

Original Research Article

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Isolation, Molecular and Electron Microscopic Detection of PPR Virus from Suspected Outbreaks of PPR in Goats of North – Eastern Karnataka, India

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ABSTRACT

Region wise monitoring of the PPR virus by active surveillance is essential as it aids in strategic planning of measures of disease control and prevention. The North Eastern Karnataka with sizable sheep and goat population experiences the outbreaks of PPR but the attempts to isolate and characterize the prevailing PPR virus is limited. Hence, the present research work was carried out with the objective of isolation and characterization of PPR virus isolates circulating in North Eastern Karnataka by microscopic (TEM) and molecular (RT-PCR) methods. A total of 15 suspected PPR outbreaks were attended and different types of clinical samples like nasal, oral, ocular, rectal swabs and blood from ailing kids and goats were collected. The samples were initially screened for the presence of PPR virus by observing the infected Vero-cells for development of characteristic CPE. The isolates showing CPE were further confirmed for the virus by RT-PCR using N and F genes of PPR virus. The PCR confirmed isolates were subjected to transmission electron microscopy (TEM). A total of six PPR isolates were obtained representing five districts of North Eastern Karnataka which were confirmed by RT-PCR. The TEM images showed pleomorphic virion particles with varying sizes indicative of Morbilli-virus. These findings provide the first documentation of PPR presence by microscopic and molecular characterization in the region.

Keywords

PPR, TEM,
Morbilli-virus, N, F
gene etc.

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Introduction

Peste des Petits Ruminants (PPR) is an economically important disease of sheep and goats. World Organization for Animal Health (OIE-previous name) identified as notifiable infectious disease of sheep and goat caused by

virus belonging to Morbilli virus genus of family Paramyxoviridae (Gibbs *et al.*, 1979) threatening food security and livelihood of small and marginal farmers across the world. Recent estimate of Food and Agriculture Organisation (FAO), indicated the economic loss due to PPR in India alone, is \$2569.00

million/year during 2012-2017. Realising the economic importance of the PPR disease and successful eradication of antigenically related Rinder pest virus from globe in June 2011, the International organizations (OIE and FAO) have set target of global elimination of the disease by 2030. Though disease is endemic in India, caused by single serotype involving lineage-IV (Asian), recent outbreaks of lineage (IV) PPR virus in Africa and European part of Turkey where outbreaks due to lineage I, II, III were common, had necessitated the continuous surveillance of the disease in an endemic region (Banyard *et al.*, 2010). Hence continuous monitoring of the type of virus circulating in the region by active surveillance is necessary in understanding the epidemiology of disease and planning the measures of disease eradication. Considering the above facts and figures, present study reports about isolation, molecular and electron microscopic detection of PPR virus from suspected outbreaks of the disease recorded in goats of North eastern region of Karnataka comprising five districts.

Materials and Methods

Sample collection

Different clinical samples like nasal, oral, ocular, rectal swabs and blood in viral transport medium (VTM, Hi-media) from ailing kids and adult goats were collected from 15 different outbreaks recorded in North eastern region of Karnataka during the period from Jan-2017 to Dec-2017. Using the clinical score card the clinical signs of the disease were classified in to mild, moderate and severe form (Balamurugan *et al.*, 2014).

A total of 85 nasal swabs, 85 ocular swabs, 85 oral swabs, 50 rectal swabs and 78 anti-coagulated blood samples were collected aseptically in VTM and brought to laboratory in ice packs.

Virus isolation

Swab samples collected in VTM were initially freeze thawed thrice and used directly for isolation. From the whole blood, buffy coat is extracted and eluted in 1X PBS and used as inoculum for infection (OIE manual). Swabs from same place and species of outbreaks were pooled and syringe filtered using 0.45µ pore sized membrane filter (Gomes *et al.*, 2016). One to one and half ml of filtrate was used as inoculum to infect the monolayer (80% confluency) of Vero cells (African green monkey kidney cells at passage 140-160) grown in T-25cm² culture flask by pre-adsorption method (Nanda *et al.*, 1996) with minor modifications. Inoculated cells were incubated at 37°C for one hour with intermittent shaking to allow adsorption of virus. Decant the virus inoculum followed by washing of infected cells with Minimum Essential Medium (serum free DMEM) and then added with maintenance medium (DMEM with 2% serum) for further incubation at 37°C for 6-8 days with change of maintenance medium on alternate days. The cells were monitored daily under inverted microscope for cytopathic effects due to viral replication. The samples were processed up to 6th passage. The specificity of the isolated virus was determined by RT-PCR. Total RNA isolated from cell culture-adapted isolates was subjected to reverse transcription to synthesize complementary DNA followed by its PCR amplification of N and F gene as described below.

Reverse transcription polymerase chain reaction (RT-PCR)

It was performed as per the protocol given in OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2017. The total RNA was extracted from clinical materials and Vero-cell adopted isolates using RNA easy kit (RNeasy®Minikit Qiagen Inc, Valencia, CA,

USA). RT-PCR was performed using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit for c-DNA synthesis followed by PCR amplification using multiple sets of PPR virus genes (N and F) specific primers (Table 1), master mix reagents (Qiagen Inc, Valencia, CA, USA) and PCR conditions (Table 2).

Detection of virus by Transmission Electron Microscopy (TEM)

The concentrated and purified virus pellet of each of the isolate was reconstituted in PBS and 3 µl of each one of them were pipetted out on a paraffin wax strip fixed on a glass slide. The samples were then deposited onto glow-discharger carbon coated copper grids (200 mesh Sigma Aldrich, India) by floating it on the respective samples present on wax strip and incubating it for 1 minute at room temperature. Excess sample was blotted using filter paper after incubation and the grid was washed twice with Milli-Q water. Samples were negatively stained with 2% uranyl acetate for 1-2 minutes (Brenner and Horne, 1959), excess stain was blotted away with filter paper and the grids were allowed to dry. The grids were finally examined on Transmission Electron Microscope HT7700 series at Indian Institute of Horticulture Research (IIHR), Bengaluru operated at 80KV and images were captured at different magnifications (5k, 10k, 15k, 20k, 25k and 30k)

Results and Discussion

Epidemiological observations

A total of 15 outbreaks were attended in the region during the period under study. The clinical signs like elevated temperature, mucous/muco-purulent nasal and ocular discharge, congested nasal and ocular mucous membrane, crusts around nostrils, respiratory

distress, severe mucosal erosion, salivation, ulcers on tongue, watery diarrhoea with soiled hind quarters were observed in the outbreaks and clinical cases attended. Based on the clinical score card the disease was classified into mild, moderate and severe form (Balamurugan *et al.*, 2014). The mild form of disease was characterized by serous nasal and ocular discharge, ulcers in the mouth, respiratory distress (Fig.1A-C) and mild diarrhea, while moderate form showed clinical signs like moderate pyrexia, mucous nasal and ocular discharge, ulcers and bran like depots in the mouth, and watery diarrhoea (Fig.2D-F). The severe form showed signs characterized by hyperaemia, mucopurulent nasal and ocular discharge, sloughing of oral mucosa, formation of crust in and around nostril and eyes, severe watery diarrhoea with soiled hind quarters (Fig.3G-I). Severe form of the disease was recorded more in the kids than adults which experienced mild to moderate forms of the disease.

Virus isolation

A total of six isolates were obtained from the samples subjected for isolation among which five isolates were recovered from nasal swabs, one from ocular and none from blood and rectal swabs. The isolates obtained showed two patterns of characteristic cytopathic effects like rounding and detachment from surface, later on aggregation of cells followed by fusion mass and in other type, CPE was characterized by cells of increased refractivity, rounding and ballooning of cells, clumping into grape like clusters and appearance of fine spindle cells with elongated processes [Fig.4 (A-D)] (Hamby and Dardiri, 1976).

The recovery of virus isolates was observed at different passage levels in which one isolate was recovered on 3rd day of post infection (dpi) in the first passage, two isolates on 2nd and 4th dpi in 2nd passage, two isolates on 3rd

and 5th dpi in 3rd passage and one isolate on 2nd dpi in 5th passage indicating specifically the load of the initial virus in the clinical samples.

The analysis of the isolation results indicates that the relative recovery of virus using nasal swabs and ocular swab was better than faecal and blood samples suggesting to consider nasal swab and ocular swab be better choice of sample for disease diagnosis in ailing animals by isolation (Forsyth and Barrett, 1995; Diop *et al.*, 2005; Harshad *et al.*, 2014). The failure of isolation from blood and rectal swab could be attributed to presence of certain inhibitory or cytotoxic factors present in blood or rectum and labile character of the virus, initial concentration of virus in the clinical material, sampling and delivery time of the sample to laboratory (Harshad *et al.*, 2014). Though isolation of virus is laborious and time consuming, yet is considered to be gold standard test for confirming any aetiological agent in an outbreak which is a mandatory method for declaring disease free status in any region.

RT-PCR Detection

RNA viruses are known to be subjected to high nucleotide substitution error frequencies (Steinhauer *et al.*, 1989). Therefore for routine detection of the virus from different origins, there is a risk of a false negative result if the PCR is carried out with two primers that were designed according to the gene sequences of only one virus strain which could be minimized if two sets of primers used to amplify two different fragments or if a set of primers is synthesized after taking into consideration of the sequences from several strains of different origins (Couacy-Hymann *et al.*, 2002; Kerur *et al.*, 2008 and Ularamu *et al.*, 2012).

Hence all the isolates obtained during the study were tested by using four different sets of primer targeting different regions of genes

from different strains which yielded expected amplicon size of 351bp, 463bp, 447bp and 371bp with primer sets of NP3 and NP4, N1 and N2, F1b and F2d and F1 and F2 respectively confirming the PPR virus (Fig. 5, 6, 7 and 8).

In order to confirm further identity of the virus the N gene specific 351 bp PCR product was gel purified and submitted for direct sequencing (EUROFFINS Bengaluru).

The sequences when searched for its similarity using NCBI BLAST, they were found to be PPR virus.

TEM Detection

Negative staining of RT-PCR confirmed isolates, when observed under TEM showed pleomorphic virion particles with varying sizes characteristic of Morbilli-virus (Losos 1989).

The detailed examination of the isolates by TEM under different magnification revealed the presence of pleomorphic virions mostly circular and elliptical in shape with diameter of intact particles ranging from 100 to 250 nm approximately (Fig. 9A, B, C, D) (Zahur *et al.*, 2009; Sumit *et al.*, 2013).

Review of literature indicate that work related to detection of virus by TEM using negative staining is scanty in India and hence present study could be a first report from state of Karnataka and one among few reports reported across the country till date.

Though immunologic and molecular diagnostic tests have high specificity in detecting an etiological agent than Electron microscopy, sometime these assays may fail to identify agents and hence sensitivity may equal that of Electron microscopy (Hammond *et al.*, 1984; Jiang *et al.*, 1996).



Fig 1.Mild form of PPR: Serous nasal ocular and oral discharge, respiratory distress (A,B,C)

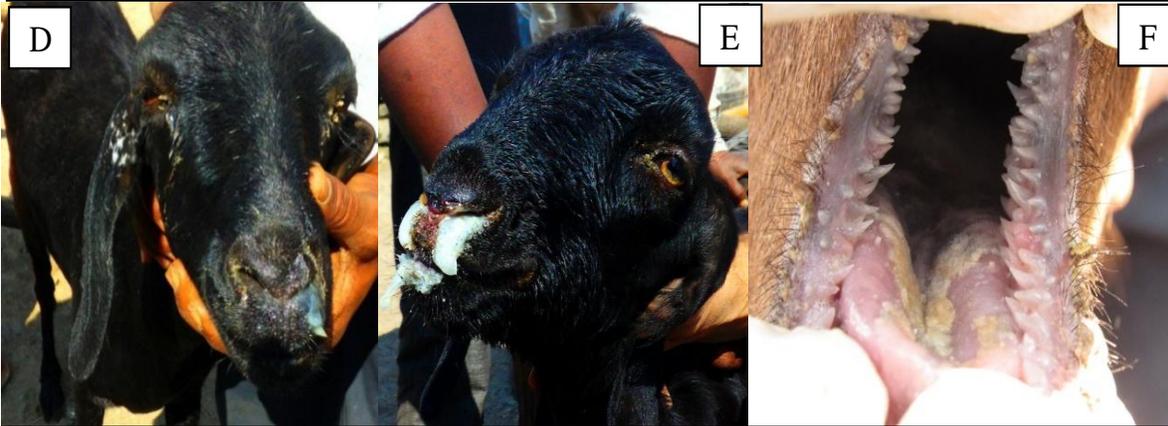


Fig 2.Moderate form of PPR:Mucous nasal and ocular discharge (D & E),congestion of mucous membrane, ulcers and bran like depots in the mouth (F)



Fig.3. Severe form of PPR: Mucopurulent nasal and oral discharge (G), sloughing of oral mucosa (H), formation of crust in and around nostril and eyes (I)

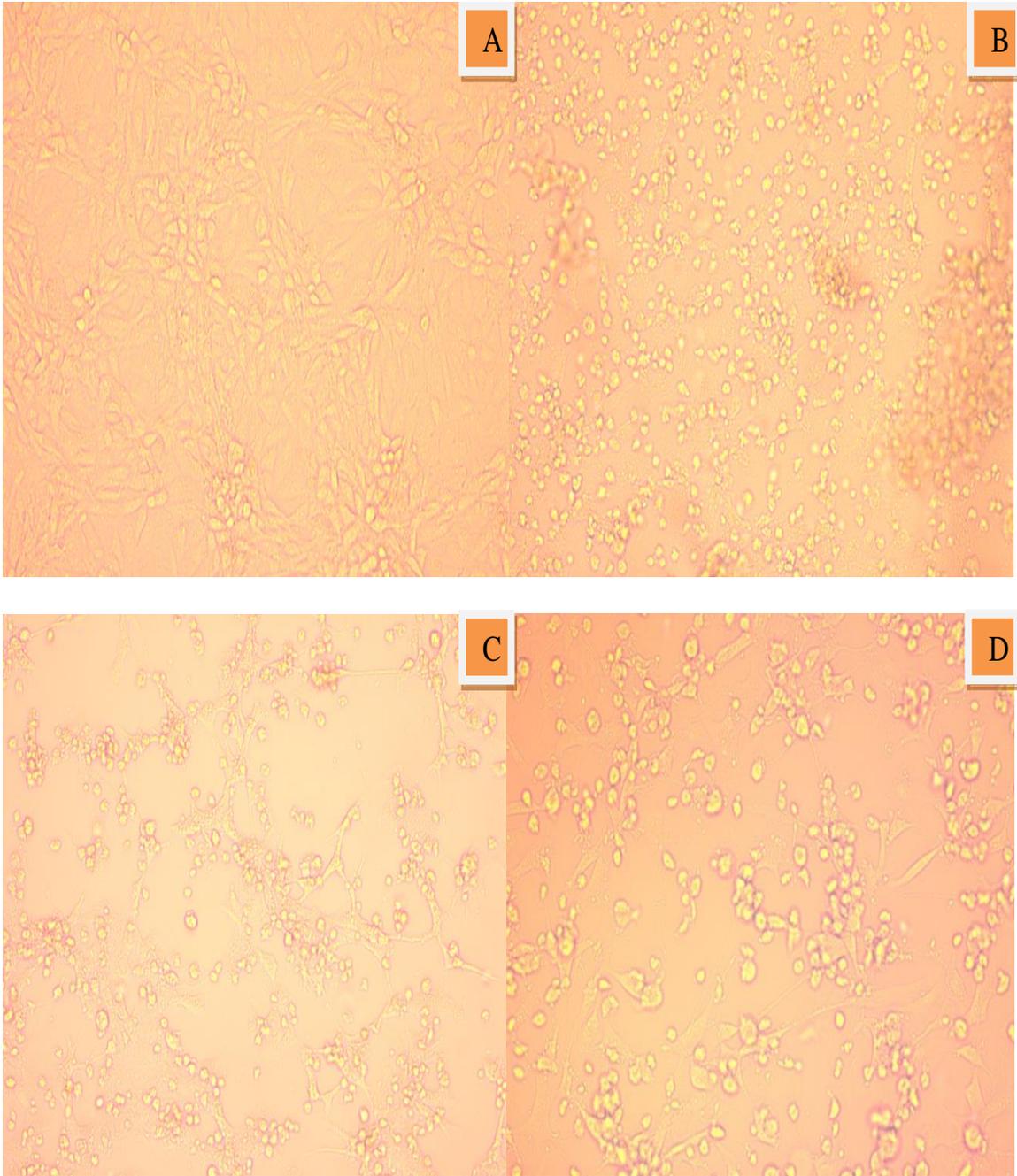


Fig.4. Pattern of CPE produced by field isolates **A.** Healthy Vero Cells (10X) **B.** Rounding and ballooning of cells detachment from surface, later on aggregation of cells followed by fusion mass (10X) **C.** Rounding and ballooning, clumping into grape like clusters increased refractivity and appearance of fine spindle cells with elongated processes (10X) **D.** Similar to C but of different isolate under 15X magnification

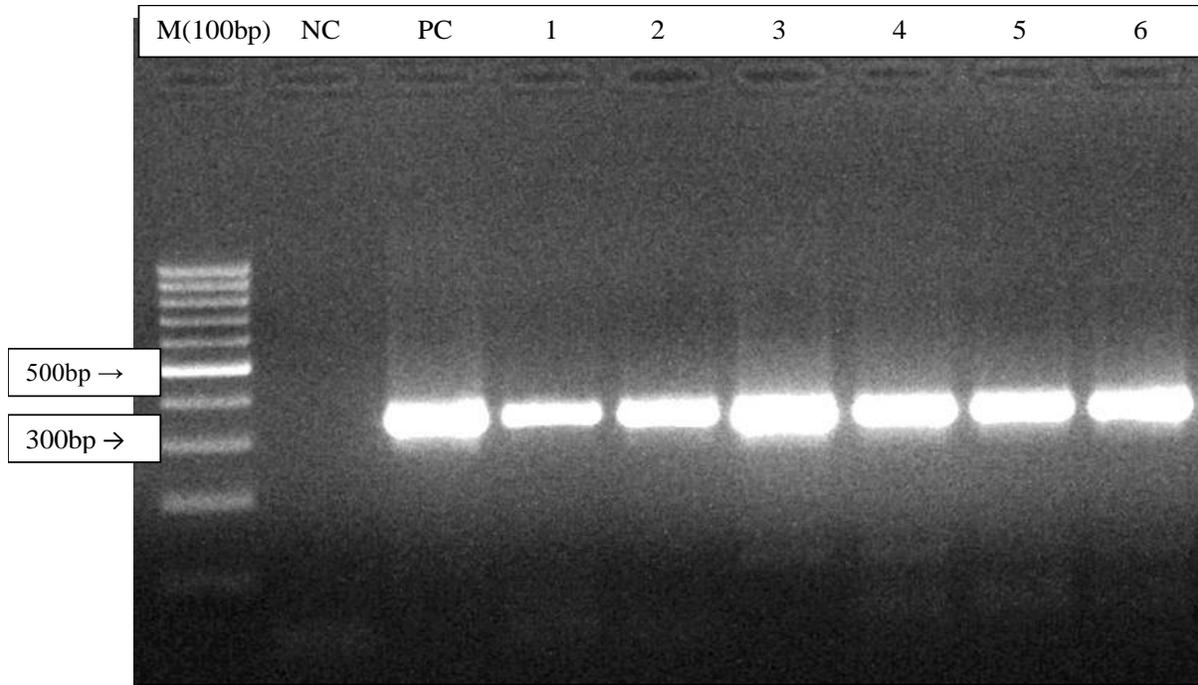


Fig: 5. PCR detection of PPR with primer set NP3&NP4(Amplicon size-351bp) Lane M- Marker, Lane NC- Negative control, Lane PC - Positive control, Lane 1- Sample 1, Lane 2- Sample 2, Lane 3- Sample 3, Lane 4- Sample 4, Lane 5-Sample 5, Lane 6-Sample 6

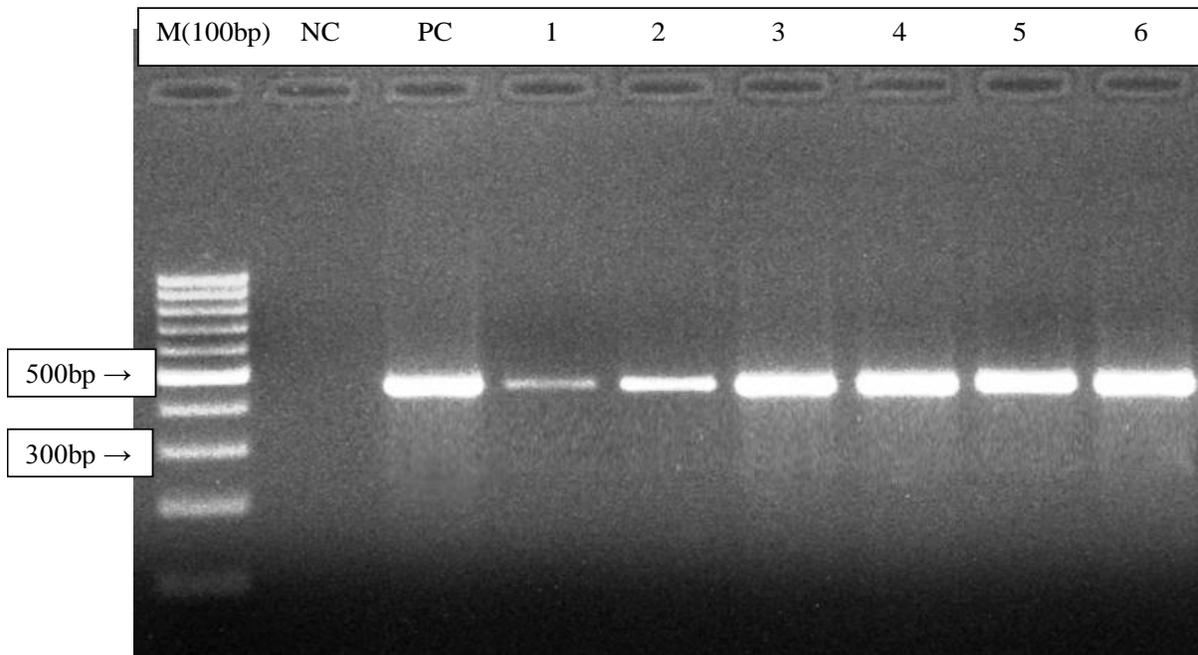


Fig: 6. PCR detection of PPR with primer set N1 & N2 (Amplicon size-463bp) Lane M- Marker, Lane NC- Negative control, Lane PC - Positive control, Lane 1- Sample 1, Lane 2- Sample 2, Lane 3- Sample 3, Lane 4- Sample 4, Lane 5-Sample 5, Lane 6-Sample 6

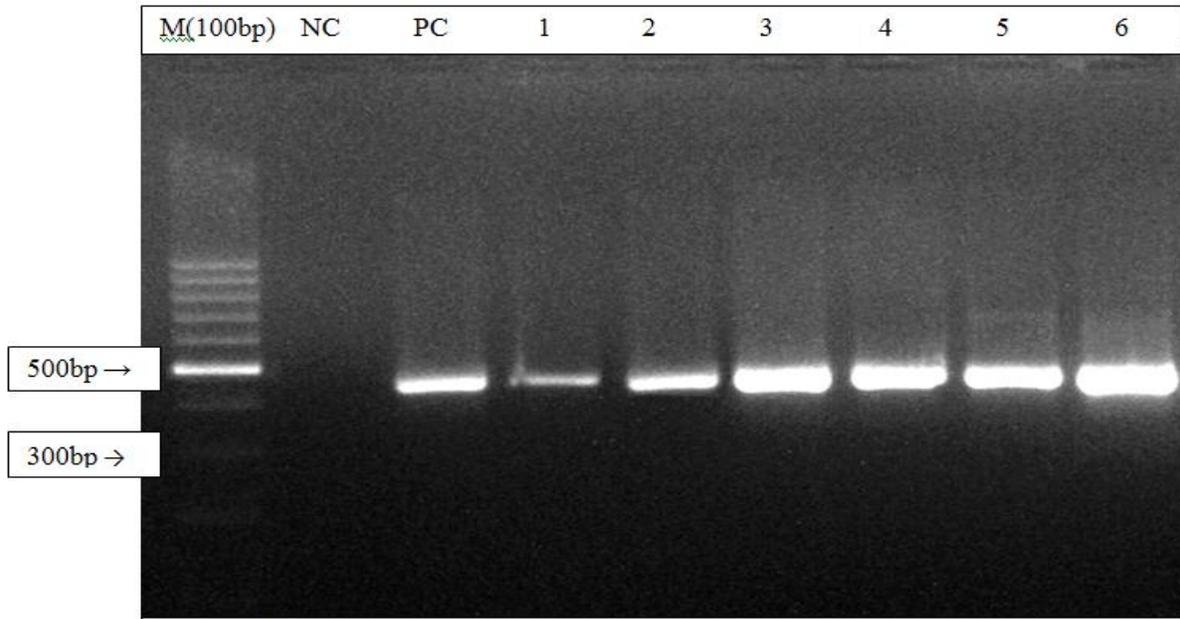


Fig: 7. PCR detection of PPR with primer set F1b & F2d (Amplicon size-447bp)
Lane M- Marker, Lane NC- Negative control, Lane PC - Positive control, Lane 1- Sample 1, Lane 2- Sample 2, Lane 3- Sample 3, Lane 4- Sample 4, Lane 5-Sample 5, Lane 6- Sample 6

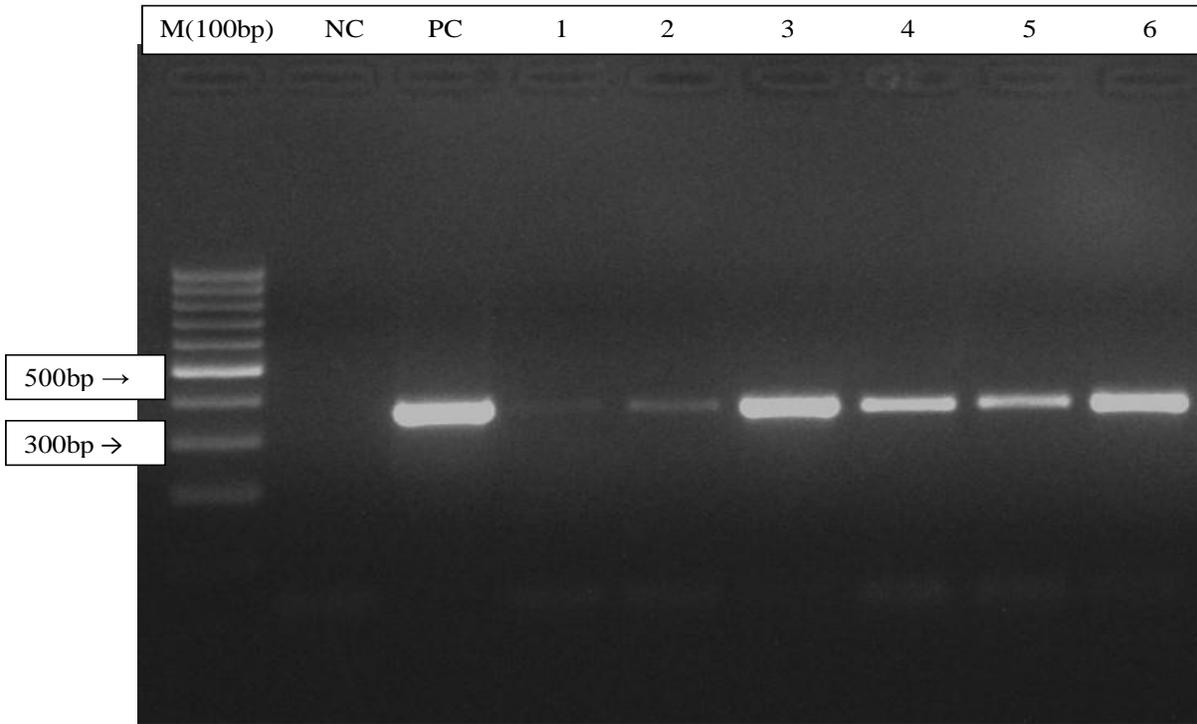


Fig: 8. PCR detection of PPR with primer set F1 & F2 (Amplicon size-371bp)
Lane M- Marker, Lane NC- Negative control, Lane PC - Positive control, Lane 1- Sample 1, Lane 2- Sample 2, Lane 3- Sample 3, Lane 4- Sample 4, Lane 5-Sample 5, Lane 6-Sample 6

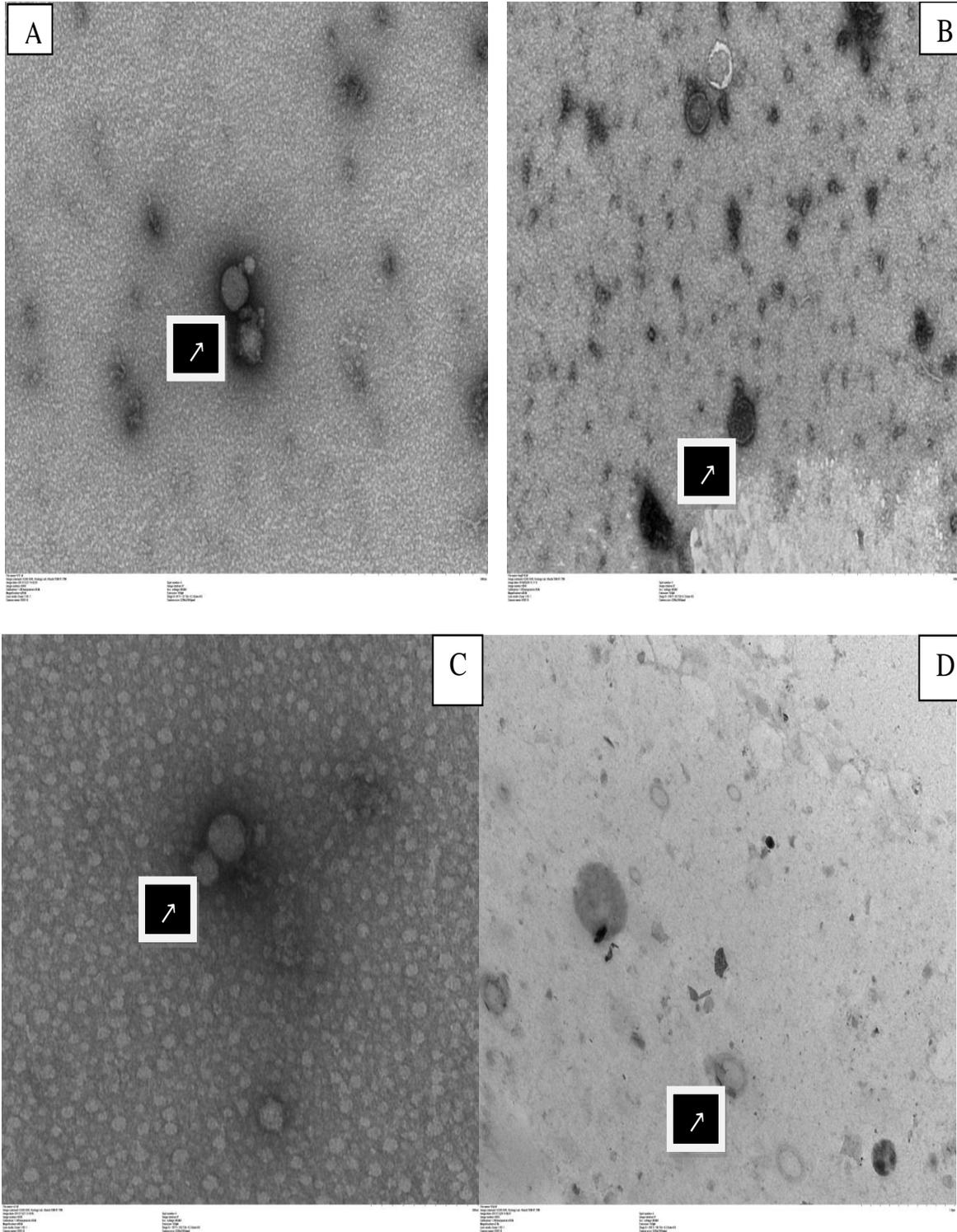


Fig.9 (A, B, C D) TEM of PPR virus: Negative staining showing pleomorphic virion particles under different magnification.(Magnifications:A-20K,B-20K C-10K and D-7K

Table.1 PPR virus specific diagnostic primer sequences

Sl No	Name of the Primer	Primer sequences	Amplicon Size	Reference
1	NP3-F	5'-GTC-TCG-GAA-ATC-GCC-TCA-CAG-ACT-3'	351 bp	COUACY-HAYMANN <i>et al.</i> , (2002)
	NP4-R	5'-CCT-CCT-CCT-GGT-CCT-CCA-GAA-TCT-3'		
2	N1-F	5'-GAT-GGT-CAG-AAG-ATC-TGCA-3'	463 bp	KERUR (2008)
	N2-R	5'-CTT-GTC-GTT-GTA-GAC-CTGA-3'		
3	F1b-F	5'-AGT-ACA-AAA-GAT-TGC-TGA-TCA-CAGT-3'	447 bp	DHAR <i>et al.</i> , (2002)
	F2d-R	5'-GGG-TCT-CGA-AGG-CTA-GGC-CCG-AATA-3'		
4	F1-F	5'-ATC-ACA-GTG-TTA-AAG-CCT-GTA-GAGG-3'	371 bp	FORSYTH and BARETT (1995)
	F2-R	5'-GAG-ACT-GAG-TTT-GTG-ACC-TAC-AAGC-3'		

Table.2 PCR Conditions

Sl. No.	Primer Name	PCR conditions			Remarks
		Temp & Time	Cycle	Step	
1	NP3&NP4	50 ⁰ C for 30 min	1	Reverse Transcription	
		95 ⁰ C for 15 min	1	Inactivates RT & activates Polymerase	
		94 ⁰ C for 30 sec	40	PCR Amplification of c-DNA	
		60 ⁰ C for 30 sec			
		72 ⁰ C for 1 min			
		72 ⁰ C for 5 min	1	Final Extension	
2	N1 & N2	50 ⁰ C for 30 min	1	Reverse Transcription	
		94 ⁰ C for 30 sec	35	PCR Amplification of c-DNA	
		50 ⁰ C for 30 sec			
		72 ⁰ C for 45 sec			
		72 ⁰ C for 10 min	1	Final Extension	
3	F1b & F2d	50 ⁰ C for 30 min	1	Reverse Transcription	
		94 ⁰ C for 2 min	1	Inactivates RT & activates Polymerase	
		94 ⁰ C for 1 min	35	PCR Amplification of c-DNA	
		55 ⁰ C for 1 min			
		72 ⁰ C for 1 min			
		72 ⁰ C for 7 min	1	Final Extension	
4	F1 & F2	94 ⁰ C for 3 min	1	Activates Polymerase	PCR product of F1b&F2d is used as template
		94 ⁰ C for 1 min	34	PCR Amplification of c-DNA	
		55 ⁰ C for 1 min			
		72 ⁰ C for 1 min			
		72 ⁰ C for 10 min	1	Final Extension	

Change in the sequences of primer target region on a viral genome due to mutations may sometime decrease the sensitivity and effectiveness of primer set and thus the ability of nucleic acid amplifications tests like PCR. The use of such tests may not able to identify certain sub-viral components such as empty virions which may be seen late in an infection and which could be identified by Electron microscopy.

Hence practical level of sensitivity of molecular and immunological tests cannot always exceed that of Electron microscopy (Ando *et al.*, 1995; Vinje *et al.*, 2000; Green *et al.*, 2002). The TEM results of our study had endorsed the confirmation of PPR virus by PCR. Hence TEM technique could also be used as a frontline diagnostic method in parallel with other diagnostic tests for diagnosis of viral infection if facility of TEM is available.

Confirmation of outbreak

Based on above results the aetiology of outbreaks were confirmed to be PPR virus indicating its circulation in the region in spite of having a very good vaccine which many investigators have justified with introduction of new animals into flocks and movement of animals from one place to other for food, playing a key role in the transmission and maintenance of PPRV in the nature.

Global target of PPR eradication can only be achieved by prompt reporting of the outbreak, rapid and accurate diagnosis and implementation of prompt control measures. Monitoring the clinical prevalence of the virus in different geographical areas of the country with varying agro-climatic conditions may be helpful for establishing disease control strategies and helps to the know the actual infection rate (Balamurugan *et al.*, 2012).

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