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Detection and confirmation of PPR virus antigen in sheep and goats by sandwich-ELISA and RT-PCR in Andhra Pradesh, India

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ABSTRACT

Peste des petits ruminants (PPR) is a highly contagious disease of domestic and wild small ruminants. Rapid and accurate laboratory assay are essential to enable the implementation of appropriate control strategies to restrict the spread of PPR. The present study was designed to detect the PPR virus (PPRV) antigen (*N*-gene) in nasal swabs and tissue samples. A total of 195 samples comprising of 138 nasal swabs from PPR suspected sheep (n=72) and goats (n=66), and 57 tissue samples comprising of lymph nodes from dead sheep (n=39) and goats (n=18) were collected from certain parts of Andhra Pradesh. The samples were subjected to sandwich-ELISA followed by RT-PCR for confirmatory diagnosis. In this study, PPRV could be detected in 27.53% (n=38/138) nasal swabs and 49.12% (n=28/57) tissue samples. Data showed that PPRV infection is widespread in the Andhra Pradesh, India.

Keywords

Goat, *N*-gene, RT-PCR, sandwich-ELISA, Sheep

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INTRODUCTION

Peste des petits ruminants (PPR) is an acute highly contagious viral disease of sheep and goats. The occurrence of PPR is associated with high morbidity and mortality in susceptible animals. The disease was first described in the Ivory Coast, West Africa (Gargadennec and Lalanne, 1942), and later from sub-Saharan Africa, the Arabian Peninsula, the Middle East, southwest Asia, and other countries. In India, PPR was first reported from Arasur, Villupuram district (Tamilnadu State) during 1987 (Shaila et al., 1989). Right now, PPR is enzootic in India causing significant economic losses, and therefore, the infection is a major constraint for small animal production.

The causative agent, PPR virus (PPRV) is a member of the genus Morbillivirus of the family Paramyxoviridae. The genome is a single-stranded negative sense RNA, and is approximately 16-kb in size. PPRV is classified into four lineages (I, II, III, and IV) based on partial F gene sequence analysis. The present study was conducted to detect the PPRV antigen (*N*-gene) in small ruminants of Andhra Pradesh, India.

MATERIALS AND METHODS

Nasal swabs (n=138) collected from forty two unvaccinated flocks from various places of Andhra Pradesh, India were used for detecting PPR antigen. The swabs were collected from sheep and goats which were showing clinical signs of PPR. Besides, tissue samples (n=55) mostly lymph nodes were collected from sheep and goats died during suspected PPR outbreaks. The tissue materials were triturated using pestle and mortar using PBS to prepare 10-20% suspension, and kept at -20^{0}C until used. Contents of nasal swabs were extracted thoroughly in 500 µL of PBS and stored at -20^{0}C until used. Sandwich-ELISA kit for PPRV antigen detection manufactured from Rinderpest Laboratory, Division of Virology, Indian Veterinary Research Institute (IVRI), Mukteswar, India was used. The lymph node tissue and nasal swabs which were found to be positive by sandwich-ELISA were used as a source of antigen for the detection of PPR by RT-PCR. One reference vaccine virus (sungri) and a nasal swab from an apparently healthy animal

were taken as positive and negative controls, respectively.

A 100 µL of diluted capture antibody (1:4000) was dispensed in all the wells of ELISA plate covered the plate and incubated for 1 h at 37^{0}C with continuous shaking on orbital shaker. Then, the plate was washed three times with wash buffer and dried. After washing, 50 µL of blocking buffer was added in all the wells, 50 µL of additional blocking buffer to antigen blank (B) wells (A1-H1), 50 µl of clinical samples in vertical duplicates as per the template provided with the kit (A3/B3, C3/D3 and so on), 50 µL of positive reference (C+) antigen in four designated wells (A2/B2, C2/D2), 50 µL of negative reference (C-) antigen in four designated wells (E2/F2, G2/H2) were added, and incubated for 1 h on an orbital shaker.

After washing, diluted detection antibody (100 µL) was added in all the wells and incubated for 1 h on an orbital shaker. After second washing, diluted (1:1000) anti-mouse conjugate (100 µL) was added in all the wells and incubated for 1 h on an orbital shaker. After third washing, a freshly prepared OPD substrate solution (100 µL) was added in each well and the plate was incubated for 10 to 20 min at 37^{0}C without shaking or till the color was developed in positive reference (C+) wells. Stopping solution (100 µL) was added to all the wells and the plate was gently tapped to mix the fluids. The plate was read in an ELISA plate reader at 492 nm.

Detection of PPRV by RT-PCR

Primes: Oligonucleotide primers were obtained from Eurofins Genomics India Pvt. Ltd. (formerly known as MWG Biotech Pvt. Ltd.) Bangalore, India.

RNA extraction and cDNA Synthesis (Reverse Transcription): RNA extraction was carried out by single step method described by Chomczynski and Sacchi (1987) with trizol reagent (invitrogen). The forward and reverse primers used were: PPR-NP3a 5'-CT CGG AAA TCG CCT CGC AGG CTG-3' and PPR-NP4 5'-CCT CCT CCT GGT CCT CCA GAA TCT-3', respectively, as described by Balamurugan et al. (2012). The RNA isolated from positive clinical samples and vaccine virus were reverse-transcribed using the primers.

Polymerase chain reaction (PCR) of *N*-gene: The synthesised cDNA was used to amplify the *N*-gene of PPRV by PCR. For 25 µL master mix, the concentration of reagents were 10XPCR Buffer 2.5 µL, 50 mM $MgCl_2$ 1.0 µL, 10 mM dNTPs 0.5 µL, forward primer (10 pMole/µL) 0.5 µL, reverse primer (10 pMole/µL) 0.5 µL, cDNA 2.0 µL, *Taq* DNA Polymerase 1.0 µL, and nuclease free water 17.0 µL. The total volume of the reaction mixture was made as 25 µL using nuclease free water. Then the tubes were spinned for 10 sec and PCR was carried out in an eppendorf Master cycler. The reaction conditions were standardized for the amplification of 351-bp of *N*-gene are as follows: an initial denaturation step of 4 min at 94^{0}C, followed by 32 cycles, each of denaturation for 30 sec at 94^{0}C, primer annealing for 30 sec at 55^{0}C, and primer extension for 30 sec at 72^{0}C. A final elongation step was carried out for 10 min at 72^{0}C. Negative control (no template) was run simultaneously, and the reaction mixtures were discarded when any DNA appeared in the control. A 10 µL of the reaction mixture was examined in 1.5% agarose gel electrophoresis, followed by staining with ethidium bromide, to check the size of amplified fragments by comparison to a DNA molecular weight marker.

RESULTS AND DISCUSSION

Peste des petits ruminants are the French nomenclature for this disease of small ruminants, which recalls "peste bovine" (rinderpest), is indicative of its clinical resemblens to rinderpest. It was also known as stomatitis pneumoenteritis complex.

In the present study, out of 57 total tissue samples, the PPR antigen could be detected in 49.12% (n=28/57) samples. Of the total positives, 48.71% (19/28) were of sheep, and 50% (n=9/28) were of goats (**Table 1**). The present findings are in accordance with reports of Abubakar et al. (2008). Rao et al. (1998, 2001) confirmed the outbreaks of PPR by sandwich-ELISA at different locations in Andhra Pradesh. However, as an additional, we exmployed RT-PCR for confirmation.

Table 1: Species wise prevalence of PPR virus antigen in tissues.

Species	Tissues tested (N)	Positive (%)
Sheep	39	19 (48.71)
Goat	18	9 (50.00)
Total	57	28 (49.12)

Table 2: Species wise prevalence of PPR virus antigen in nasal swabs.

Species	Swabs tested (N)	Positive (%)
Sheep	72	18 (25.00)
Goat	66	20 (30.33)
Total	138	38 (27.53)

Out of 138 nasal swabs tested by sandwich-ELISA, the PPR antigen could be detected in 27.53% (n=38/138)

samples. Of the 38 positive cases, 25% (n=18/38) nasal swabs belonged to sheep, and 30.33% (n=20/38) nasal swabs belonged to goats (**Table 2**). The present findings are in agreement with observations of Kumar et al. (2002) in India.

In the present study, RT-PCR targeting *N*-gene was used for the confirmation of PPR. Recently, RT-PCR has emerged as highly specific and sensitive test for molecular characterization of the virus. It has become the most popular tool for diagnosis as well as molecular epidemiological studies (Shaila et al., 1996). Among the structural proteins, N protein is antigenically most conserved among morbilliviruses, and is highly immunogenic inspite of its internal location (Libeau et al., 1995). So, in the present investigation, *N*-gene was targeted for the detection of PPRV.

In the present study, the clinical samples which were positive by sandwich-ELISA were subjected to RT-PCR, and positive amplification was found for both the samples and reference vaccine virus. Reference vaccine virus as well as sandwich-ELISA positive samples, including lymph node and nasal swab, yielded an approximately 351-bp amplicons which was specific to the primers used, as described by Couacy-Hymann et al. (2002).

CONCLUSION

The present investigation reveals no difference in the severity of PPRV infection in sheep and goats. Molecular analysis of the virus isolates originating from the same geographical region and involving both sheep and goats is necessary to detect differences at the genomic level.

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