DEVELOPMENT OF A N GENE-BASED PCR-ELISA FOR DETECTION OF PESTE-DES-PETITS-RUMINANTS VIRUS IN CLINICAL SAMPLES

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Summary. – A highly sensitive N gene-based PCR-ELISA for the detection of Peste-des-petits-ruminants virus (PPRV) was developed. The RT-PCR yielded a digoxigenin (DIG)-labeled product of 336 bp comprising a sequence from PPRV N gene, which was then detected by ELISA. The assay could detect the viral RNA in PPRV-infected tissue culture fluids with a titer as low as 0.1 $TCID_{50}$/ml. The assay is 10,000 times more sensitive than a classical RT-PCR combined with agarose gel electrophoresis. The assay could detect the virus in the clinical samples, which were negative by conventional sandwich ELISA (S-ELISA). The percentage positivity of the assay in detecting the virus in clinical samples was 66.2% compared to 48.6% for S-ELISA. The assay was more sensitive than S-ELISA also in detecting the virus in early as well as late phases of the disease. In addition, the assay could also be used for differential diagnosis of PPRV and Rinderpest virus (RPV).

Key words: clinical samples; diagnosis, differential diagnosis; PCR-ELISA; peste-des-petits-ruminants; Peste-des-petits-ruminants virus; rinderpest; Rinderpest virus; RT-PCR; sandwich ELISA

Introduction

Peste-des-petits-ruminants (PPR) is a highly contagious and devastating viral disease of small ruminants with morbidity and mortality rates as high as 100% and 90%, respectively (Abu-Elzein *et al*., 1990; Joshi *et al*., 1996). Clinical signs include dullness, high body temperature, severe nasal and ocular discharges, sores in the mouth, foul smelling diarrhea, respiratory problems and cough followed by death (Roeder and Obi, 1999). The disease is caused by an RNA virus of the species *Peste-des-petits-ruminants virus*, the genus *Morbillivirus*, the family *Paramyxoviridae* (van Regenmortel *et al*., 2000). There are four distinct lineages of PPRV circulating in the world, among which the lineage 4 is common in India (Shaila *et al*., 1996; Dhar *et al*., 2002). In order to control the disease effectively, a highly sensitive test for the diagnosis of samples collected at different stages of infection is required. The diagnosis of PPR is mainly based on conventional tests such as agar gel immunodiffusion, counter mmunoelectrophoresis and indirect-ELISA (Obi and Patrick, 1984). These tests cannot be used for the differential diagnosis of PPR and rinderpest (RP). A monoclonal antibody (MAb)-based immunocapture ELISA (Libeau *et al*., 1994) and S-ELISA (Singh *et al*., 2004) are more rapid and more specific but less sensitive. Novel molecular biological techniques like nucleic acid hybridization, RT-PCR and simple and aqueous phase hybridization ELISA (SNAP-ELISA) are highly sensitive and specific techniques for the differentiation of PPRV and RPV (Diallo *et al*., 1989; Shaila *et al*., 1989; Pandey *et al*., 1992; Forsyth and Barrett, 1995; Couacy-Hymann *et al*., 2002; Forsyth *et al*., 2003).

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Abbreviations: GTV = Edward's caprinised RPV; LRPV = lapinised RPV; MAb = monoclonal antibody; p.i. = post inoculation; PPR = peste-des-petits-ruminants; PPRV = PPR virus; RP = rinderpest; RPV = RP virus; TCRPV = tissue culture-adapted RPV; S-ELISA = sandwich ELISA; SNAP-ELISA = simple and aqueous phase hybridization ELISA

Table 1.

Oligonucleotide primers and probes

Primer/probe	Sequence 5'-3'	Length (nt)	Posittion (nt)
pprn-og1	CCCGGCCAACTGCTTCCGGAGA	22	1421–1442[a]
rpn-og2	GTA AGC TCC TCA GCT ATG ACT C	22	1500–1521[b]
pprn-fr2	ACA GGC GCA GGT TTC ATT CCT	21	1270–1290[a]
pprn-re1	GCT GAG GAT ATC CTT GTC GTT GTA	24	1606–1584[a]

[a]The numbering based on the African isolate Nigeria 75/1 of PPRV (Diallo *et al*., 1994; Aleyas, 2002), Acc. No.X74443 in the EMBL database.
[b]The numbering based on the N gene sequence of the Plowright vaccine strain of RPV (Baron and Barrett, 1995), Acc. No. Z30697.

PCR-ELISA is an advancement over these techniques as it can detect a very low amount of the virus in clinical materials. At present, this technique is of utmost importance for the confirmatory diagnosis of critical clinical samples free of false positivity. This presumption and other successful reports of use of this technique in the detection of various viruses, namely human enteroviruses (Andreoletti *et al.,* 1996), human papilloma viruses (Poljak and Seme, 1996), Epstein-Barr virus (Bazzichi *et al*., 1998), Swine vesicular disease virus (Callens and Clercq, 1999), Rabies virus and rabies-related viruses (Whitby *et al*., 1997; Black *et al*., 2000), Swine fever virus (Barlic-Maganja and Grom, 2001), and Avian influenza A virus (Munch *et al*., 2001) prompted us to develop a PCR-ELISA for the detection of PPRV in clinical samples collected at different phases of infection. The sensitivity of this assay was compared with that of classical RT-PCR and MAb-based S-ELISA. Successful attempts were also made to differentiate PPRV from the closely related RPV using this assay.

Materials and Methods

Viruses. The PPRV Sungri 96 isolate, obtained from the Rinderpest and Allied Diseases Laboratory, Division of Virology, Indian Veterinary Research Institute, was used. This virus was isolated from an PPR outbreak in the Sungri village, Rohru Tehsil, Shimla District, Himachal Pradesh, India in 1996 and has initially been adapted to B95a cells and subsequently attenuated in Vero cells (Sreenivasa *et al*., 2000). The Vero cell-adapted attenuated vaccine virus was used for RNA isolation and PCR-ELISA. Also an attenuated RBOK strain of RPV adapted to tissue cultures (TCRPV; Plowright and Ferris, 1962), the Edward's caprinised RPV (GTV; Edwards, 1928) adapted to grow in Vero cells (Parida and Bandyopadhyay, 1996), and the lapinised RPV (LRPV; Nakamura *et al*., 1938) were used.

Cells. Vero and B95a (a marmoset lymphoblastoid cell line) cell lines were used.

Clinical samples, namely swabs and tissues were collected from goats during experimental trials conducted for the development of a live attenuated homologous PPR vaccine and S-ELISA. The nasal and ocular swabs, 20 each were obtained from live animals inoculated with PPRV and postmortem tissues like spleen, lymphnode, lungs and intestine from goats and sheep suspected for PPR were used in PCR-ELISA. Also some tissue samples obtained from field cases suspected for PPR were included in this study. The swabs were prepared in 0.5 ml PBS while the tissue samples were prepared as 10% (w/v) suspensions in PBS by grinding the material with sterile sand using a pestle and mortar. All the clinical samples mentioned above were tested simultaneously by S-ELISA and PCR-ELISA.

Oligonucleotide primers and probes. The random hexanucleotide primers used for cDNA synthesis in reverse transcription of PPRV RNA and the N gene primers used for PCR amplification were obtained from Life Technologies, USA. The N gene primers pprn-fr2 and pprn-re1 were designed according to the N gene sequence of the African isolate Nigeria 75/1 of PPRV (Diallo *et al*., 1994; Aleyas, 2002). The oligonucleotides pprn-og1 (nt 1421–1442) and rpn-og2 (nt 1500–1521) were biotinylated to produce capture probes based on the internal sequence of PCR product of N gene, derived from published sequences of Nigeria 75/1 and vaccine RPV, respectively. Details of the primers and probes are listed in Table 1.

Preparation of biotinylated capture probes. Biotin labeling of the oligonucleotides pprn-og1 and rpn-og2 was done using the Biotin-Chem-Link Kit (Roche Molecular Biochemicals, Germany). Briefly, 2 µl of pprn-og1 or rpn-og2 (1 µg), 2 µl of the Biotin-Chem-Link reaction mixture (10 times concentrated) and 16 µl of nuclease-free water was mixed and the mixture was incubated at 85°C for 60 mins. The biotin labeling reaction was stopped by addition of 10 µl of the stop solution from the kit and the probe was stored at -25°C. The capture probes pprn-og1 and rpn-og2 were specific for PPRV and RPV, respectively.

Total RNA was extracted from PPRV-infected cell cultures and clinical samples (ocular, rectal, oral and nasal swabs and spleen, lymphnode, tongue, lung and intestine suspensions) with the Trizol LS reagent (Life Technologies, USA) according to the manufacturer's instructions and with necessary modifications (Chomczynski and Sacchi, 1987).

RT-PCR. The first-strand cDNA was synthesized by reverse transcription reaction using a hexanucleotide random primer and Moloney murine leukemia virus reverse transcriptase (Promega). A DIG-labeled PCR product was generated using the primers pprn-fr2 and pprn-re1 amplifying the sequences of both PPRV and RPV. Direct labeling of the PCR product with DIG was carried out using the DIG-labeling PCR Kit and following the manufacturer's protocol (Roche Molecular Biochemicals, Germany). The amplification reaction mixture (50 µl) consisted of 29.5 µl of PCR water, 5 µl of the 10x PCR-DIG labeling buffer, 3 µl of 25 mmol/l $MgCl_2$, 5 µl

of the PCR-DIG labeling mixture, 1 µl (10 pmoles) of the primers pprn-fr2 and pprn-re1, 0.5 µl (2.5 U) of *Taq* DNA polymerase, and 5 µl (5 ng) of cDNA.

The PCR cycling conditions were as follows: initial denaturation at 95°C for 3 mins was followed by 35 cycles of 94°C/30 secs, 55°C/30 secs and 72°C/1 min with final extension at 72°C for 10 mins. DNA prepared from uninfected Vero cells served as negative control.

Agarose gel (1%) electrophoresis was employed as a classical comparative technique for separation and detection of the DIG-labeled PCR product. The UVP Image Master VDS (Amersham Pharmacia Biotech, USA) gel documentation system was used. The PPRV-specific amplicon consisted of 336 bp.

PCR-ELISA, the technique developed for detection of the PPRV N gene-based PCR product, was based on the use of a commercial detection kit (Roche Molecular Biochemicals, Germany) and a few modifications. Briefly, 5 µl of PCR product was mixed with 20 µl of a denaturing solution (0.05 mol/l NaOH) and the mixture was incubated at room temperature for 10 mins. Then, 225 µl of a hybridization solution containing the biotinylated capture probe (6 pmoles) was added to the denatured PCR product. The mixture was vortexed and then 200 µl aliquote was transferred into a microtitre plate well precoated with streptavidin and incubated at 37°C for 3 hrs with shaking. The wells were then washed 5 times with a washing solution and 200 µl of an anti-DIG-horseradish peroxidase conjugate was added per well. After the incubation at 37°C for 45 mins on a shaker and 5-fold washing with a washing solution 200 µl of a substrate solution (1.9 mmol/l ABTS in 100 mmol/l phosphate-citrate buffer pH 4.4 and 3.2 mmol/l H_2O_2) was added. The color was allowed to develop at 37°C for 30 mins in dark under constant shaking and A_{405} (A_{492} as reference) was determined in an ELISA reader. The hybridization conditions were optimized with different concentrations of the biotinylated capture probe and temperatures of incubation. The cut-off value was determined as the 3-fold mean absorbance value of a negative sample (tissues from healthy animals, uninfected Vero cells) plus 2 SD.

S-ELISA was employed as a comparative classical technique for detection of PPRV in clinical samples. It was based on the use of a S-ELISA kit (Singh *et al.,* 2004). Briefly, the microtitre wells were coated with 100 µl/well of the capture antibody diluted 1:4,000 in PBS pH 7.4. After incubation at 37°C for 1hr under shaking, the wells were washed 3 times with a washing buffer (0.0025 mol/l PBS containing 0.05% Tween 20). Fifty µl of a blocking buffer (0.01 mol/l PBS with 0.1% Tween 20 and 0.5% goat serum negative for PPRV antibodies) was added to all the wells except the antigen blank well, which received 100 µl of blocking buffer. Clinical samples (50 µl per well) were tested in duplicate. Positive controls containing the Vero cell-derived purified PPRV antigen as well as negative controls containing the antigen from uninfected Vero cells (50 µl each) in quadruplicate were also included. The plates were incubated at 37°C for 1 hr under shaking. The wells were washed 3 times with the washing buffer and 100 µl/well of a detection antibody (a MAb raised against PPRV N protein) diluted 1:20 in the blocking buffer was added. After incubation at 37°C for 1 hr with shaking the wells were washed 3 times as described above. Then 100 µl/well of a rabbit anti-mouse horseradish peroxidase conjugate (Dako Patts,Denmark) diluted 1:1,000 in the blocking buffer was added and incubated at 37°C for 1 hr with shaking. After a 3-fold washing 100 µl/well of a substrate solution (0.4 mg/ml OPD in 4 ml of 3% H_2O_2) was added and the plates were kept at 37°C for 15 mins in dark. The color development was stopped by addition of 100 µl/well of 1 mol/l H_2SO_4 and A_{492} was read in an ELISA reader. A cutoff value of 0.232 was determined.

Results

Optimization of hybridization conditions for PCR-ELISA

In order to optimize the hybridization conditions, the DIG-labeled PCR product of PPRV N gene of 336 bp in size was hybridized with the PPRV N gene-specific probe pprn-og1 and RPV-specific probe rpn-og2 at different temperatures (37, 42, and 45°C) for 3 hrs and with different biotinylated capture probe concentrations (2, 4, 6, 8, 10, 12, 14 and 16 pmol/ ml). Optimal hybridization was observed at 37°C for 3 hrs and with the capture probe concentration of 6 pmol/ml for both PPRV- and RPV-specific probe. All subsequent experiments were performed at these hybridization conditions.

The mean A_{405} value of known negative sample was 0.070 with SD of 0.008. Therefore, the cutoff value for the PCR-ELISA was set as 0.226 (0.070 x 3 + 0.008 x 2). Further,

Table 2. Detection of PPRV in ocular swabs from an experimentally infected goat

Day p.i.	S-ELISA		PCR-ELISA	
	A_{492} (cutoff = 0.232)	Positive/ negative	A_{405} (cutoff = 0.226)	Positive/ negative
0	0.111	Negative	0.079	Negative
1	0.094	Negative	0.083	Negative
2	0.118	Negative	0.152	Negative
3	0.099	Negative	0.067	Negative
4	0.085	Negative	0.101	Negative
5	0.110	Negative	0.097	Negative
6	0.168	Negative	0.411	Positive
7	0.378	Positive	0.769	Positive
8	0.308	Positive	0.741	Positive
9	0.554	Positive	1.115	Positive
10	1.443	Positive	1.241	Positive
11	0.695	Positive	0.508	Positive
12	0.390	Positive	1.203	Positive
13	0.173	Negative	0.519	Positive
14	0.199	Negative	1.060	Positive
15	0.204	Negative	0.733	Positive
16	0.157	Negative	0.550	Positive
17	0.122	Negative	0.319	Positive
18	0.123	Negative	0.122	Negative
19	0.124	Negative	0.100	Negative

Absorbance values differred significantly at $P \leq 0.01$.

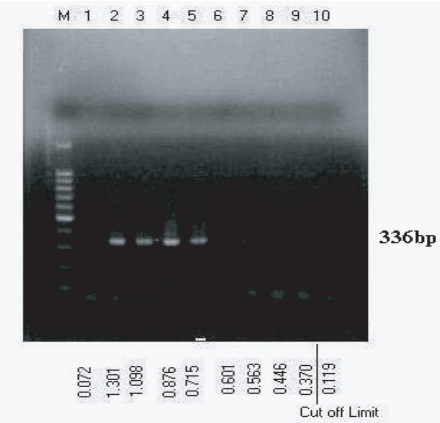

Fig. 1

Comparison of the sensitivity of detection of DIG labeled PCR product of PPRV N gene by agarose gel electrophoresis and PCR-ELISA

DNA size marker, 100 bp ladder (lane M); negative control, uninfected Vero cell cDNA (lane 1); 10^5, 10^4, 10^3, 10^2, 10, 1, 0.1, 0.01, and 0.001 $TCID_{50}$ of purified PPRV (lanes 2–10). PCR-ELISA A_{405} values are indicated below the lanes. The cutoff value was 0.226.

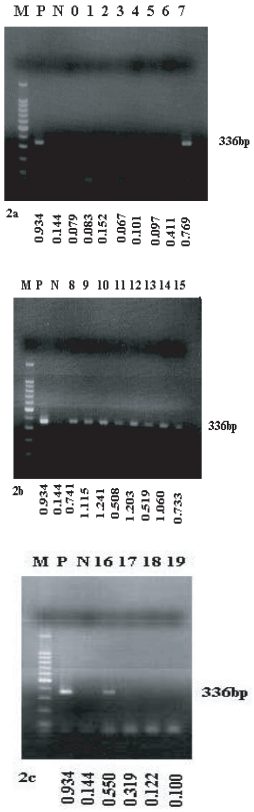

Fig. 2

Detection of PPRV in ocular swabs from experimentally infected goats by PCR-ELISA

DNA size marker, 100 bp ladder (lane M); Positive control (lane P); negative control (lane N); eye swabs collected on days 0–19 p.i. from experimentally infected goats (lanes 0–7, 8–15, and 16–19). A_{405} values are indicated below the lanes.

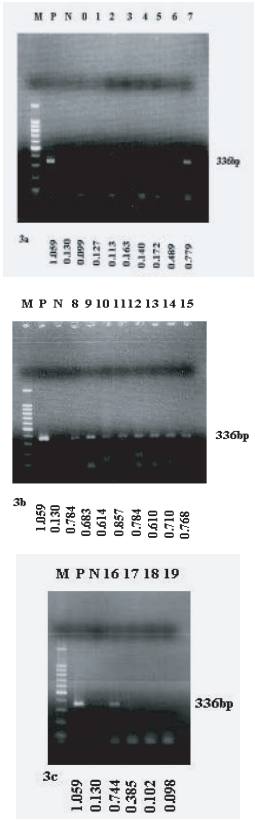

Fig. 3

Detection of PPRV in nasal swabs collected from experimentally infected goats

DNA size marker, 100 bp ladder (lane M); positive control (lane P); negative control (lane N); nasal swabs collected on days 0–19 p.i. from experimentally infected goats (lanes 0–7, 8–15, and 16–19). A_{405} values are indicated below the lanes.

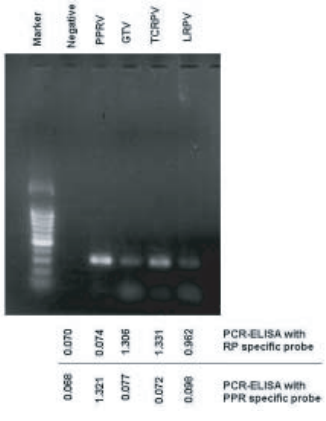

Fig. 4

Differentiation of PPRV from RPV by PCR-ELISA using PPRV- and RPV-specific biotin-labeled probes

DNA size marker, 100 bp ladder, negative control; PPRV, GTV, TCRPV, and LRPV. The same primer set was used for all samples. A_{405} values for RPV-specific (upper part) and PPRV-specific probes (lower part) are indicated below the lanes. The cutoff value of 0.226 for both probes was used.

the PCR-ELISA was tested for its sensitivity using a PPRV of known titer (10^5 $TCID_{50}$/100 µl). The virus was serially 10-fold diluted, the DIG-labeled PCR product for each virus dilution was produced and detected by agarose gel electrophoresis and PCR-ELISA. Whereas the least amount of PPRV detectable by the agarose gel electrophoresis was 10^2 $TCID_{50}$ in 100 µl, it was only 0.01 $TCID_{50}$ in 100 µl, that means 10,000 times less by the PCR-ELISA (Fig. 1).

Detection of PPRV in clinical samples

First, clinical samples from experimentally infected goats were tested. The DIG-labeled PCR products amplified from clinical samples were detected simultaneously by two different techniques: agarose gel electrophoresis and PCR-ELISA. It was found that whereas the gel electrophoresis detected PPRV in nasal and ocular swabs from day 7 to day 16 post inoculation (p.i.), PCR-ELISA did so in a wider time period, from day 6 to day 17 p.i. (Figs. 2 and 3). Then these two methods were compared with S-ELISA. The same clinical samples were positive for PPRV by S-ELISA from day 7 to day 12 p.i. (Tables 2 and 3).

Next, clinical field samples were tested for PPRV by PCR-ELISA and S-ELISA (Table 4).

The percentage positivities of PCR-ELISA and S-ELISA were 66.2% (49/74) and 48.6% (36/74), respectively.

Table 3. Detection of PPRV in nasal swabs from an experimentally infected goat

Day p.i.	S-ELISA		PCR-ELISA	
	A_{492} (cutoff = 0.232)	Positive/ negative	A_{405} (cutoff = 0.226)	Positive/ negative
0	0.131	Negative	0.099	Negative
1	0.094	Negative	0.127	Negative
2	0.138	Negative	0.113	Negative
3	0.104	Negative	0.163	Negative
4	0.124	Negative	0.140	Negative
5	0.110	Negative	0.172	Negative
6	0.154	Negative	0.489	Positive
7	0.283	Positive	0.779	Positive
8	0.460	Positive	0.784	Positive
9	0.574	Positive	0.683	Positive
10	0.748	Positive	0.614	Positive
11	0.665	Positive	0.857	Positive
12	0.562	Positive	0.784	Positive
13	0.230	Negative	0.610	Positive
14	0.141	Negative	0.710	Positive
15	0.118	Negative	0.768	Positive
16	0.153	Negative	0.744	Positive
17	0.113	Negative	0.385	Positive
18	0.130	Negative	0.102	Negative
19	0.209	Negative	0.098	Negative

Absorbance values differed significantly at $P \leq 0.01$.

Table 4. Comparison of PCR-ELISA with S-ELISA in detection of PPRV in clinical samples

Sample	No. of samples	Positive by S-ELISA	Positive by PCR-ELISA
Eye swab	22	8	12
Nasal swab	22	7	13
Rectal swab	4	4	3
Mouth swab	2	1	2
Spleen	10	6	8
Lymph node	8	4	7
Tongue	2	2	2
Lung	2	2	1
Pooled (spleen, lungs, lymph node and intestine)	2	2	1
Total	74	36	49

Differentiation of PPRV from RPV by PCR-ELISA

The DIG-labeled PCR products amplified from the samples containing TCRPV, GTV, LRPV or PPRV with the same primers were subjected to detection by PCR-ELISA using RPV- and PPRV-specific probes. The RPV-specific probe detected TCRPV, GTV and LRPV, but not PPRV. Similarly, the PPRV-specific probe detected only PPRV but not the three RPV strains (Fig. 4). The clinical samples positive for PPRV by PCR-ELISA were found to be negative for RPV.

Discussion

PCR-ELISA is a sensitive technique, exploits the advantages of higher sensitivity of PCR and nucleic acid hybridization and simplicity of ELISA. Its sensitivity could be further enhanced by the use of avidin and biotin. Although the immunocapture ELISA (Libeau *et al*., 1994) and S-ELISA (Singh *et al*., 2004) are popular, they suffer from a disadvantage of false results due to lower sensitivity. Singh *et al*. (2004) have reported that these techniques could detect the PPRV antigen as early as on day 7 p.i. and then up to day 12 p.i. in experimentally infected animals. To confirm an outbreak of PPR, samples should be collected at the peak of infection, but this is usually not ensured in developing countries like India. In fact, the samples collection is often delayed because of the lengthy chain of procedures involved and the samples collected after the peak of outbreak may result in false diagnosis using conventional tests like S-ELISA. Further, the delay in postal delivery and breaks in the cold-chain during the transportation of samples may result in the loss of epitopes leading again to false diagnosis, namely to negative S-ELISA results for samples from animals, which are e.g. clinically indicative of PPR. PCR-ELISA is a test of great importance especially for

the diagnosis of various viral diseases in such circumstances as those mentioned above (Andreoletti *et al.,* 1996; Poljak and Seme, 1996; Whitby *et al*., 1997; Bazzichi *et al*., 1998; Callens and Clercq, 1999; Black *et al*., 2000; Barlic-Maganja and Grom, 2001; Munch *et al*., 2001).

The PCR-ELISA described in this study was standardized and developed for the detection of PPRV in clinical samples. The developed assay detected the virus as early as on day 6 p.i. and as late as on day 17 p.i., inclusive of the detection of carrier stage, which is rather difficult to diagnose by a conventional test. Thus, the assay could detect PPRV in nasal and ocular swabs earlier than S-ELISA, which could detect PPRV between days 7 and 12 p.i. (Singh *et al*., 2004). The developed assay is of importance for the confirmation of controversially or doubtfully diagnosed clinical samples, and such conditions are more likely to arise after launching a disease control program (Sreenivasa *et al*., 2000; Singh *et al*., 2004).

For routine diagnosis of PPR from clinical samples and differentiation of PPR from RP we have also developed an one step multiplex RT-PCR based on the PPRV N and M genes. This test could be useful for the detection of PPRV in swabs from infected animals. This test could detect the virus in nasal and ocular swabs from experimentally infected goats between days 7 and 17 p.i. and in oral swabs between 7 and 15 p.i. (unpublished results).

The fact that the sensitivity of the developed PCR-ELISA in detecting purified PPRV was 10,000 times higher than that of RT-PCR concurs with many other findings such as those for the detection of swine vesicular disease (Callens and Clerck, 1999).

The comparison of PCR-ELISA with S-ELISA in detecting PPRV in clinical samples revealed percentage positivities of 66.2% and 48.6%, respectively. PCR inhibitors present in fecal and pooled tissue samples and hemoglobin present in lung samples (Andreoletti *et al.,* 1996), have an adverse effect on the efficacy of a PCR-based test. A similar observation was made by us in the present study with three samples (one rectal swab, one lung sample and one pooled tissue sample), which were positive by S-ELISA but negative by PCR-ELISA. A F gene-based RT-PCR-SNAP-ELISA has been developed for the lineage differentiation of PPRV from RPV (Forsyth *et al*., 2003). The N gene-based PCR-ELISA developed by us appears to be the first screening tool for the detection of PPRV in clinical samples.

The capture probes and primers designed according to the African isolate Nigeria 75/1 of PPRV (Diallo *et al*., 1994; Aleyas, 2002) worked well for the Asian lineage 4 Sungri 96 isolate of PPRV (Dhar *et al*., 2002) indicating specificity of these probes and primers for PPRV of other lineages too. Although fresh swab or tissue samples are optimal materials to be tested, long preserved and partially deteriorated samples could be screened efficiently by PCR-ELISA too, indicating the higher sensitivity of the assay for the evaluation of such critical samples, in which only a very small number of virus particles are present due to deterioration of samples.

Using PCR-ELISA, it was possible to detect and differentiate PPRV from RPV by employing the RPV-specific biotinylated capture probe (rpn-og2) and the DIG-labeled PCR product generated by N gene-specific PPRV primers. The RPV-specific probe rpn-og2 did not cross-react with PPRV-positive control samples indicating a high specificity. Further, a common set of primers could be applied to amplify the N gene of both PPRV and RPV in clinical samples, which could further be differentiated using the virus-specific probes.

Under global RP eradication program, many countries have undertaken sero-surveillance and India has not been an exception. In such a circumstance, standardization of a confirmatory test like the presently described PCR-ELISA would solely depend on available vaccine viruses (TCRPV, GTV and LRPV) due to non-availability of clinical samples, as India is provisionally free from RP (Sinha, 1998).

Summing up, the PCR-ELISA reported here is a novel rapid and highly sensitive technique for the detection of PPRV in clinical samples from early as well as late phases of infection with meager amounts of the virus, which would rather be hardly detectable by other conventional assays.

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References

Abu-Elzein EME, Hassanien MM, Al-Afaleq AI, Abd-Elhadi MA, Housain FMI (1990): Isolation of peste des petits ruminants virus from goats in Saudi Arabia. *Vet. Rec.* **127**, 309–310.

Aleyas AG (2002): Comparative evaluation of different gene targets for PCR diagnosis of peste des petits ruminants. *MVSc Thesis* submitted to Indian Veterinary Research Institute, Deemed University, Izatnagar, Bareilly, UP, India

Andreoletti L, Hober D, Belaich S, Lobert PE, Dewilde A, Wattre P (1996): Rapid detection of enterovirus in the clinical specimens using PCR and microwell capture hybridization assay. *J. Virol. Methods* **62**, 1–10.

Barlic-Maganja D, Grom S (2001): Highly sensitive one tube RT-PCR and microplate hybridization assay for the detection and for the discrimination of classical swine fever virus from other pestiviruses. *J .Virol. Methods* **95**, 101–110.

Baron MD, Barrett T (1995): Sequencing and analysis of the nucleocapsid (N) and polymerase (L) genes and the terminal extragenic domains of the vaccine strain of rinderpest virus. *J. Gen.Virol*. **76**, 593–602.

Bazzichi A, Guidi FV, Rindi L, Incaprera M, Garzelli C (1998): PCR ELISA for the quantitative detection of Epstein-Barr virus genome. *J. Virol. Methods* **74,** 15–20.

Black EM, McElhinney LM, Lowings JP, Smith J, Johnstone P, Heaton PR (2000): Molecular methods to distinguish between classical rabies and the rabies-related European bat lyssa viruses. *J. Virol. Methods* **87**, 123–131.

Callens M, Clercq K (1999): Highly sensitive detection of swine vesicular disease virus based on a single tube RT-PCR system and DIG-ELISA detection. *J. Virol. Methods* **77**, 87–99.

Chomczynski P, Sacchi N (1987): Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem*. **162**, 156–159.

Couacy-Hymann E, Roger F, Hurard C, Guillou JP, Libeau G, Diallo A (2002): Rapid and sensitive detection of peste des petits ruminants virus by a polymerase chain reaction assay. *J. Virol. Methods* **100**, 17–25.

Dhar P, Sreenivasa BP, Barrett T, Corteyn M, Singh RP, Bandyopadhyay SK (2002): Recent epidemilogy of peste des petits ruminants virus (PPRV). *Vet. Microbiol*. **88**, 153–159.

Diallo A, Barrett T, Barbron M, Shaila MS, Lefevre PC (1989): Differentiation of rinderpest and peste des petits ruminants viruses using specific cDNA clones. *J. Virol. Methods* **23**, 127–136.

Diallo A, Barrett T, Barbron M, Meyer G, Lefevre PC (1994): Cloning of the nucleocapsid protein gene of peste des petits ruminants virus: relationship to other morbilliviruses. *J. Gen. Virol*. **75**, 233–237.

Edwards JT (1928): Rinderpest active immunization by means of the serum simultaneous method, goat virus. *Agric. J. India* **23**, 185–189.

Forsyth MA, Barrett T (1995): Detection and differentiation of rinderpest and peste des petits ruminants viruses in diagnostic and experimental samples by polymerase chain reaction using P and F gene-specific primers. *Virus Res*. **39**, 151–163.

Forsyth MA, Parida S, Alexandersen S, Belsham GJ, Barrett T (2003): Rinderpest virus lineage differentiation using RT-PCR and SNAP-ELISA. *J. Virol. Methods* **107**, 29–36.

Jacobson RH (1998): Validation of serological assays for diagnosis of infectious diseases. *Rev. Sci. Tech. Off. Int. Epizoot*. **17**, 469–486.

Libeau G, Diallo A, Colas F, Guerre L (1994): Rapid differential diagnosis of rinderpest and peste des petits ruminants using an immunocapture ELISA. *Vet. Rec*. **134**, 300–304.

Munch M, Nielson LP, Handberg KJ, Jorgenson PH (2001): Detection and subtyping (H5 and H7) of avian type A influenza virus by reverse transcription–PCR and PCR-ELISA. *Arch. Virol*. **146**, 87–97.

Nagamura J, Wagatuma S, Fukusho K (1938): On the experimental infection with rinderpest virus in the rabbits:I.Some fundamental experiments. *J. Japanese Vet. Sci*. **17**, 185–204.

Obi TV, Patrick D (1984): The detection of peste des petits ruminants (PPR) virus antigen by agar gel precipitation test and counter immunoelectrophoresis. *J. Hyg*. **93**, 579–586.

Pandey KD, Baron MD, Barrett T (1992): Differential diagnosis of rinderpest and PPR using biotinylated cDNA probes. *Vet. Rec*. **131**, 199–200.

Parida MM, Bandyopadhyay SK (1996): Adaptation of caprinised rinderpest virus to grow in vero cell in vitro. *Acta Virol.* **40**, 45–48.

Plowright W, Ferris, RD (1962): Studies with rinderpest virus in tissue culture. The use of attenuated culture virus as a vaccine for cattle. *Res. Vet. Sci*. **3**, 172–182.

Poljak M, Seme K (1996): Rapid detection and typing of human papilloma viruses by consensus polymerase chain reaction and enzyme-linked immunosorbent assay. *J. Virol. Methods* **56**, 231–238.

Roeder PL, Obi TU (1999): *Recognizing Peste des Petits Ruminants: A Field Manual.* FAO, Rome, Italy, 17, 75–81.

Shaila MS, Purushothaman V, Bhavasar D, Venugopal K, Venkatesan RA (1989): Peste des petits ruminants of sheep in India. *Vet. Rec*. **125**, 602.

Shaila MS, Shamaki D, Forsyth MA, Diallo A, Groatley L, Kitching RP, Barrett T (1996): Geographic distribution and epidemiology of peste des petits ruminants viruses. *Virus Res*. **43**, 149–153.

Singh RP, Sreenivasa BP, Dhar P, Bandyopadhyay SK (2004):A sandwich-ELISA for the diagnosis of peste-des-petits-ruminants (PPR) infection in small ruminants using anti-nucleocapsid protein monoclonal antibody. *Arch. Virol*. (in press).

Sinha NK (1998): Rinderpest in India: the delegate declares the peninsular zone 'provisionally free' from the disease. *Dis. Inform.* **11**, 61.

Sreenivasa BP, Dhar P, Singh RP, Bandyopadhyay SK (2000): Evaluation of an indigenously developed homologous live attenuated cell culture vaccine against peste des petits ruminants infection of small ruminants. Abstract in the *Proceedings of "XX annual conference of Indian association of veterinary microbiologists, immunologists and specialists in infectious diseases*". October, Pantnagar, Uttaranchal, India, pp. 84.

van Regenmortel MHV, Fauquet CM, Bishop DHL (2000): *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses.* Academic Press, San Diego-San Francisco-New York-Boston-London-Sydney-Tokyo.

Whitby JE, Heaton PR, Whitby H E, O'Sullivan E, Johnstone P (1997): Rapid detection of rabies and rabies-related viruses by RT-PCR and enzyme-linked immunosorbent assay. *J. Virol. Methods* **69**, 63–72.