P' gene 2nd Fragment



a. RT- PCR amplified PPRV 'P' gene 3rd Fragment

Plate 1: RT-PCR amplification of PPRV P gene overlapping fragments.

5'AGGACCCAGGTCCAAGCAACCACACATCGACACCCCAGCCAATCGAGTAGAGACCACCG**ATG**GCAGA
AGAACAAGCATACC**ATG**TCAACAAGGGGCTGGAATGTATCAAGTCCCTCAAAGCCTCTCCCCCGGATCTA
TCCACCATTAGAGATACCATCGAGAGCTGGAGAGAGGGGCTTAGCCCCTCGGGCCGTGCAACACCGAAC
CCTGATACGTCCGAGGGAGATCATCAGAATATCAACCAATTCAGCTCACCAGCAATCGGACCAAACAAAG
TCTACTTGTCTCCTGAAGATAATCTCGGATTTAGAGAGGTCACTGGCAACGACTGTGAGGCTGAGTTCGG
AGGAGTCCAGGGAAAAGGATCCAACTCTCAAGTACAGCGTTACTATGTTTATAGCCACGGGGGTGAAGA
GATTGAAGGACTCGAGGATGCTGACTCTCTCGTGGTTCAAGCAGATCCTCCAGTTGCTAACATATTCAAT
GGAGGAGAGGATGGATCTGACGACAGCGATGTGGACTCTGGCCCAGATGATCCCAGCAGAGATACTCTA
TATGACCGGGGACCTGCTGCCGGCAATGATGTCGCTAGGTCCACAGATGTCGAAAAAC**TAG**AAGGTGCT
GATATTCAAGAAGTTCTCAACTCCCAGAAAGGCAGAGGAGGAAGATTCCAAGGCGGGAAAACCTTACGA
GTCCCGGAAGTACCCGATGTCAAGAACTCCAGACCATCAGCCCAATCAA**TTAAAAAGGGCACAG**ACGGG
AACTCAGTCTCATCTGGAACGGTGACAGAGTGTTTATCGATAAGTGGTGCAACCCAAGCTGTGCCAGAGT
CAAGATGGGAGTCATCAGAGCAAAATGCGTCTGTGGGGAGTGTCCTCAAGTCTGCGAAGAGTGCAAAG
ACGATCCAGGGGTCGACACAAGAATCTGGTACCACAGCATCACCGAGTCAACC**TAA**AGAGAATGACTCC
GAGTATGAGTATGAGGATGACCTATTTACAGAGATTCAGGACATCCGTGCAAGCATTGCCAAGATCCATG
ATGACAACAAGTCTATCCTCTCAAAGCTTGATTCTATACTGTTATTGAAAGGAGAAGTCGACACTATTAAG
AAACAAATCAGCAAGCAGAATATAAGTATATCCACCATTGAGGGCCATCTCTCCAGTATAATGATAGCCA
TCCCGGGCTTTGGGAAGGATATCAAGGACCCAACATCCGAGGTTGAGTTGAACCCAGATTTAAGACCTAT
AATAAGCCGTGACTCTGGCAGAGCTCTCGCGGAGGTCCTCAAGAAACCCGCTGTGGATAGGTCTCAGAA
AACTGGAACCAAAGCCAACTCCAACTCAAAGGGTCAGCTTCTTAAGGACCTCCAGCTAAAACCTGTCGAC
AAGCAGGCAAGCTCTGCAATCGGGTTTGTCCCATCCGACCATGAATCATCCAGAAATGTCATCCGCTCCAT
AATCAAGTCGAGCAAGCTAAACATTGATCACAAGGACTATCTTCTAGATTTACTGAATGATGTGAAAGGC
TCCCACGATCTTAAGGAATTGCACAAGATGCTAACAGCAATTCTCGCCAAGCACCCG**TAA**CACATCCTATA
ATCAACATCTCATACTCGGTTGAAAACATCCTCTCAATCAGGCTATTACAAAAAA3'

ATG –TAA: P gene coding region, **ATG –TAG**: C gene coding region, **ATG – TAA:V gene** coding region, **TTAAAAAGGGGCACAG:** Editing site

Fig 1: Complete coding sequence 'P' gene of PPRV/IND/TN/Apampattu/2014 strain (1530 nucleotides).

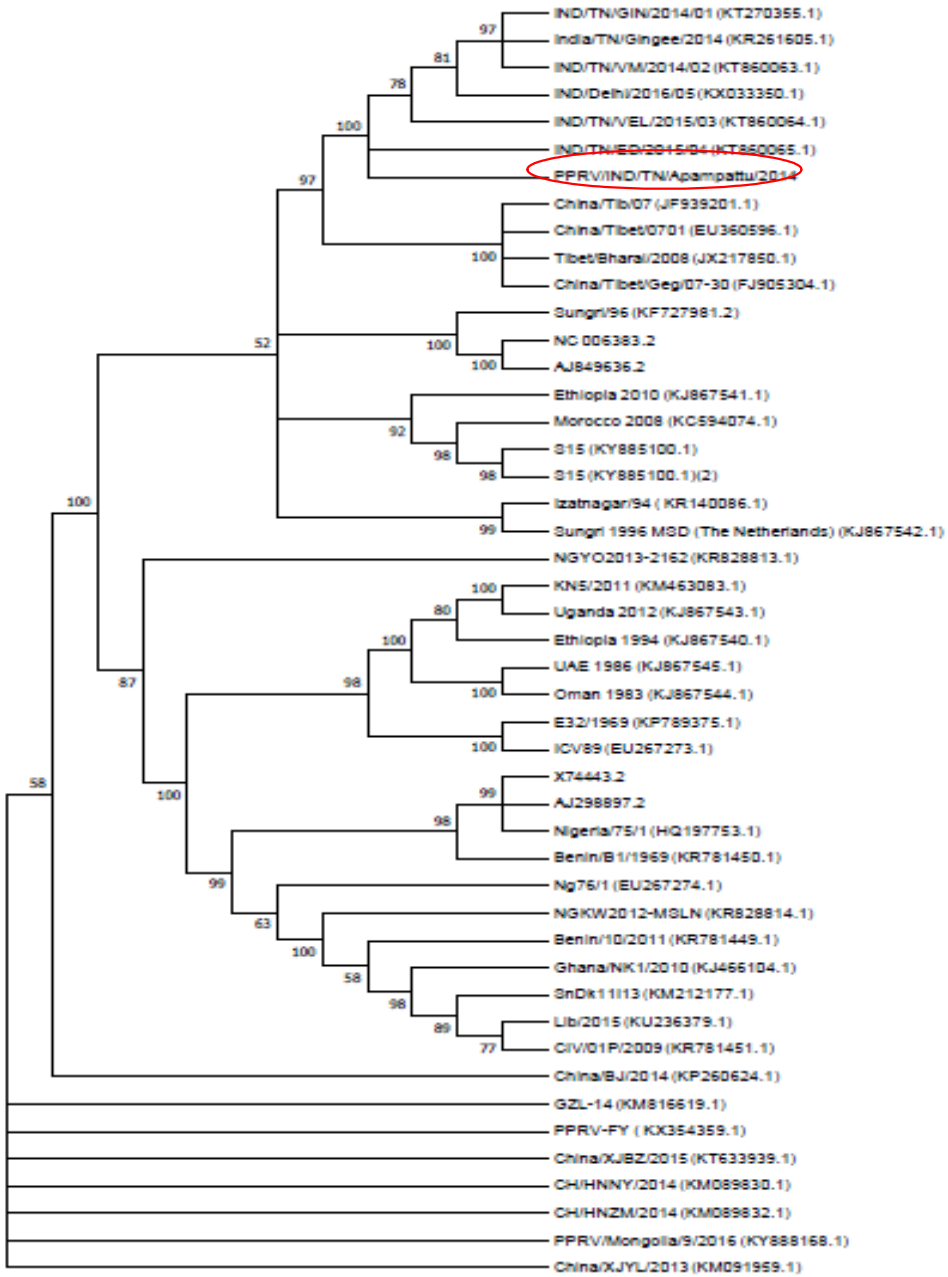


Fig 2: Molecular Phylogenetic analysis by Maximum Likelihood method.
The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [1]. The bootstrap consensus tree inferred from 500 replicates [3] is taken to represent the evolutionary history of the taxa analyzed [3]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [3]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.6110)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 34.50% sites). The analysis involved 47 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1529 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [2].

The P gene was found to be 1,655 nucleotides long with two overlapping open reading frames (ORFs), as depicted in Fig 1. The first ORF is 1530 nucleotides long and would produce P protein of 509 amino acid residues, as reported for other PPRV strains (Muthuchelvan *et al*. 2006 and Sahu *et al*. 2017). The second ORF is 534 nucleotides long and would produce C protein of 177 amino acid residues. The first ORF codes for a second mRNA transcript of 896 nucleotides long and would produce a V protein of 298 amino acid residues. The nucleotide and deduced amino acid sequence were compared with other PPRV isolates by blastn and blastx programme of NCBI, respectively. It was found that nucleotide sequence of the given isolate shows 98 per cent identity with recent isolates of Tamil Nadu, India and 95 per cent identity with other isolates from north India (e, g, PPRV/IND/Delhi/2016/05) and Sungri/96 vaccine virus. At amino acid level the identity was 97-98 % with recent Indian PPRV isolates and 95 % with Sungri/96. An alternate reading frame starting at nucleotide position 23 of first ORF, coding a 177 amino acid of PPRV 'C' protein, is present as previously reported (Mahapatra *et al*. 2003 and Muthuchelvan *et al*. 2006).

The phylogenetic tree developed by including all the 47 complete PPRV ' P' gene sequences using maximum likelihood (ML) algorithm with a distinct gamma distribution model (+G) for P gene are provided in Fig 2. It was found that our isolate, PPRV/INDIA/TN/Apampattu/2014, is more closely related to all Indian isolates of Tamil Nadu region with boot strap values of more than 78 %. Meanwhile, some of the other Indian isolates showed separate lineage with low boot strap value. Another interesting observation could be made from the phylogenetic tree that instead of four lineages, it resulted in multiple ones. It has been reported that there are genetically four distinct lineages of PPR virus circulating all over the world based on molecular epidemiological studies of partial 'F' and 'N' gene sequences (Kumar *et al*. 2014) and also as per with whole genome sequence analysis. However, in the present study using the complete PPRV 'P' gene sequences, the four lineages could not be determined properly, which is totally not in compliance with the standard F' and 'N' gene based analysis. This finding leads to a major conclusion that the PPRV 'P' gene based analysis may not be suitable for lineage detection. It has been reported that 'H' gene is the most preferred candidate gene for phylogenetic study with 93% identity set as a nucleotide cutoff (Sahu *et al*. 2017). Therefore, in compliance with earlier literatures, our study further suggests that 'P' gene based phylogenetic analysis may not be useful in deciding the PPRV lineage, rather could be of help to cluster into close related groups. In conclusion, PPRV 'P' gene based phylogentic analysis was found not suitable for lineage detection.

Nucleotide sequence accession number: The complete P gene sequence of PPRV isolate PPRV/INDIA/TN/Apampattu/2014 has been deposited in GenBank under the accession no. MH607414.

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