COMPARISON OF VIRUS ISOLATION AND POLYMERASE CHAIN REACTION FOR DIAGNOSIS OF PESTE DES PETITS RUMINANTS

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Summary. – Oculonasal swabs and tissue samples collected from peste des petits ruminants (PPR) suspected sheep and goats were tested for presence of the virus of peste des petits ruminants (PPRV) or its RNA by reverse transcription–PCR (RT-PCR) and virus isolation (VI). Of 44 samples 31.8% and 40.9% were positive by VI and RT-PCR, respectively. The RT-PCR-positive samples were subjected to the nested PCR. Three of six samples positive by RT-PCR but negative by VI were negative by the nested PCR. The specificity and accuracy of the nested PCR were higher than those of the RT-PCR although the sensitivity of both tests were similar. Nucleotide sequencing of one nested PCR product revealed a 92% homology with the sequence available in the GenBank (Acc. No. Z37017).

Key words: peste des petits ruminants; reverse transcription–polymerase chain reaction; virus isolation; nested PCR

Introduction

PPR is an acute, highly contagious and fatal disease of small ruminants. The causative agent, PPRV (genus *Morbillivirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*), is closely related to its counterpart – the rinderpest virus (RPV) (Saliki *et al.*, 1993). Generally, RPV causes the disease in large ruminants, such as cattle and buffalo, while PPRV in small ruminants such as goats and sheep. However, in India, RPV causes clinical and subclinical infection in small ruminants too (Taylor, 1986), which can then be transmitted to cattle causing a more severe disease (Anderson *et al.*, 1990) while PPRV is known to cause only a subclinical infection in cattle (Diallo *et al.*, 1989).

Shaila *et al.* (1989) first reported PPRV in India and several PPR outbreaks have been reported since then in almost all the states of the country (Chandran Choudhary *et al.*, 1995; Kulkarni *et al.*, 1996; Nanda *et al.*, 1996). As differential diagnosis of rinderpest and PPR by host species and clinical signs is not always correct, laboratory diagnosis is necessary, especially when small ruminants may be a source of infection of cattle. The laboratory techniques available to differentiate these diseases include virus neutralization (Taylor, 1979; Chandran *et al.*, 1995), hybridization with cDNA probes (Diallo *et al.*, 1989; Pandey *et al.*, 1992), immunocapture enzyme-linked immunosorbent assay (ELISA) (Libeau *et al.*, 1994), immunofluorescence (IF) test (Sumption *et al.*, 1998) and RT-PCR (Forsyth and Barrett, 1995). RT-PCR can also produce a DNA product which can be sequenced.

Before using RT-PCR in routine diagnostic testing of field samples, it has to be compared with the "gold standard", the VI. The latter cannot always be done as a routine diagnostic assay owing to its time-consuming, cumbersome procedure and requirement of cell culture facilities. Forsyth and Barrett (1995) have successfully used RT-PCR to diagnose PPRV and differentiate it from RPV in samples

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Abbreviations: CPE = cytopathic effect, p.i. = post infection; PPR = peste des petits ruminants; PPRV = PPR virus, RPV = rinderpest virus, RT-PCR = reverse transcription–PCR; VI = virus isolation

from natural and experimental infections. However, they have not compared the RT-PCR assay with the VI test. This study was primarily undertaken to compare the sensitivity and specificity of the RT-PCR assay with those of the VI test using samples of tissues or swabs collected from PPRV-suspected sheep and goats.

Materials and Methods

Field samples collected in and around Chennai, India, from PPR-suspected sheep and goats included the lymph node, spleen and lungs from dead animals or oculonasal swabs from live ones. Field samples were processed in duplicate.One half of swabs was stripped off into a barrel of syringe with 1 ml of phosphate buffered saline pH 7.2 (PBS) and squeezed by a plunger to collect the eluted virus. The eluate was centrifuged at 3000 rpm for 10 mins and the supernatant complemented with antibiotics was stored at -70°C. Another half of swabs was subjected to vortexing in the D solution and used for RNA extraction following the method of Chomcyznski and Sacchi (1987). Tissue specimens were washed in PBS, minced with scissors and ground in liquid nitrogen with a pestle in mortar to a fine suspension. The latter were centrifuged and the supernatants were used for VI and RT-PCR.

RT-PCR. RNA extraction, cDNA synthesis and PCR were done according to the methods described by Forsyth and Barrett (1995). cDNA was synthesized using the Thermoscript RT-PCR kit (Life Technologies, USA) from total RNA extracted. For the PCR, two sets of F gene-specific primers were used, F1 and F2, which were shown to amplify a 372 bp PCR product. For the nested PCR, the primers F3 and F4 amplifying a 308 bp product were used The primer sequences are given in Forsyth and Barrett (1995). The PCR was performed in a thermal cycler (MJ Research Inc , USA) using the Taq PCR Core kit (Qiagen, USA) and the following program: step 1, 1 cycle, 94°C for 5 mins; step 2, 35 cycles, 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; step 3, 1 cycle, 72°C for 7 mins. A 10 µl sample of the PCR product was electrophoresed on a 1.5% agarose gel containing 2 5 µg/ml of ethidium bromide and DNA bands were visualized under UV light. Controls included (i) cDNA replaced with RNA to rule out DNA contamination in the extracted RNA and (ii) cDNA replaced with nuclease-free water to test the reliability of the PCR reagents A sample was considered positive if a band of specific size and no bands in the controls were seen. A few samples were tested with the ß actin gene primers to check the quality of the RNA isolated (Forsyth and Barrett, 1995).

Nested PCR The PCR-positive samples only were subjected to the nested PCR. The products were purified using the microspin SH-400R columns (Pharmacia, USA) and then used as templates for the nested PCR. One nested PCR product was sequenced using the automated ABI Prism module at Bangalore Genei, Bangalore, India The sequence obtained was compared with the PPRV F gene sequence available at the GenBank (Acc. No Z37017).

VI Isolation of PPRV from samples was done in Vero cells grown in 24-well microplates (Saliki *et al.*, 1994). The supernatants obtained by processing the swabs or tissues (described above) were used to infect one day-old cell monolayers (0.1 ml per well). After virus adsorption for 2 hrs at 37°C, the inoculum was removed and replaced with Eagle's Minimum Essential Medium without serum. The plates were examined for cytopathic effect (CPE) and frozen on day 5 post-infection (p i) After repeated freezing and thawing they were used for inoculation of fresh monolayers. Two blind passages were done and the third passage was done in wells containing coverslips. The coverslips were fixed in 70% acetone, blocked in PBS containing 2% bovine serumalbumin, incubated with a PPRV-specific monoclonal antibody against the virus nucleoprotein (Dhinakar Raj *et al.*, 2001) followed by fluorescein-conjugated anti-mouse IgG (Sigma) at predetermined dilutions. The VI test was considered positive if the infected cells showed cytoplasmic fluorescence.

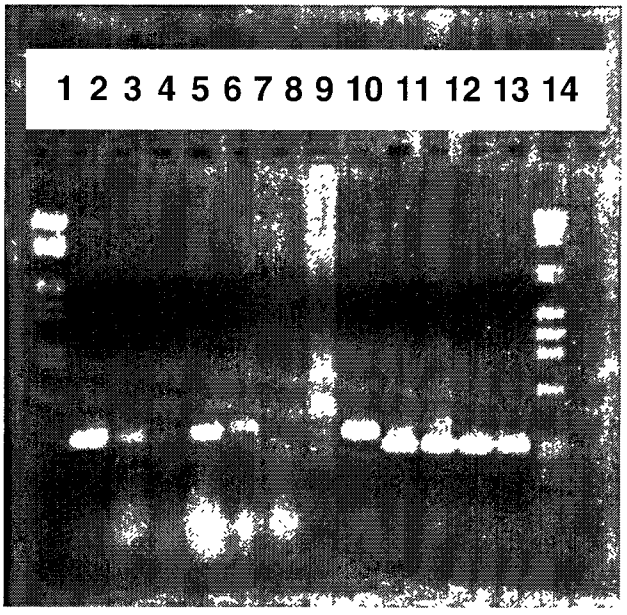

Fig. 1
Agarose gel electrophoresis of RT-PCR products amplified from field samples
Samples of swabs and tissues (lanes 2–7) The samples in lanes 4 and 7 are negative, others are positive. Negative control (lane 8) and positive control (lane 9) Products of nested PCR from positive samples seen in lanes 2, 3, 5 and 6, respectively (lanes 10–13) DNA size markers (22,130, 9,416, 6,557 4,361, 2,322, 2,027, 1,353, 1,078, 872, 603 and 564 bp (lane 1) DNA size markers (21,226, 51,48, 4,973, 4,268, 3,530, 2,027, 1,904, 1,584, 1,575 947, 831, 564 and 125 bp)

Results

Samples were collected from sheep and goats showing symptoms of fever, ocular and nasal discharges, diarrhoea and oral lesions. A total of 44 samples were collected, 6 from sheep and 38 from goats. The most consistent symptoms seen in PPR-positive cases were oculo nasal discharges and necrotic stomatitis. In total, 31.8% and 40.9%

of the samples were positive by VI and RT-PCR, respectively (Table 1; Fig. 1). When the RT-PCR-positive samples (18 samples) were tested by the nested PCR, 3 of the 6 samples, positive by PCR but negative by VI, were negative by the nested PCR.

In the VI test, the CPE produced by the virus included cell rounding and clumping, clock-faced syncytia and cell death. To confirm the identity of the causative agent producing the CPE, an immunofluoresence test using monoclonal antibody against the PPRV nucleoprotein was used. A clear cytoplasmic fluorescence was seen in positive cases (results not shown).

The results obtained by the PCR and nested PCR were compared with those of VI, the "gold standard", and the results are summarized in Table 2. The nested PCR had a slightly higher specificity and accuracy than the PCR although the corresponding sensitivities were similar.

One nested PCR-positive product was sequenced and upon comparison with the existing sequence in GenBank using the BLAST analysis produced a 92% homology. This further confirmed the identity of the PCR product amplified in the nested PCR.

Table 1. Comparison of PPRV-positivity obtained by virus isolation (VI) and RT-PCR

Species	VI	RT-PCR
Sheep	16.7% (1/6)	33.3% (2/6)
Goats	34.2% (13/38)	42.1% (16/38)
Total	31.8% (14/44)	40.9% (18/44)

Discussion

The most consistent clinical symptoms of oculonasal discharges and oral lesions encountered in PPR-positive cases were similar to those reported by Kulkarni *et al.* (1996). The "gold standard" for any diagnostic test is the isolation and identification of the causal agent of the specific disease. In this study, the causative agent, namely PPRV, was isolated in Vero cells by three blind passages and identification was done by immunofluoresence using the monoclonal antibody which was specific for PPRV and did not cross react with RPV (Dhinakar Raj *et al.*, 2001). Sumption *et al.* (1998) have also used an immunofluoresence test on conjunctival smears for specific diagnosis of PPRV.

RT-PCR assays using the F gene primers have been shown to be specific for PPRV by Forsyth and Barrett (1995) and have been used for PPR diagnosis from field samples (Shaila *et al.*, 1996; Diallo *et al.*, 1995). The PCR test detects the presence of viral genome while VI detects infectious virus particles. Hence, before using RT-PCR for routine diagnosis from field samples it needs to be compared to VI. This study revealed good agreement between the two tests. Presently an immunocapture ELISA is available for specific diagnosis of PPR (Libeau *et al.*, 1994). However, in India, the necessary kits are not readily available and are exorbitantly priced. Thus RT-PCR could be the diagnostic test of choice due to its high sensitivity, specificity and rapidity. It also yields the product for further analysis of the genetic nature of the viruses involved in the outbreaks by sequencing.

A nested PCR is used for further confirmation of PCR products since it amplifies the PCR product using a set of internal primers. The mere appearance of a band of expected size does not always correlate with the presence of the virus genome. Hence confirmation of the PCR positivity needs to be performed by one of the several methods available such as nested PCR, southern blot and hybridization, sequencing and so on. The nested PCR increased the specificity of the diagnosis. Three samples found to be positive by PCR were negative by nested PCR. This could also be attributed to some change in the primer binding sites of the internal primers in these three viral genomes. This seems less likely since these samples were negative by VI although this theory cannot be ruled out completely. Moreover, negative results in VI are not due to the poor quality of the samples since the tissues were properly stored and processed immediately for RNA isolation. The quality of the RNA isolated was also checked using ß actin primers. Thus nested PCR can also be used for diagnosis and may be

Table 2. Data analysis of the results of virus isolation (VI), RT-PCR and nested PCR

Test	VI-positive	VI-negative	Total	Sensitivity	Specificity	Accuracy
RT-PCR-positive	12	6	18			
RT-PCR-negative	2	24	26	85.7%	80%	81.8%
Total	14	30	44			
Nested PCR-positive	12	3	15			
Nested PCR-negative	2	27	29	85.7%	90%	88.6%
Total	14	30	44			

preferred over RT-PCR due to its higher specificity. However care should be taken for possible laboratory contamination of the cDNA while using such a highly sensitive test for routine diagnosis. As a further confirmation, one nested PCR product was sequenced and it also revealed a 92% homology. Although sequencing cannot be undertaken as a routine diagnostic method, a few positive PCR products can be sequenced every year to keep track of the molecular and genetic nature of the PPRV circulating in the field.

The samples of swabs collected from live animals forms a non-invasive method of collection and they can be easily transported to the laboratory for further testing. The lower mortality rates caused by PPR do not facilitate easy availability of post mortem specimens for diagnosis.

Thus, the RT-PCR test can be used as a routine diagnostic tool for PPR diagnosis, which is in good correlation with the VI test provided proper controls are included in the test.

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