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Development and Validation of Filter Paper Method of RT-PCR for the Detection of 'N'gene of Peste Des Petits Ruminants Virus

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Abstract

Peste des petits ruminants (PPR) is an acute viral disease of goats and sheep. The disease causes a huge loss of small ruminant production per year in Bangladesh. Diagnosis of PPR from suspected field samples is not properly carried out due to unreliable transportation and poor infrastructure. This study was planned to develop and validate a hazard free, time saving and specific sampling technique for the diagnosis of PPRV (Peste des petits ruminants vaccine). Two categories of samples including bronchial lymph node and viral suspensions from nasal swab (from dead and live animals) were collected from 25 goats and from each case corresponding nasal swab smeared filter paper was also taken as sample. All the samples were subjected to RT-PCR with (lymph node and viral suspension from nasal swab) or without (smeared filter paper) RNA extraction using N gene specific primer. Twenty three goats found PPR positive for both categories of samples. One found positive where extracted RNA is used as template but negative to smeared filter paper RNA. On the other hand, one found negative to extracted RNA but positive to filter paper RNA. One goat found negative for both the cases. The result of the study has suggested that sampling through filter paper could be easiest, less time consuming and hazardless for transportation of samples from the field through which diagnosis of PPRV can be done by RT-PCR.

1. Introduction

Peste des petits ruminants (PPR) is a major disease problem of small ruminants of Bangladesh. It is a highly contagious viral disease of sheep and goats that causes high morbidity and mortality in these species [1]. In Bangladesh, outbreaks of Peste des petits ruminants (PPR) have been occurring in goats since 1993 [2, 3]. The disease was first described at Cote d'Ivore of West Africa in 1942. Since then the disease has been reported in several parts of the worlds such as Middle East [4], the Arabian Peninsula [5] and Southern Asia [6] including India, Bangladesh, Pakistan Nepal and Afganistan. Peste des petits ruminants (PPR) is an economically important transboundary viral disease of goats in Bangladesh [7]. The disease causes a huge loss of small ruminant production per year in Bangladesh. Although the epidemiology of the disease is not well understood and very limited studies have been done in Bangladesh [3, 8].

The causative agent of PPR disease is a Morbillivirus under the family of Paramixoviridae. PPR virus is an envelope and pleomorphic in shape with single stranded negative sense non segmented RNA genome [9, 10, 11]. PPR virus is mostly spread through aerosol. The clinical features of PPR disease is associated with high fever (106°F-107°F), nasal and ocular discharges, excessive salivation with erosive stomatitis and ulceration of the gastro-intestinal tract leading to severe diarrhoea [12]. The occulo-nasal discharge becomes mucopurulent followed by pneumonia with cough, abdominal breathing and pleural rales.

Diagnosis of PPR in the field is mainly based only on clinical signs, symptoms and post mortem findings. The confirmatory diagnosis of the PPR virus is based on the laboratory techniques. Samples can be collected from live animals include swabs from nasal secretions, mouth and rectal lining, conjunctival sac, clotted and whole blood (with EDTA anticoagulant). At post-mortem, samples of tonsil, tongue, spleen, lymph nodes, affected areas of the alimentary tract mucosa may be collected (OIE, 2004). A conventional method of transportation of the samples for diagnosis is usually done by maintaining a cool chain environment. The diseases are not always properly diagnosed in the field because of inadequate laboratory facilities, although rapid and confirm diagnosis is necessary to control the disease. The conventional method of collection, preservation and sending of samples to the laboratory is too much hazardous, unreliable and time consuming because of poor infrastructure in Bangladesh. Maintaining of cool chain for sending samples is difficult. So in many cases, diagnosis of field samples is not properly carried out. Sampling through filter paper, can reduce these problems. Maintaining of cool chain is not needed in case of filter paper method of sampling. Besides this, using this method sample can be preserved for long time and it can be sent easily through an envelope to the laboratory. For confirmatory diagnosis of PPR, there are several laboratory tests are being used such as virus neutralization test, AGID or ELISA that are time consuming, difficult or sometimes non-specific. RT-PCR is now being used to detect the virus rapidly and specifically [13, 14, 15, 16, 17]. For molecular detection of viruses, the filter papers have been used with [18] or without RNA extraction [19, 20, 21, 22].

Therefore, this research was conducted to

a. Develop the filter paper method of RT-PCR for the detection of PPR virus and
b. Validate the filter paper method of sampling for the detection of PPR viruses through RT-PCR.

2. Material and Methods

The research work was performed in the Department of Pathology, Bangladesh Agricultural University, Mymensingh.

2.1. Materials

2.1.1. Reagent

The reagents used in this experiment are given bellow-

I. Phosphate Buffer Saline

Dulbecco's phosphate buffered saline solution was prepared as 1X concentration as follows:

NaCl	8 gm
KCl	0.2 gm
Na_2HPO_4, (anhydrous)	1.4 gm
KH_2PO_4	0.2 gm
H_2O q.s. to make	1 liter

All ingredients were dissolved by stirring, adjust pH to 7.4. Sterilized by autoclaving. Stored at RT. Before use, 50ml PBS (1x) was mixed with 450ml sterile double distilled water, to which one ml gentamicin (50mg/ml) (Sigma, USA) was added to give a final concentration of 100μg per ml.

II. Viral Transport Medium

PBS-glycerine solution	49 volume
Antibiotic mix.(Amphotericin-B+ Penicillin+Streptomycin)	1 volume

PBS-Glycerine solution was made by adding equal volume of 50% of PBS to 50% glycerine.

2.1.2. Equipment and Supplies

Balance (Mettler PM400, Mettler, Germany); microcentrifuge (Eppendorf, Germany) bench centrifuge (Heraeus, Hanau, Germany); class II biosafety cabinet (Esco, Singapore); micropipettes (Japan, Italy, Germany); pH meter (Schott, UK); vortexer (VELP Scientifica, Italy); bench top autoclave (Tuttnauer Systec, Germany); refrigerators, freezers, ice box, thermomixer.

2.1.3. Plastic Ware and Consumables Materials

Pipette tips (Eppendorf, Germany); eppendrof tubes: 50 ml, 15 ml (Becton Dickinson Labware, USA) 1.5 ml (Eppendorf, Germany), PCR tubes, falcon tubes, RNeasy mini spin column; sterile filter tips (Eppendorf, Germany); syringe and needle (Opsonin, Bangladesh); Collection tubes (1.5ml and 2ml) etc.

2.1.4. Kit

Following kits are used in this study:

RNeasy mini Kit (RNA extraction kit) (Qiagen, Germany)

Qiagen one step RT-PCR kit (Qiagen, Germany)

2.1.5. Filter Paper

Whatman 3mm sized filter papers (What man International Ltd., United Kingdom) were used for this study.

2.1.6. Primer

The oligonucleotides used in the present study were obtained from Integrated DNA Tecnologies (Japan). Details of the primer are summarized in Table 1.

Table 1. *Primers used for amplification of N gene of PPRV in the study.*

Gene	Primer	Sequence	Position	Size	References
N	NP3	5′-TCTCGGAAATCGCCTCACAGACTG -3′	1232-1255	351 bp	Couacy-Hymann, *et al.,* 2002
	NP4	5′-CCTCCTCCTGGTCCTCCAGAATCT -3′	1583-1560		

2.1.7. DNA Size Marker

100bp DNA ladder obtained commercially (Invitrogen, USA) were used for this study.

2.1.8. Electrophoresis Buffer and Reagents

I. TAE buffer (50X)

Tris base	242 gm
Glacial acetic acid	57 ml
0.5 M EDTA, pH 8.0	100 ml
H_2O q.s. to make	1 liter

Ingredients were dissolved by stirring and stored at room temperature.

1X working solution was prepared by mixing 1 part stock buffer with 49 parts water.

II. Electrophoresis grade Agarose

Obtained from Sigma, USA

III. Loading dye: Ultra pure Agarose Gel Loading dye Bio Basic Inc., USA

IV. Ethidium bromide (10 mg/ml): Obtained from Sigma, USA.

2.2. Methods

2.2.1. Cleaning and Sterilization of Glassware and Appliances

Glassware, forceps and mortar and pestle were soaked in a household dishwashing detergent solution ("Trix", Reckitt and Benckeser Bangladesh Ltd.) for at least one hour. Contaminated items were disinfected overnight in 2% sodium hypochlorite solution prior to immersing in detergent. The items soaked in detergent were cleaned by brushing, washed thoroughly in running tap water and rinsed four items in distilled water. Finally glassware, forceps and mortar and pestle were wrapped with brown paper and sterilized by dry heat in a hot air oven at 160°C for an hour. Glass bottles having plastic caps or rubber seals and disposable plastic ware (Eppendrof tubes, micropipette tip [s etc.) were sterilized by autoclaving for 15 minutes at 121°C under 15 lbs pressure per sq. inch. Autoclaved items were dried in a hot air oven at 50°C. The graduated glass pipettes were taken in a large cylinder filled with water, washed thoroughly by continuous filling and removal of water from the cylinder. Finally washed 4 items in the same manner using distilled water. The pipettes were dried in an oven at 50°C. The graduated pipettes were cotton plugged at the neck, placed in a canister and sterilized by dry heat in a hot air oven at 160°C for an hour.

2.2.2. Collection of Local Isolates of PPRV

I. Post-mortem samples

Lungs, mesenteric and bronchial lymph nodes, spleen were collected aseptically from PPR suspected dead goats. Dead animals were showing sign and symptoms suggestive for PPR. Samples were stored in falcon tube at -70°C.

II. Virus suspensions from nasal swab of live animal

Nasal secretions were taken by swab sticks from the PPR suspected goats. Swab sticks then emerged into 2.5 ml micro centrifuge tube containing 1 ml viral transport medium. Then the upper part of the swab stick was broken up to the 2 mark of the micro centrifuge tube. Then the tubes were capped along with the broken swab sticks. The samples were stored at -70°C for further use.

III. Filter paper sample

Nasal secretion was collected by swab stick and then it was rubbed into a piece of Whatman 3MM filter paper. Then the filter paper was air dried. Dried by direct sun light was strictly prohibited. Filter papers are then collected into envelopes and were carried to the laboratory. The samples were stored at -70°C in the laboratory for long time storage. Detail information of collected samples is given in Table 2.

Table 2. *Detail of collected samples.*

Sl. No.	Species		Age (month)	Source	Sample collected
1	Dead	Goat	16	Netrokona, Bangladesh	Bronchial Lymph node and Filter paper sample from nasal swab
2		Goat	24		
3		Goat	14		
4		Goat	11		
5		Goat	5	Narayangonj, Bangladesh	
6		Goat	16		
7		Goat	12		
8		Goat	15		
9		Goat	12		
10	Live	Goat	8	Narayangonj, Bangladesh	Viral suspension from nasal swab and Filter paper sample from nasal swab
11		Goat	14		
12		Goat	10		
13		Goat	4		
14		Goat	4		
15		Goat	2.5		
16		Goat	6		

Sl. No.	Species	Age (month)	Source	Sample collected
17	Goat	18		
18	Goat	12		
19	Goat	12		
20	Goat	5		
21	Goat	9		
22	Goat	16		
23	Goat	12	Comilla, Bangladesh	
24	Goat	10		
25	Goat	months		

2.2.3. RNA Extraction from Field Sample

Viral RNA was extracted from the post mortem tissue samples and viral suspension from nasal swab sample using RNeasy Kit (Qiagen, Germany) as manufacturer's instruction. In case of smeared filter paper samples, there is no need for RNA extraction. Dried and soaked filter paper was directly used for RT-PCR.

Procedure:

"(a)" 250 µl viral sample was taken in a eppendrof tube.

"(b)" 450 micro liter RLT buffer was added to the eppendrof tube containing sample.

"(c)" The mixture was vortexed and allowed for 2 minutes incubation for room temperature.

"(d)" 700 µl of 70% ethanol was added in each tube containing sample and RLT mixture and mix gently

"(e)" 700 µl was transferred from the mixture to the special RNeasy mini spin column in a 2 ml collection tube

"(f)" Centrifuge the tube for 30 seconds at 10,000 rpm.

"(g)" Then flow through was discarded and remaining 700 micro liter mixed solution was again transferred into the mini spin column and was centrifuged for 30 seconds at 10,000 rpm.

"(h)" Flow through was discarded and 700 microliter of RW1 buffer was added in the spin column and centrifuged at 10,000 rpm for 30 seconds.

"(i)" Flow through was discarded and 500 micro liter of RPE buffer was added in the spin column and was centrifuged at 10,000 rpm for 30 seconds.

"(j)" Again 500 µl of RPE buffer was added in the spin column and centrifuged finally at 10000 rpm for 30 seconds.

"(k)" Spin column was then transferred in the eppendroff tube and 50 µl nuclease free water was added to the tube and centrifuged for 2 minutes at 10,000 rpm.

"(l)" Finally spin column was discarded and eppendrof tube containing RNA was labeled and stored at -20°C or -80°C for short term and long term storage, respectively.

2.2.4. RT-PCR

Procedure:

Following the description of Rahman *et al.,* 2012, the protocol of RT-PCR was used. N gene specific primer set of PPR virus used in this protocol.

1. Soaked and dried filter paper was taken as samples. Filter paper was cut into 4-5 micron sized pieces and 3-4 pieces were taken as sample for RT-PCR.
2. Filter paper pieces were taken into 0.2 ml PCR tube.
3. Then 33.5 µl of nuclease free water was added to the tube.
4. Filter paper containing tube was then heated for 10 minutes at 95 degree centigrade. Immediate after heating the tube was placed into ice for snap freezing for 5 minutes.
5. Then 16.5 µl of master mix was added to the tube (Table 3).

Table 3. *Composition of reaction mixture for RT- PCR.*

Component		Volume/ reaction
Master mix		20 µl
Nuclease free water	12 µl	
5x Qiagen OneStep RT-PCR buffer	5 µl	
dNTP Mix (10 mM of each dNTP)	1 µl	
Primer Forward (100 pmol/ul)	0.5 µl	
Primer Reverse (100 pmol/ul)	0.5 µl	
Qiagen OneStep RT-PCR Enzyme Mix	1.0 µl	
Template RNA		5 µl
Total		25 µl

Thermal profile was set in the PCR machine as below (Table 4):

Table 4. *Thermal profiles for RT-PCR reaction.*

Gene	Primers pair	Thermal profile							Expected product size
		Reverse trans-cription	Initial denaturation and activation of *Taq* polymerase	PCR cycles				Final elonga-tion	
				Denaturation	Annealing	Elongation	No. of cycles		
N gene	NP3	50°C 30 min	95°C 15 min	94°C 30 s	55°C 30 s	72°C 30 s	35	72°C 10 min	351 bp

2.2.5. Analysis of PCR Products by Agarose Gel Electrophoresis

Agarose gels were prepared by dissolving agarose powder in 1x TAE Buffer to a final concentration of 1.5% (w/v). The slurry was heated in a microwave oven until the agarose was dissolved. After that ethidium bromide was added to the agarose solution @ 0.5 µg/ml. The agarose solution containing ethidium bromide was poured into the gel casting tray to which the comb was properly positioned. After the gel was completely set, the comb was removed and the gel was transferred into the electrophoresis tank and covered with 1x

TAE buffer. DNA samples were mixed with DNA loading buffer and loaded to individual slots of the gel. As a size standard, a DNA size marker was also loaded to one slot. Electrophoresis was run at 90 V for 30 min. When the electrophoresis was completed, the gel was briefly washed with running water and placed on the UV transilluminator in the dark chamber of the image viewing and documentation system. The result or expected band (351 bp) was viewed on the monitor; the image was printed as well as saved electronically.

3. Results and Discussion

The study was carried out in goats sample collected from different district of Bangladesh in order to develop and validate the filter paper method of RT-PCR for the detection of PPR viruses. The experiment was conducted in the Pathology Department, Faculty of veterinary Science, BAU, Mymensingh.

Development of Filter Paper Method of RT-PCR

A rapid, safe, economic and specific diagnostic technique has been developed in this study for molecular detection of PPR virus using filter paper sample. A fragment of N gene of PPR virus was amplified from the sample soaked filter paper directly without prior extraction of RNA. Filter papers have been shown to be suitable for the conservation of either DNA or RNA viruses for extended period of time (up to 4-11 years) at moderate or tropical temperatures [23, 24]. The virus genome can be detected after extraction of the genomic material [18] or by direct RT-PCR without extraction [19, 20, 21]. Recently, this method has been applied more widely for the diagnosis of other infectious diseases as well, since this approach offers the feasibility of collecting large numbers of field specimens. All the samples were tested by RT-PCR and efficacy of paper method of RT-PCR was validated using these field samples. No significant differences were found whatever the sample used.

In this study, samples were collected from 25 goats, of which 6 from dead goats and 19 from live goats. Out of 6 dead goats, all lymph node samples and the corresponding filter paper samples found PPR positive (Table 5). Thus no significant difference in sensitivity of the methods was observed for the two samples (Figure 1 and 2).

Table 5. *Comparative study of RT-PCR from bronchial lymph node and nasal swab smeared filter paper samples.*

S.N. of animals	Species	Source	Extracted RNA used as template	Filter paper used as template
1	Goat	Netrokona, Bangladesh	+	+
2	Goat	Netrokona, Bangladesh	+	+
3	Goat	Netrokona, Bangladesh	+	+
4	Goat	Netrokona, Bangladesh	+	+
5	Goat	Narayangonj, Bangladesh	+	+
6	Goat	Narayangonj, Bangladesh	+	+

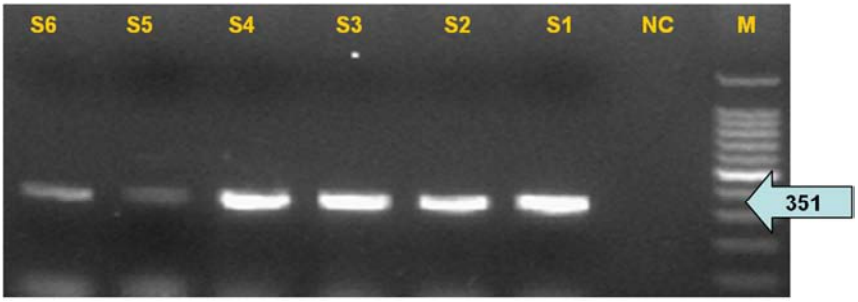

Figure 1. *PCR picture of PPR N gene amplification using RNA extracted from bronchial lymph node as template. S1-S6=Samples (RNA from bronchial lymph node), NC= Negative control, M=100 bp DNA Marker.*

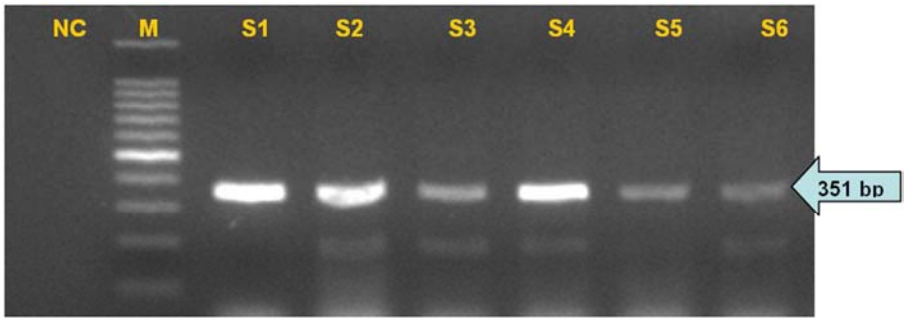

Figure 2. *PCR picture of PPR N gene amplification using filter paper directly as Template, NC=Negative control, M=100bp DNA Marker, S1-S6=Samples (Filter papers smeared with nasal swab).*

Nasal swab and nasal swab smeared filter paper samples were collected from 19 goats. All samples were subjected to RT-

PCR with or without RNA extraction. Out of 19, 17 goats found RT-PCR positive where extracted RNA from nasal swab and filter paper smeared RNA used as template. One goat (No. 12) showed RT-PCR negative with extracted RNA, but positive with corresponding filter paper samples. On the other hand, 1 goat (No. 24) found positive in case of extracted RNA sample but found negative with filter paper sample. 1 animal (No. 16) found negative in both cases (Table 6) (Figure 3 & 4).

Table 6. *Comparative study of RT-PCR from nasal swab and nasal swab smeared filter paper.*

S.N. of animals	Species	Source	Extracted RNA used as template	filter paper used as template
7.	Goat	Netrokona, Bangladesh	+	+
8.	Goat	Netrokona, Bangladesh	+	+
9.	Goat	Netrokona, Bangladesh	+	+
10.	Goat	Netrokona, Bangladesh	+	+
11.	Goat	Narayangonj, Bangladesh	+	+
12.	Goat	Narayangonj, Bangladesh	-	+
13.	Goat	Narayangonj, Bangladesh	+	+
14.	Goat	Narayangonj, Bangladesh	+	+
15.	Goat	Narayangonj, Bangladesh	+	+
16.	Goat	Narayangonj, Bangladesh	-	-
17.	Goat	Narayangonj, Bangladesh	+	+
18.	Goat	Narayangonj, Bangladesh	+	+
19.	Goat	Narayangonj, Bangladesh	+	+
20.	Goat	Narayangonj, Bangladesh	+	+
21.	Goat	Narayangonj, Bangladesh	+	+
22.	Goat	Comilla, Bangladesh	+	+
23.	Goat	Comilla, Bangladesh	+	+
24.	Goat	Comilla, Bangladesh	+	
25.	Goat	Comilla, Bangladesh	+	+

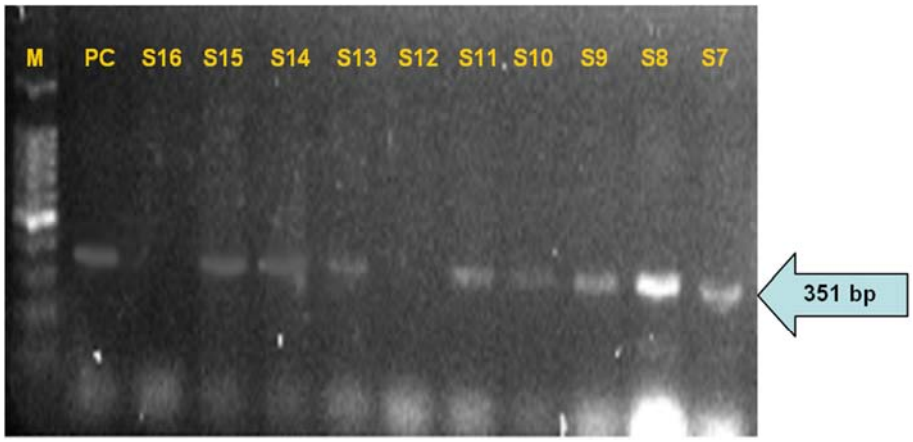

Figure 3. *PCR picture of PPR N gene amplification using RNA extracted from Nasal swab samples as template. S1-S10=Samples (RNA extracted from nasal swab), PC=Positive control, M =100bp DNA Marker.*

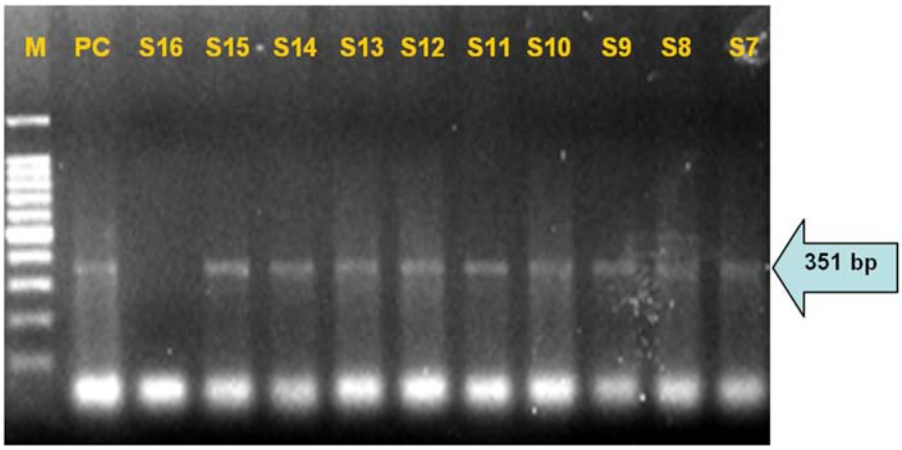

Figure 4. *PCR picture of PPR N gene amplification using filter paper smeared with Nasal swab directly as template. S1-S10=Samples (Nasal swab smeared filter paper), PC=Positive control, M=100 bp DNA Marker.*

In this study, a total of 25 samples from each group were subjected to RT-PCR using N gene primer, where extracted RNA and smeared filter paper RNA used as template. 24 samples found positive to PPRV. Among which 23 samples found RT-PCR positive for both cases. This result relates with the findings of Rahman *et. al*., 2011 [25] and Ularamu *et al.,* 2012 [26]. The primers NP3 and NP4 produced an amplicon of 351 bp amplifying a region of N gene.

In this study filter paper was used directly into the PCR tube without extraction. Filter papers were used experimentally for detection of PPRV after long-term storage at high temperatures in a direct PCR test without any previous extraction of nucleic acids with satisfactory sensitivity [22]. Whatman 3M filter papers are not specifically designed for nucleic acids preservation, but they proved to be efficient in this study and others [21]. Moreover, this method of sampling saves time and money as extraction of RNA needs extraction kits or reagents.

4. Conclusion

Transportation of samples from the remote area to the reference laboratory is time consuming and hazardous due to poor infrastructure of Bangladesh. Maintaining of cool chain is needed to transfer the samples, which is not always properly carried out. This study was conducted to ease the transportation hazard and detection of PPRV effectively and specifically. In this study, samples were collected from 25 goats from different area of Bangladesh. Tissue sample and nasal swab smeared filter paper samples were collected from each individual goats. RT-PCR was done to detect PPRV from these sample using N gene specific sets of primer. The filter paper method of sampling was validated whether it is effective or not. In this study, it was found that, the filter paper method of sampling for the detection of PPRV through RT-PCR is almost 100% effective and sensitive.

From the above summary, it is concluded that filter paper method of sampling could be a effective tool for the rapid and specific detection of PPRV through RT-PCR. Filter paper method of sampling could play revolutionary role to ease sample transportation hazards. Using of N gene specific primer for the detection of PPRV through RT-PCR, could show better results.

Further study should be taken to investigate more detailed molecular epidemiology of PPRV through RT-PCR by using filter paper samples. Diagnosis of other infectious or non-infectious diseases of animals may be done by adopting this type of sampling method.

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