Original Research

Detection of Peste des petits Ruminants Infection in Goats in Assam by Reverse Transcription Polymerase Chain Reaction (RT-PCR)

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Abstract

The present investigation was undertaken to diagnose (PPR) Peste des petits ruminants, a viral disease belonging to Genus Morbillivirus and family Paramyxoviridae by molecular technique i.e., RT-PCR. These will help clinician, pathologist and scientists for better control and prevention of the disease. The tissue materials like lung, spleen, lymph node and intestine were collected in sterile containers during post mortem examination and preserved at -20°C. The samples were further processed for RNA isolation by both TRIsol method and by using commercially available RNA isolation kit. Out of 16 samples collected from natural cases, 9 samples were diagnosed positive by RT-PCR. Among the positive samples N-gene was amplified producing 365 bp amplicon size and H-gene was amplified in eight cases producing 347 bp amplicon size. The results indicated that diagnosis of Peste des petits ruminanats can be perform from necropsy material using molecular technique like PCR, which showed promising results.

Key words: H-gene, N-gene, Peste des petits ruminants, Reverse Transcription, Polymerase Chain Reaction

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Introduction

PPR (Peste des petits ruminants) is an acute or sub-acute viral disease. The disease is caused by a member of Morbillivirus genus and family Paramyxoviridae in goat and sheep. PPR is characterized by fever, necrotic stomatitis, gastroenteritis, pneumonia and death. The disease is transmitted by close contact, fomites and by all secretion and excretion of the sick animals. The acute form of PPR is accompanied by a

sudden rise in temperature to 40-41°C, with congested mucous membrane, lack of appetite, erosive lesion in the buccal cavity and crust formation in the muco-cutaneous junction. Morbidity and mortality is high in young animals than in adults (Chauhan *et al*., 2011). India has the second largest population of goat in the world. Goat rearing is an inseparable component of mixed farming system and is practiced by the rural people in developing countries. Due to low investment and high feed conversion efficiency, goats play an important role in income generation, capital storage, employment generation and improving household nutrition. Goats are reared by men and women with diverse background. Goats are considered to be the main meat producing animals in India (FAOSTAT, 2008 and Chakrabarty *et al*., 2014). As Assam is an agranian state, where goat is reared mostly in the rural society and mortality due to PPR outbreaks leads to heavy economic loss.

RT-PCR is a technique which is commonly used to detect RNA expression. The technique is used for qualitative detection of gene expression through production of complementary DNA transcripts from RNA. The technique is used to clone expressed genes by reverse transcribing the RNA of interest into its DNA complement through the use of reverse transcriptase. The process works on exponential amplification via reverse transcription polymerase chain reaction provides for a highly sensitive technique in which a very low copy number of RNA molecules can be detected (Freeman *et al*., 1999). Considering the importance of goat rearing in rural Assam with devastating effect of Peste des petits ruminant's outbreaks in goat population and efficiency of RT-PCR in diagnosis of infectious disease, the present study was made, where both natural suspected cases of PPR and experimentally produced cases of PPR was screened.

Materials and Methods

Animals died of suspected PPR and as well as experimental model of PPR were subjected for detailed post mortem examination and suitable samples like lung, mesenteric lymph nodes, tongue, spleen and intestine were collected in sterile vial without adding any preservative for confirmation of PPR by performing PCR.

Preparation of Tissue Sample

The collected tissue samples were homogenised in 1x PBS and subsequently centrifuged at 4000 rpm for 15 minutes. Supernatant fluid was subjected to RNA extraction by TRIsol reagent.

RNA Isolation from Tissue Samples using TRIsol Reagent

250 µl of supernatant was mixed with 750 µl TRIsol reagent in 1.5 ml eppendorf tube and mixed vigorously by vortexing.

1. 200 µl of chloroform was added to the homogenised lysate and kept at room temperature for 15 min and vortexed.
2. The mixture was then centrifuged at 12000 rpm for 15 min at 4^{0}C.

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3. The supernatant was collected carefully in 1.5 ml vial.
4. 600 µl of isopropanol (chilled) was added to the collected supernatant and kept at -20^0 C for 30 min to allow precipitation of RNA.
5. The precipitated RNA was centrifuged at 12000 rpm for 15 min at 4^0 C in a refrigerated centrifuge.
6. The supernatant was discarded and the pellet was washed with 70% ethanol and air- dried.
7. The RNA pellet was dissolved in 50 µl nuclease free water by keeping at 55-60^0 C in water bath.
8. Finally it was labelled properly and stored at -80^0 C till further use.

RNA Isolation using QIAmp Viral RNA kits-Qiagen

RNA was extracted using commercially available RNA extraction kits (QIAmp viral RNA Kits-Qiagen) following the standard procedure recommended by the manufacturer. The extracted purified RNA was stored at -80^0 C till further use.

Reverse Transcription Polymerase Chain Reaction

For Preparation of Complementary DNA

RNA isolated was reverse transcribed with random hexamer primer using reverse transcriptase as per the following reaction-

Component	Amount
Random Hexamer primer	1.0 µl
RNA	9.0 µl
Total volume	**10.0 µl**

The reaction mixture was incubated at 65^0 C for 5 minutes and snap cooled on ice for 5 minutes. The following reagents were added to the mixture keeping the tube in ice-

Component	Amount
5X RT Buffer	4.0 µl
10mM dNTPs	1.0 µl
MMLV-RT (200U)	1.0 µl
Nuclease free water	4.0 µl
Total volume	**10.0 µl**

The reaction mixture was mixed thoroughly and carefully by pipetting for 5 seconds. Then the tube was put in thermal cycler with the following temperature-

1. Primary annealing at 25^0 C for 5 minutes.
2. For extension at 42^0 C for 1 hour.
3. Inactivation of enzymes at 70^0 C for 10 minutes.
4. The final holding at 4^0 C.

The cDNA prepared was labelled properly and stored at -20^0 C for further use.

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Polymerase Chain Reaction

The cDNA samples were screened for PPRV based on H and N region of this virus by PCR. The sequence of the primers and thermal condition were maintained in the Table-

Detail Sequence of H and N Gene Primers

Gene	Primer sequence	Expected amplicon size
H gene	F- TGT CAT GTT CTT ATA GAG TT	347 bp
	R- GAC TGG ATT ACA TGT TAC CT	
	Balamurugan (2006).	
N gene	F- ACA GGC GCA GGT TTC ATT CTT	365 bp
	R- TGA TTT GGA CGG AGG GTG	
	(Saravanan,2010)	

Enzymes

Dream Taq Green, PCR Master Mix (2X), Thermoscientific

The above reaction mixture was mixed thoroughly and then placed in a thermocycler for amplification of H and N gene using specific primer. The amplification condition are as follows-

Step 1: Initial Denaturation	2.00 min	94^0C
Step 2: Denaturation	30 sec	94^0C
Annealing	30 sec	55^0C
Extension	30 sec	72^0C
Step 3: Final Extension	10.00 min	72^0C

Visualization and Confirmation of PCR Product

To confirm the targeted PCR amplicons, 5 µl of PCR amplicons from each tube were mixed with 1 µl of 6X loading dye and then subjected to electrophoresis in 1.5% agarose gel containing 10 mg/ml concentrated Ethidium bromide as per the following procedure.

1. 1.5% molten agarose was prepared from 1X TAE buffer in microwave oven by boiling.
2. Molten agarose was then allowed to cool to 45^0 C and then Ethidium bromide was added.
3. Molten gel was poured in to a gel holder of horizontal electrophoresis apparatus and comb was inserted.
4. The gel was allowed to solidify at room temperature for 30 minutes.
5. The electrophoresis tank was then filled with 1X TAE buffer containing 0.5% ethidium bromide.
6. The gel holder then fitted in to the electrophoresis apparatus and comb was removed leaving the well in the gel, submerged in the buffer.
7. The sample was prepared on parafilm by mixing 2 µl of PCR amplicons and 1 µl of 6X loading dye.
8. 100 bp DNA ladder (Gene Ruler 100 bp Plus, Thermo Scientific) was run parallel to the samples loaded in the wells.
9. Electrophoresis was carried out at 80 volts for 60 minutes.
10. The gel was viewed under gel documentation system (UVITEC Cambridge, GeNei™) and the image was captured under UV mode.

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Result and Discussion

A total of 16 natural cases was screened by RT-PCR, of which nine samples were confirmed to be PPR. All the samples were subjected specifically to N gene and H gene based primers. N gene was detected in all the nine positive cases. Confirmation of N gene was done by the formation of the desired amplicon size, 365 bp (Fig.1). Similar findings were recorded by earlier workers Saravanan *et al*. (2010).

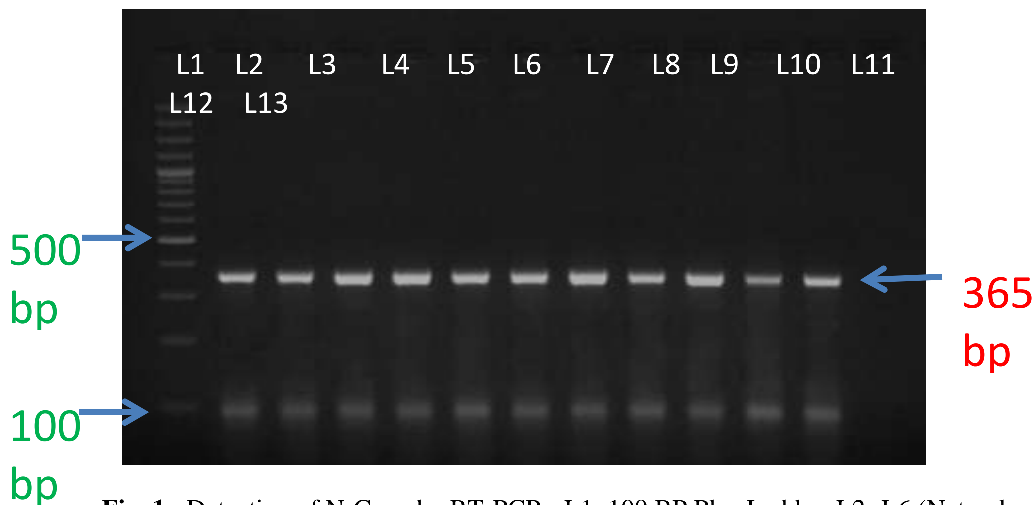

Fig. 1: Detection of N-Gene by RT-PCR: L1: 100 BP Plus Ladder; L2- L6 (Natural Cases); L7-L11: (experimental cases); L12: Positive Control; L13: Negative Control

While the H gene was detected in eight positive samples of natural cases and was confirmed by the formation of amplicon size of 347 bp (Fig. 2). This finding is in agreement with the results of previous workers (Balamurugan *et al*., 2006).

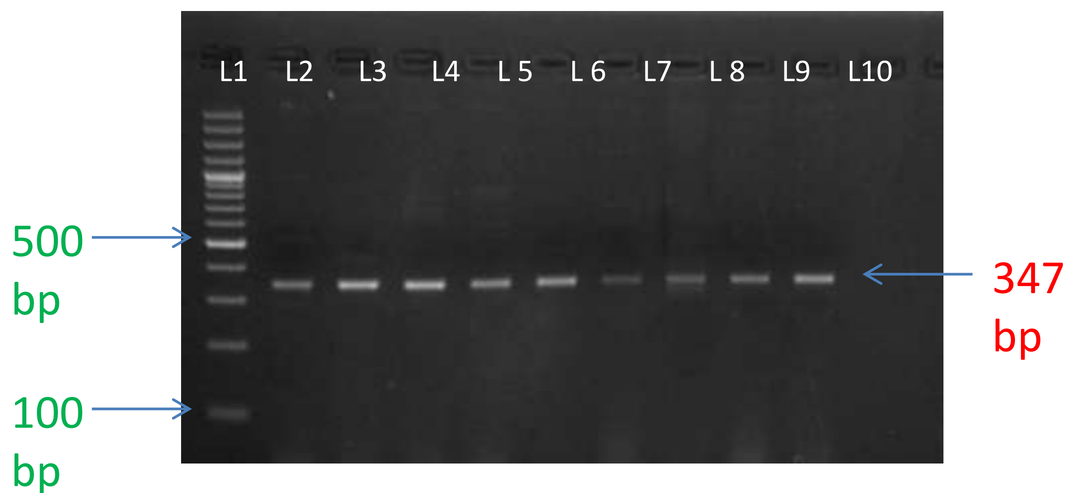

Fig. 2: Detection of H-Gene by RT-PCR, L1: 100 BP Plus Ladder; L2-L5 (Natural Cases); L6-L9 (Experimental Cases); L10: Positive Control; L11: Negative Control

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Tissue samples collected from experimentally infected animals were found to be positive by RT-PCR. Both the N-gene and H- gene were detected in all the cases. In the present study, N-gene has been detected in more number of sample as compared to H-gene. This might explain the presence of abundant nature of N-gene than the other genes (Steinhauer *et al*., 1986).Considering the abundance N gene are more sensitive nature to N-based primer than H-gene (Bhuiyan *et al*., 2012). This might be the reason for amplication and development of specific bands in more number of samples to that of H-genes.

Conclusion

From the present investigation it can be inferred that detection RT-PCR is a sensitive and reliable diagnostic technique which can be used to detect Peste des petits ruminants from necropsy tissue material .Thus based on the results of the present findings it is established that RT-PCR is an useful diagnostic method, which will be helpful in early detection of PPR outbreaks.

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