

# Evidence of mixed infection of peste des petits ruminants virus and bluetongue virus in a flock of goats as confirmed by detection of antigen, antibody and nucleic acid of both the viruses

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**Abstract** A mixed infection with peste des petits ruminants virus (PPRV) and bluetongue virus (BTV) occurred in goats which exhibited symptoms characteristic of PPR. A number of samples were collected from ailing or dead goats for laboratory diagnosis. Antibody to BTV and PPRV was detected in sera samples by competitive ELISA. No PPRV antigen was detected in tissue samples like lung and spleen, however, presence of PPRV antigen in some sera samples was confirmed by sandwich ELISA. All the blood samples collected from the ailing animals were found positive for BTV antigen by a sandwich ELISA. BTV- and PPRV nucleic acids were amplified from the pooled blood and tissue samples respectively by RT-PCR assays. The identity of the amplicons was confirmed by cloning and sequencing. All these tests confirm that the goats were infected with PPRV and BTV simultaneously. Isolation of viruses from the clinical samples is underway.

**Keywords** Peste des petits ruminants virus · Bluetongue virus · Mixed infection · ELISA · RT-PCR

## Introduction

Peste des petits ruminants (PPR) is a highly contagious viral disease of sheep and goats which is caused by the *peste des petits ruminants virus* (PPRV). The virus has been classified in the genus *Morbillivirus* within the family *Paramyxoviridae* (Van Regenmortel et al. 2000). PPR is endemic in India (Shaila et al. 1996; Dhar et al. 2002) and regular outbreaks in small ruminants occur throughout the country (Aruni et al. 1998; Kulkarni et al. 1996) since its first report from the Southern peninsula in late 1980s (Shaila et al. 1989).

Bluetongue (BT) is an arthropod-borne viral disease primarily of sheep caused by the bluetongue virus (BTV), which is the prototype species of the genus *Orbivirus* in the *Reoviridae* family (Mertens et al. 2004). BT is endemic in India. The first outbreak of BT in India was reported in the early 1960s and thereafter regular outbreaks have been recorded in different parts of the country (Jain et al. 1986; Mehrotra et al. 1996; Sreenivasulu et al. 1999; Aruni et al. 2006; Ilango 2006). The virus can infect most species of domestic and wild ruminants such as goat, cattle, buffalo, camel, deer and other artiodactyls (McLachlan 1994). BTV infection in goat is asymptomatic and infected goats serve as reservoir of virus.

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Seroprevalence of BTV has been reported in goats from many parts of India (Sreenivasulu et al. 2004; Ravishankar et al. 2005).

Mixed infections with PPRV and BTV or PPRV and orf virus in small ruminants are occasionally seen in India, but in most cases mixed infections remain undiagnosed or diagnosed as a disease caused by a single virus ignoring the other viral agents. In this paper we report a mixed infection with PPRV and BTV in goats on the basis of detection of antigen, antibody and nucleic acids of both the virus in the clinical samples submitted for diagnostic investigations.

## Materials and methods

### Field samples

A number of samples were collected from ailing or dead goats (Table 1) and sent to our laboratory under cold chain for diagnostic investigations. The flock of goats, in the district of Mathura, Uttar Pradesh, was suffering from a disease with symptoms of severe pneumonia and coughing, high body temperature (about 41.1°C), oral gession with erosive mucosa and diarrhea. Upon receiving, the samples were stored at -20°C and then examined for antigen, antibody and nucleic acid of PPRV and BTV.

### Competitive ELISA for PPRV antibody detection

For detection of PPRV antibody in goat sera, a diagnostic competitive ELISA (c-ELISA) was used as described by Singh et al. (2004a). The test uses murine monoclonal antibody to a neutralizing epitope of hemagglutinin protein of the virus. Briefly, ELISA plate (Nunc Maxisorp, Hamburg, Germany) was coated with the PPRV antigen and after washing proper blocking was done. After washing, test sera were added along with the monoclonal antibody.

Following incubation and washing, conjugate and substrate were added for color development. Absorbance was read at 492 nm and interpretation of the test results was made according to calculations mentioned in the paper.

### Sandwich ELISA for PPRV antigen detection

PPRV antigen was detected in the samples by a diagnostic sandwich ELISA (s-ELISA) developed by Singh et al. (2004b). The test uses monoclonal antibody to PPRV N protein as the detection antibody. Briefly, ELISA plate was coated with capture antibody (anti-PPRV antibody raised in rabbit). After incubation at 37°C for 1 h, plate was washed with washing buffer. The plate was then blocked with the blocking buffer and simultaneously, test samples were added to the wells and incubated at 37°C for 1 h. Plate was washed and the detection antibody (anti-PPRV N murine monoclonal antibody) was added in blocking buffer. Incubation was done at 37°C for 1 h. After washing, conjugate and substrate were added. The plate was read at 492 nm on an ELISA reader.

### Reverse transcription polymerase chain reaction (RT-PCR) for amplification of PPRV nuclei acid

The PPRV nucleic acid was detected in the clinical tissue material by RT-PCR following the method described by George et al. (2006) which ensures amplification of PPRV N gene using the primers NP1 (5'-ACA GGC GCA GGT CTC CTT CCT-3') and NP2 (5'-CAC TGA TTT CGA CAG AGG GTG-3'). These primers were designed on highly conserved sequence of PPRV N gene to amplify a 368 bp product (from nucleotide 1291 to 1658). Amplification was done on a thermal cycler under the following conditions: initial denaturation at 95°C for 3 minute, then denaturing at 94°C for 30 second, annealing at 55°C for 30 second and extension at 72°C for 30

**Table 1** List of samples collected from goats and assays performed

Sample No	Materials	Test conducted for
1-10	Sera	PPRV antibody and antigen, BTV antibody
11-13	Nasal swab	PPRV antigen
14-15	Piece of lung and spleen	PPRV antigen, nucleic acid
18-26	Blood in anticoagulant	PPRV antigen, BTV antigen and nucleic acid

second for 35 cycles. Final extension was done at 72°C for 6 min. The PCR products were checked in 1.2% agarose gel electrophoresis.

Competitive ELISA (c-ELISA) for detection of BTV group-specific antibody

BTV group-specific antibody was detected in sera by a monoclonal antibody-based commercial competitive ELISA kit (Bluetongue Antibody Test Kit, VMRD, Pullman WA, USA). The test was performed as per the instruction of the manufacturer. Test sera are positive if they produce an OD 650 nm less than 50% of the mean of the negative controls.

Sandwich ELISA for BTV antigen detection

BTV (or its group-specific antigen) was detected in the goat blood by a polyclonal antibody-based s-ELISA developed recently in our laboratory (Chand 2008, details of the test communicated elsewhere). Briefly, capture antibody (rabbit hyperimmune serum, HIS, against purified whole BTV) diluted in carbonate-bicarbonate buffer, pH 9.6 (Sigma, St. Louis, MO, USA) was coated on to wells of ELISA plate (Maxisorp) for 1 h at 37°C. Plate was washed three times with washing buffer (PBS plus 0.05% Tween 20). Blocking was done with blocking buffer (4% skimmed milk, 2% gelatin in PBS) at 37°C for 1 h. After washing, control and test antigens were added to the plate and incubated. After further washing, detection antibody (guineapig HIS against BTV core particles) was added to the plate and color reaction was developed by adding conjugate-substrate solution which was read on an ELISA plate reader at 492 nm. A sample was classified as positive when the OD was greater than twice the OD of negative control ( $P/N \geq 2$ ).

RT-PCR for amplification of BTV nuclei acid

BTV nucleic acid was amplified from the blood samples by RT-PCR described in the Manual of OIE (2004) with some modifications. The oligonucleotide primers were designed on highly conserved sequence of NS1 (RNA 6) gene of BTV (Dangler et al. 1990). The primer sequences are as follows: first stage PCR primers (to amplify RNA6 from nucleotide 11 to 284), Primer A: 5'-GTT CTC TAG TTG GCA ACC ACC-3' and Primer B: 5'-AAG CCA GAC TTG TTC

CCG AT-3'; nested PCR primers (to amplify RNA6 from nucleotide 170–270), Primer C: 5'-GCA GCA TTT TGA GAG AGC GA-3' and Primer D: 5'-CCC GAT CAT ACA TTG CTT CCT-3'.

RNA was extracted from pooled or individual blood sample. About 100 µl of blood was taken to prepare washed RBCs that was lysed by sonic vibration and centrifuged at 10000 g for 10 min. The supernatant was treated with sodium dodecyl sulfate (SDS, 0.5% final concentration) at 37°C for 30 min followed by Protinase K (200 µg/ml final concentration) treatment at 56°C for 1 h. The RNA was extracted with Tri Reagent (Sigma). The cyclic conditions in first stage PCR (with Primer A and Primer B) were: initial denaturation at 95°C for 5 min, then denaturing at 95°C for 20 second, annealing at 53°C for 20 second and extension at 72°C for 20 second for 35 cycles. Final extension was done at 72°C for 6 min. For nested PCR, 2 µl aliquot from the first PCR was used with the nested primers C and D, and the cyclic conditions were same except the annealing temperature was 58°C. The PCR products were checked in 2% agarose gel electrophoresis.

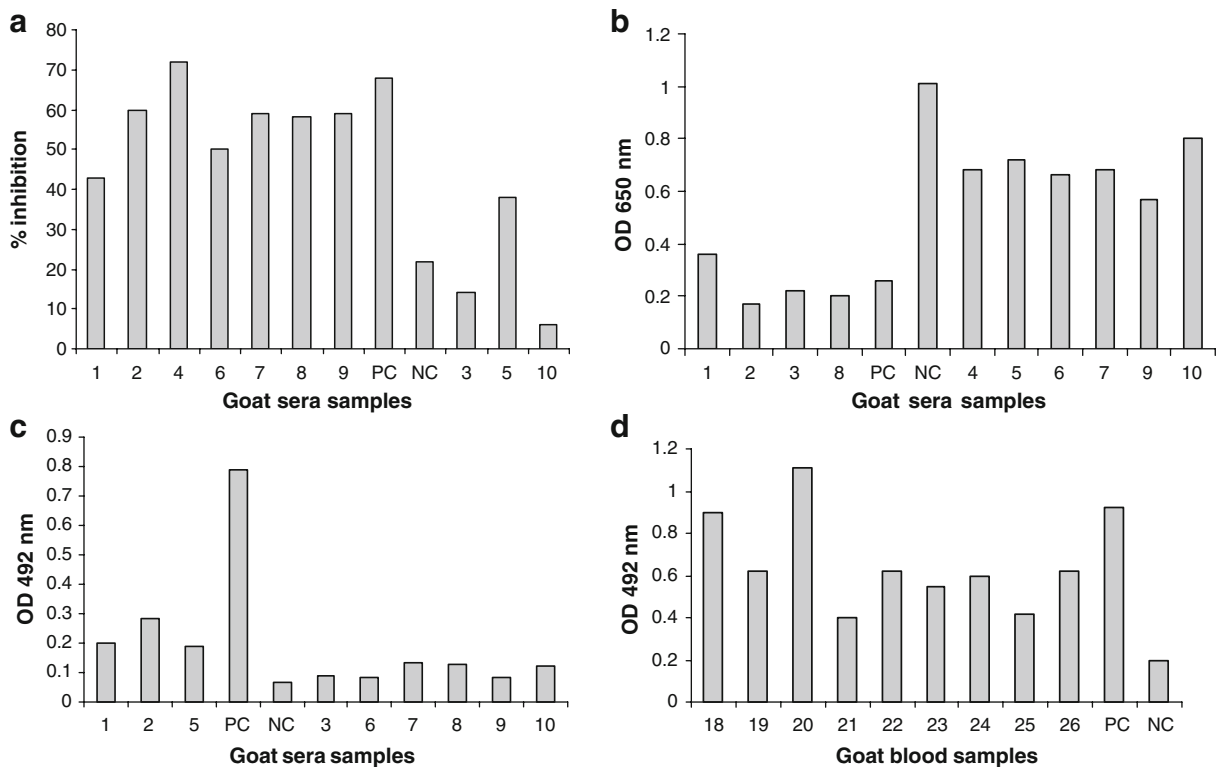
## Results

Detection of PPRV- and BTV antibody in goat sera

Antibody to PPRV was detected by c-ELISA in 7 (sera No. 1, 2, 4, 6, 7, 8 and 9) of the 10 goat sera tested and few sera (No. 2 and 4) were strongly positive. Sera No. 3 and 10 were negative and No. 5 was marginally negative (Fig. 1a). BTV group-specific antibody was detected by c-ELISA in 4 sera (No. 1, 2, 3 and 8) (Fig. 1b). An indirect ELISA based on recombinant VP7 (Pathak et al. 2008) was also used to detect BTV group-specific antibody in these sera. The test results of this assay were similar to that of c-ELISA (VMRD Kit) except in the indirect ELISA the serum No. 9 was found positive (data not shown) otherwise it was negative by c-ELISA. Three sera samples (No. 1, 2 and 8) were positive for antibody to both PPRV and BTV.

Detection of PPRV- and BTV antigen

No PPRV antigen was detected in nasal swab, lung and spleen tissue. Both sera and blood (in anticoagulant)



**Fig. 1** (a) Detection of PPRV antibody in goat sera by c-ELISA. An inhibition more than 40% was considered positive test result. Positive sera samples are shown on the left side of the positive control (PC) bar and negative samples on the right side of the negative control (NC) bar. (b) Detection of BTM group-specific antibody in goat sera by a commercial c-ELISA kit (VMRD, USA). Test sera considered positive if they produced an OD less than 50% of the mean of negative controls. Positive sera are on the left side of the positive control (PC) bar and negative samples are on the right side of the negative control (NC) bar. (c) Detection of PPRV antigen in the

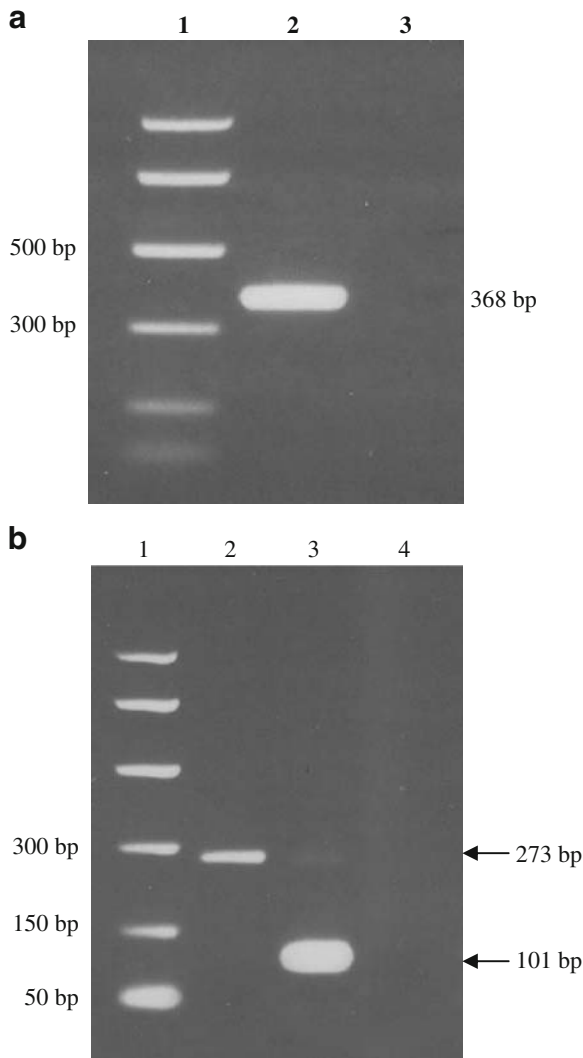
goat sera by an s-ELISA. PPRV antigen was present in three goat sera samples shown at the left side of the positive control (PC) bar. The negative samples are at the right side of the negative control (NC) bar. OD more than double the mean of the NC was considered positive. (d) Detection of BTM in the anti-coagulated goat blood samples by a polyclonal antibody-based s-ELISA. All the samples were found to contain BTM (or antigen) by the assay. Test sera considered positive if they produced an OD more than twice the mean value of the negative control (NC). PC denotes positive control

samples were examined for the presence of PPRV antigen by s-ELISA. Blood samples were negative and sera No. 1, 2 and 5 were positive for PPRV antigen (Fig. 1c). All the blood samples (No. 18–26) were found positive for BTM by s-ELISA (Fig. 1d) however, these samples were negative for PPRV antigen. The antigen- and antibody-detection assays revealed that sera No. 1 and 2 were positive for PPRV antigen and antibody, and BTM antibody.

#### Detection of PPRV and BTM nucleic acid

PPRV nucleic acid could not be amplified from sera, which were positive for antigen, however, N gene was

amplified from the pooled material of lung and spleen tissue and a product of expected size (368 bp) was observed in agarose gel electrophoresis (Fig. 2a). BTM nucleic acid was amplified from the pooled blood samples initially. PCR products of expected size were observed in the agarose gel electrophoresis both by the first PCR (273 bp) and nested PCR (101 bp) (Fig. 2b). Subsequently, individual blood samples were tested by PCR and all were found positive for BTM nucleic acid (data not shown). The PCR products (368 bp and 273 bp of PPRV and BTM respectively) were gel-purified, cloned in pGEMT Easy vector (Promega) and sequenced. Nucleotide sequences were analyzed to verify the identity of the



**Fig. 2** (a) Amplification of PPRV N from pooled tissue material of goat by RT-PCR. PCR products were resolved in 1.5% agarose gel electrophoresis. Lane 1: DNA molecular weight marker (# G316A, Progema); Lane 2: N gene PCR product (368 bp) generated with primers NP1 and NP2; Lane 3: No template control. (b) Amplification of BTV NS1 gene (RNA 6) from pooled blood sample of goat by RT-PCR. PCR products were resolved in 2% agarose gel electrophoresis. Lane 1: DNA molecular weight marker (# G316A, Progema); Lane 2: First PCR product (273 bp) generated with Primer A and B; Lane 3: Nested PCR product (101 bp) generated with nested primer set C and D; Lane 4: No template control

PCR products. Upon matching with the sequences available in the public data base it was observed that the nucleotide sequence of the 368 bp and 273 bp products are of PPRV N gene and BTV NS1 gene respectively (data not shown).

## Discussion

In the present study we report a mixed infection in goats with PPRV and BTV on the basis of detection of antigen, antibody and nucleic acid of both the viruses in clinical samples. In the same sera samples PPRV antigen and antibody, and BTV antibody have been detected which indicates that these animals had infected with both the viruses. Although the disease could readily be diagnosed as PPR by clinical signs, the etiology was confirmed by various laboratory tests performed on the field materials. The morbidity, mortality and other epidemiological data of the present outbreak are not known, but it could be assumed that goat(s) had died of the disease since morbid materials were sent for laboratory examinations, and death was, probably, due to PPR. The flock of goats sampled was not vaccinated against PPR or BT. Therefore, the seroprevalence of antibody and antigen only could have resulted from field infection with PPRV and BTV since these viruses are endemic in the northern part of India (Prasad et al. 1994; Nanda et al. 1996).

Goat is considered sub-clinical reservoir for BTV and potential source of virus for sheep and other animals (Luedke and Jones 1984). Natural BT in goats is rare and the most obvious clinical signs are sharp drop in milk production and high fever (up to 42°C). Sometimes, goats may show oedema of the lips and head, some nasal discharge and scabs on the nose and lips (Dercksen et al. 2007). Clinical signs of BT in experimental goats have been described which are milder than sheep and consisting of generalized illness, high fever (above 40.5°C), apathy, dysphagia, diarrhea and lameness (Backx et al. 2007). A serological study was conducted in the goats of Haryana, Himachal Pradesh and Rajasthan, which revealed that more than 60% population harbor anti-BTV antibody (Naresh and Prasad 1995). These goats generally do not show clinical symptoms but act as source of virus with unknown status of viremia or antigenemia. All the blood samples, in this study, were positive for BTV and high level antigen (or virus) has been detected in some samples. This confirms active multiplication of virus in goats, although, symptoms were more suggestive of PPR than clinical BT. The surge of BTV antigen or virus may be due to acute infection or flare up of virus that was already present in the animals because of stress or immunosuppressive

conditions. The role of PPRV for suppressing the immune system of the goats should not be ignored in this case. A few cases of mixed viral infections in sheep and goats involving PPRV and orf virus or goat pox virus or BTV have been reported in India and other countries (Mehrotra et al. 1995; Martrenchar et al. 1997; Saravanan et al. 2007). Morbilliviruses in general have been shown to cause suppression of host immune responses (Yanagi et al. 1992; Wohlsein et al. 1995). Recent studies show that experimental PPRV infection in goats can lead to marked suppression of host immune response accompanied by severe leucopenia (Rajak et al. 2005).

It is interesting to note that PPRV antigen was not present (or s-ELISA could not detect) in the nasal swabs, lung and spleen material and blood samples. But as expected, N gene was amplified from the pooled lung and spleen material because RT-PCR is more sensitive than s-ELISA. The failure of antigen detection in the above samples prompted us to test the sera and some of the sera have been found to contain low level of PPRV antigen even though, they were positive for the PPRV antibody. How PPRV co-exists in the serum or plasma with its cognate antibody is a matter of further investigations. But our observations for the past several years suggest that PPRV can exist in serum probably in the presence of antibody. In last few years, more than 1500 sera from sheep and goats (received as diagnostic submissions) were tested by s-ELISA in our laboratory and more than 50% sera were found positive for PPRV antigen (unpublished observations). Presence of virus or nucleic acid in serum or plasma has been reported in some morbillivirus infections such as, CDV and MV (Krakowka et al. 1979; Nakayama et al. 1995; Frisk et al. 1999).

Based on the clinical signs and laboratory tests, it may be concluded that the outbreak was due to mixed infection of PPRV and BTV. Differential assays for this type of mixed infections should be developed in diagnostic laboratories. Initially, pooled blood sample was used for detection of BTV nucleic acid to understand whether these samples contained BTV or not. The amplicon derived from the pooled sample was sequenced to genetically confirm as BTV. After confirmation of BTV in the pooled sample, all the samples were tested by RT-PCR to know the presence of BTV in the individual sample. Although the nucleotide sequences of the PCR products confirmed the genetic materials of PPRV and BTV, it is very

difficult to ascertain the strain or serotypes of the viruses because the sequence information is too little and that too from highly conserved regions of non-variable genes. Further studies should be done to isolate and determine the lineage of PPRV. Isolation of bluetongue viruses from blood samples is also important to ascertain the involvement of multiple serotypes.

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