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Detection of peste des petits ruminants virus in goats in a private farm in Kottayam by real time polymerase chain reaction

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Abstract
An acute and economically significant disease, peste des petits ruminants (PPR) is caused by PPR virus (PPRV), a Small Ruminant Morbillivirus of the family Paramyxoviridae. The disease is characterised by fever, serous to mucopurulent oculo-nasal discharges, respiratory distress, cough, necrosis and erosions on oral mucosa and diarrhoea. It causes heavy mortality and morbidity among small ruminants, especially goats. The present study aims at molecular detection of N gene of PPRV from the clinical samples collected from goats showing clinical signs of PPR like ocular and nasal discharges, fever, cough and diarrhoea. Nasal swabs, faecal swabs and whole blood in EDTA vials from 15 animals were collected. The N gene of PPRV was detected in two nasal swabs when all the samples were subjected to both real time RT-PCR and conventional RT-PCR. All other samples were negative by both conventional RT-PCR and real time RT-PCR.

Keywords: peste des petits ruminants, goats, Kerala, RT-PCR, real time RT-PCR

1. Introduction
A devastating viral disease, peste des petits ruminants (PPR), affects small ruminants, especially, goat. The highly contagious nature of the disease pose a major threat to farmers by incurring severe economic loss. The disease is caused by PPR virus (PPRV), a *Small Ruminant Morbillivirus*. According to ICTV (2019), peste des petits ruminants virus (PPRV), a member of the family *Paramyxoviridae*, belongs to the order *Mononegavirales* subfamily *Orthoparamyxovirinae*, genus *Morbillivirus* and species *Small Ruminant Morbillivirus*. The disease is widespread in Africa, Arabian Peninsula, Middle East and Asia. A single serotype is detected with four distinct lineages for the virus, lineages I, II, III and IV (Wang *et al.*, 2009; Kwiatek *et al.*, 2011; Parida *et al.*, 2016) [20, 9, 13]. Cattle act as carriers of the virus without showing any clinical signs (Lembo *et al.*, 2013) [10].
The disease is characterised by fever, oculo-nasal discharges, erosions in oral mucosa, respiratory distress and diarrhoea. Post-mortem examination revealed thickening and blackening of mucosal folds of the large intestine resembling zebra stripes (Taylor, 1984) [18]. Morbidity rate can reach 90-100 per cent and mortality rate can reach 50-100 per cent.
In India, the disease was first reported in1987 in South India from Arasur village of Villupuram district of Tamil Nadu (Shaila *et al*., 1989) [17]. A tentative diagnosis can be made based on clinical signs which are often confused with those of other viral and bacterial diseases like orf, blue tongue, foot and mouth disease, contagious caprine pleuropneumonia and pasteurellosis. In laboratories, confirmation of the disease can be done by detection of the virus or the antigen, its nucleic acid or antibodies against it.
The current study involves detection of PPRV using real time RT-PCR from a private farm in Kottayam district of Kerala which is highly sensitive and specific.

2. Materials and Methods
2.1 Collection of Samples
A total of 45 samples were collected from an organised farm in Kottayam, Kerala. Out of 15 animals maintained in the farm, two were showing fever, ocular and nasal discharges and diarrhoea. From the animals, nasal swabs, faecal samples and whole blood were collected. Whole blood from jugular vein was collected in EDTA vials for the separation of buffy coat. All the samples were processed for the detection of viral RNA.

2.2 RNA extraction
The RNA was extracted from all the samples (buffy coat, nasal and faecal swabs) as well as PPR vaccine (IAH&VB, Palode) which was used as the positive control, using TRI reagent as per manufacturer's protocol. To the samples taken in microcentrifuge tubes, 750 µL of TRI reagent was added. It was mixed thoroughly and kept at room temperature for five minutes. After adding 200 µL of chloroform to the lysate, it was vigorously shaken for 15 sec. The mixture was kept at room temperature for 10 min. and then centrifuged at 12000 xg for 15 min. at 4 ºC. The upper aqueous phase was carefully transferred into a fresh microcentrifuge tube to which 500 µL of isopropanol was added. It was kept at room temperature for five minutes and then centrifuged at 12000 xg for eight minutes at 4 ºC. The RNA pellet was washed twice with one millilitre of 75 per cent ethanol. The pellet was air dried and dissolved in 50 µL of DEPC treated RNase free water.

2.3 Complementary DNA (cDNA) synthesis
The cDNA was synthesised using Revert Aid First strand cDNA synthesis kit (Thermo Scientific, USA). The reaction mixture was prepared as given in the table. It was subjected to an initial incubation at 25 ºC for five minutes followed by 42 ºC for one hour followed by 70 ºC for five minutes. The cDNA thus synthesised was stored at -80 ºC until further use.

2.4 Polymerase chain reaction (PCR)
The reaction mixture was prepared as per table 2. The PCR was conducted using specific primers targeting the N gene of PPRV (forward primer-5' TCTCGGAAATCGCCTCACAGACTG - 3' and reverse primer- 5' CCTCCTCCTGGTCCTCCAGAATCT -3'). Amplification was done as per table 3. The PCR products were detected by submarine electrophoresis in 1.5 per cent agarose gel in 1X TBE buffer. The gel was analysed in a gel documentation system (BIORAD).

Table 1: Reaction mixture

Sl. No.	Item	Volume (µL)
1	Template RNA (500 ng/µL)	6.0
2	Random hexamer primer	1.0
3	5X reaction buffer	4.0
4	Ribolock RNase inhibitor	1.0
5	dNTP mix (10m*M*)	2.0
6	Revert Aid M-MuLV RT (200U/µL)	1.0
7	Water (Upto)	5.0
	Total	20.0

Table 2: PCR mixture

Components	Quantity (µL)
PCR master mix (2X, Thermo scientific)	6.25
DNA template	2.5
Primer: F(10 p*M*/µL)	0.5
Primer: R(10p*M*/µL)	0.5
PCR Grade Water	2.75
Total	12.5

Table 3: Amplification cycle for N gene

Step	Temperature	Time	No. of cycles
Initial denaturation	95 ºC	3 min.	1
Denaturation	95 ºC	2 min.	30
Annealing	70.5 ºC	30 sec.	
Extension	72 ºC	1 min.	
Final extension	72 ºC	7 min.	1

2.5 Real-time RT-PCR
All the samples were subjected to real time SYBr based RT-PCR using animal PPR detection kit (HiMedia). The reaction mix was prepared and real time RT-PCR was done according to the manufacturer's protocol. The mixture contained 5 µl of Hi-SYBr master mix, 0.5 µl each of one step RT enzyme mix and PPR primer mix, 3 µl of template RNA or negative control and made upto 10 µl using molecular biology grade water. The recommended PCR program include an initial denaturation at a temperature of 95 ºC for 10 min. It was then followed by denaturation at 95 ºC for 15 sec.
Annealing was done at a temperature of 60 ºC for 30 sec. Finally, extension was carried out at 72 ºC for 30 sec. Melt curve analysis was done at 95 ºC for 15 sec. 60 ºC for 1 min. and 95 ºC for 15 sec.

3. Results and Discussion
The pathogens associated with respiratory infections in small ruminants include PPRV, capripox virus, *Pasteurella multocida* and *Mycoplasma capricolum* ssp. *capripneumonia*. The clinical manifestations are often similar. In the present study, the affected animals showed fever, cough, purulent oculo-nasal discharges and diarrhoea (Fig. 1). Similar symptoms were reported by Aruni *et al*. (1998) [3] in goats with PPR. Babiuk *et al*. (2008) [4] reported symptoms of fever, pox lesions on skin and mucous membrane, conjunctivitis and respiratory distress in case of capripox infection. Prominent respiratory symptoms were also reported in case of pasteurellosis and contagious caprine pleuropneumonia (Thiaucourt and Bolske, 1996; Odugbo *et al.*, 2006) [19, 12]. There are also reports of mixed infection with PPR and orf (Saravanan *et al*., 2007) [16]. Due to the similarity in the symptoms caused by these agents, it is often difficult to differentially diagnose the disease from clinical signs alone. A tentative diagnosis can be made but laboratory diagnosis is mandatory to confirm the disease.

Fig 1: Animal showing purulent nasal discharge

Isolation of the virus is considered as the gold standard test for diagnosis of PPR (Saeed *et al.*, 2004) [15]. However, several factors such as poor quality of the samples, less amount of the virus as well as sampling and delivery time involved may lead to failure in isolation of virus. Moreover, it was found to be more labourious and time consuming making them unsuitable for routine diagnosis.
According to several studies, RT-PCR was found to be more sensitive than virus isolation techniques (Forsyth and Barrett, 1995; Brindha *et al.*, 2001; Couacy-Hyman *et al.*, 2002) [7, 5, 6].

The type of samples collected is critical in accurate molecular diagnosis. Luka *et al*. (2012) [11] reported that the viral nucleic acid can be detected in oculo-nasal swabs and buffy coats and these samples are used in endemic areas due to the ease of collection. According to Forsyth and Barrett (1995) [7], oculo-nasal swabs and tissues yielded positive results on RT-PCR. However, in the present study, out of 15 nasal swabs tested, 2 were found to be positive for N gene of PPRV. The nasal swabs revealed amplicons of 351 bp on RT-PCR (Fig. 2) and all the buffy coat samples were found to be negative.

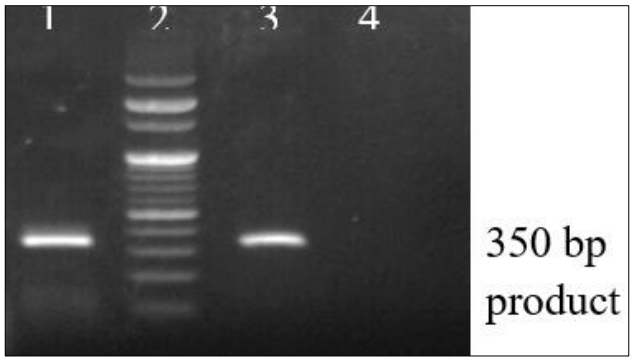

Fig 2: Amplification of N gene of PPR (lane 1- positive control, lane 2- 100bp ladder, lane 3- positive sample and lane 4- negative sample)

Parida *et al*. (2019) [14] reported that nasal swab was considered as an appropriate sample to be collected for molecular studies since it can detect the nucleic acid in both early and later stages of disease. In our present study, no viral nucleic acid could be detected in faecal samples. It may be due to the fact that the viral RNA can be detected in faeces in later phase of infection and may be absent early during the infection (Parida *et al*., 2019) [14].

Several other studies also reported more positivity rates from nasal swabs and tissues (Albayrak and Alkan, 2009; Gurcay *et al.*, 2013) [2, 8]. They also reported RT-PCR as a sensitive and reliable diagnostic technique.

Abera *et al.* (2014) [1] developed a real-time RT-PCR assay that used SYBR Green and it was found to be more sensitive and specific technique for the rapid detection and quantification of PPRV nucleic acid. This is more easy and less time consuming than conventional RT-PCR since the additional steps like gel preparation and electrophoresis can be avoided. In the present study, two samples were found to be positive for N gene in RT-PCR and also in real-time RT-PCR. All the samples positive by conventional RT-PCR were also found to be positive in real-time RT-PCR. No additional positives could be detected using the real time RT-PCR.

As per the previous studies, more sensitivity was reported in case of real time RT-PCR assay than conventional RT-PCR, since additional positive results could be detected from the clinical samples (Abera *et al.*, 2014) [1]. But, in the present study, real time RT-PCR was found as sensitive as conventional RT-PCR. This may be due to limited sample size. However, real-time RT-PCR is preferred over the conventional technique as it is found to give results faster and also consumes less amount of reagents.

4. Conclusion

Since, the clinical signs of PPR are confused with that of other bacterial and viral diseases, confirmatory diagnosis can be made using molecular techniques. Real time RT-PCR is more rapid and preferred over RT-PCR. Early and rapid diagnosis of the disease helps to minimise the economic loss of farmers.

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