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Article

Sero-surveillance of circulating PPR virus and its molecular analysis in selected areas of Bangladesh

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Abstract: Peste des Petits Ruminants (PPR) is a highly fatal viral disease of goat and sheep. This research work was done in 2017-2018 by executing, surveillance and epidemiological studies to determine present status of circulating PPR virus and its molecular characterization in different areas of Bangladesh. cELISA was conducted to detect the PPR antibody and RT-PCR also used for identification of N gene PPRV. Sera samples and nasal swabs were collected from eight (8) selected villages under Meherpur sadar upazila of Meherpur district on questionnaire basis. Considering two villages as control and six villages as treatment villages. The total 1860 sera were collected at pre vaccination, 21 days, 3 months, 6 months of post vaccination at these selected areas that tested by cELISA and 8035 goat and sheep were received locally produced PPR Vaccine. Baseline study showed that a total of 950 household rear goats in selected 8 villages where number of goats per household ranges from 4.0-5.0. Deworming was done before vaccination in the treatment villages. Pre-vaccination status of six (6) treatment villages were 55.95%, 50.76%, 37.68%, 41.12%, 44.62% and 43.26% in Chakshamnagar, kola, Amjupi, Amdah, Gopalpur and Chadbill respectively, whereas in the control villages (2) seropositive were 40.00% and 42.57% in Doforpur and Mayamari, respectively. Overall 44.90% goats were seropositive against PPR Virus in treatment villages before vaccination. The Sera was analyzed from 21 days, 3 months and 6 months of post-vaccinated goat and sheep from the treatment (6) villages showed the average herd immunity level of goats and sheep rose to 89.10%, 93.25% and 93.37% respectively whereas in the control villages seropositive goats was 38.14%, 43.98% and 35.64% respectively. Awareness building campaigns with villagers have been conducted involving both men and women through the training, meeting, regular visit of household, distribution of poster and leaflet. The mortality and case fatality rate recorded were 7.4% and 18.8%, respectively due to PPR outbreaks. In clinical case, total 59 nasal swabs were molecular characterized by RT-PCR and 41 (69.49%) samples were N gene positive. Among them, the highest presence of PPR virus was recorded at Meherpur sadar upazila 80.77% (21 out of 26) samples was positive. The result of RT-PCR indicates the PPR virus circulating in the different regions of Bangladesh. It is reflected that locally produced PPR vaccine confers sufficient herd immunity that can protect PPR disease in goat and sheep which helps to meet global PPR control programme.

Keywords: PPRV; goat; surveillance; vaccine; antibody

1. Introduction

Peste des Petits Ruminants (PPR) is one of the diseases of major economic importance and imposes a significant constraint upon sheep and goat production owing to its high mortality rate. It is an acute, highly contagious and frequently fatal disease of sheep and goats caused by PPR virus (PPRV), a member of genus morbillivirus of family Paramyxoviridae, Zohari *et al.* (2008). The disease is mostly present in developing countries which often rely heavily on subsistence farming of small ruminants for trade and food supply, De Nardi *et al.* (2012). Since 2007, more than one billion small ruminants in Africa and Asia have been considered at risk of being infected with the PPRV (FAO, 2009). Because of the dramatic clinical incidence and associated restrictions on animal and product movements, PPR is considered as a disease of major economic impact and has to be notified to the World Animal Health Organization, Albina *et al.* (2013). The clinical signs of PPRV infection are always associated with high fever (106°-107.7°F), discharges (nasal, ocular and oral), erosive stomatitis and excessive salivation. The oculo-nasal discharges become mucopurulent followed by pneumonia accompanied with coughing, pleural rales and abdominal breathing. A watery blood stained diarrhea is common in the later stage of infection, which is followed by death.

Gradually, it was realized that several clinically similar diseases occurring in other parts of West Africa shared the same cause. The virus now called Peste des petits ruminants virus (PPRV). Investigators soon confirmed the existence of the disease in Nigeria, Senegal and Ghana. For many years, it was thought that it was restricted to that part of the African continent until a disease of goats in Sudan, which was originally diagnosed as rinderpest in 1972, was confirmed to be PPR. The disease is endemic in Bangladesh since 1993, Islam *et al.* (1996). Generally 100% morbidity and 80-90% mortality were recorded in goat, Hamdy *et al.* (1976). The etiological agent of PPRV is a member of the genus morbillivirus under the family of the paramyxoviridae. Other members of the genus are rinderpest, measles, cannine distemper, seal distemper and dolphin distemper viruses. PPRV although serologically related to rinderpest virus but can be differentiated using cDNA probes, Diallo *et al.* (2007), monoclonal antibodies Anderson *et al.* (1991) and blocking ELISA Saliki *et al.* (1993). PPR virus is enveloped with helical pleomorphic shape containing negative sense single stranded non-segmented RNA molecules.

The most effective way to control PPR is mass immunization of small ruminants as often, farmers in areas where the virus is endemic are unable to afford and implement the strict sanitary control measures, including the stamping out policy, required to contain the virus. Therefore, the control of PPR requires an effective vaccine and for this purpose several vaccines including both homologous and recombinant vaccines have been developed, Abu bakar *et al.* (2011).

A progressive control campaign based on repeated inoculation of all susceptible small ruminants is unaffordable to be implemented. Hence, an epidemiologically based targeting of endemic populations and high-risk zones will be essential. Despite an expansion of PPR to previously unreported area, very little work exists in the country to clearly reveal the epidemiology of the disease, Abraham *et al*. (1991); Abraham *et al*. (2005). Therefore; additional epidemiological and socio-economic studies are needed to support the current initiative towards controlling the disease. Hence, the objectives of the study were design as to detect the PPR antibody and RT-PCR also used for identification of N gene of the circulating PPR virus.

2. Materials and Methods

This research was conducted in the SAARC Regional Leading Diagnostic laboratory for PPR, Bangladesh Livestock Research Institute (BLRI) during the period of 2017 to 2018. The detailed outline of materials and methods are given below:

2.1. Study area

The study was conducted on sero-surveillance of Peste des Petits Ruminants (PPR) for specific antibodies in goat and sheep. For this purpose, the total 1860 (one thousand and eight hundred sixty) sera was collected from 8 (eight) selected villages of Meherpur district namely six (6) treatment villages were Chakshamnagar, kola, Amjupi, Amdah, Gopalpur and Chadbill and the control villages (2) seropositive goats were Doforpur and Mayamari.

In case of treatment villages, the total 8035 goats and sheep were provided PPR Vaccination campaigns by local PPR vaccine (Livestock Research Institute, Dhaka, Bangladesh). The sera were collected from the pre vaccination, 21 days, 3 months and 6 months of post vaccination (PV) in treatment villages. On the other hand, the control villages (2) were considered as non vaccinated village only sera were collected at different interval.

This study also carried out clinical investigation for detection of viral nucleic acid against PPR Virus in goat and sheep. For this purpose, the total 59 (fifty nine) nasal swabs was collected from different areas of Bangladesh

such as Rajshahi (n=6), Sirajganj (n=8), Meherpur sadar upazila (n=26) and Chaudanga (n=19) with active passive base line survey was conducted for indemnified the incidence rate of morbidity, mortality and case fatality in selected goat population.

2.2. Collection and storage of samples

A total of 1860 serum samples were collected from goat and sheep in the study periods. The aim was to determine the level of antibody in the serum/herd immunity in vaccinated areas as well as the sero-prevalence in high risk areas of infection in non-vaccinated areas. Initially, blood samples were collected by jugular-vein puncture (Figure 1) with (3-5) ml sterile syringe. The suspected nasal swabs collected for molecular study (Figure 2). Then labeling as a specimen type/name. (e.g. serum), Unique identification number, Place of collection (location), Date of collection etc. Finally, the sera samples were transported in an ice box chilled on ice packs to the SAARC Regional Leading Diagnostic Laboratory for PPR, Bangladesh Livestock Research Institute (BLRI). Savar, Dhaka, Bangladesh where serological analysis was carried out using cELISA and the collected samples were stored at −20°C until processed.

2.3. Active field investigation and questioner survey

An epidemiological study on PPR outbreak was employed between July/2017 and June/2018 to collect epidemiological data and samples (Nasal swabs and serum). The questioner surveys were interviewed to reveal information regarding flock size, age and sex, health status, grazing management, introduction of new animals, access to veterinary services, clinical signs of disease encountered, number of diseased and dead animals. Close-ended questions were coded and entered in a excel spread sheet. In addition, an observational study on clinical cases was conducted during the occurrence of an outbreak and photographed using digital camera.

2.4. Serological study

A monoclonal antibody (MAb) based competitive Enzyme Linked Immunosorbent Assay (Diallo *et al.*, 2007; OIE, 2013) was used for the detection of antibodies directed against the nucleoprotein of the PPR virus using approved competitive ELISA kit as ID vet. Innovative Diagnostics, France. The resulting coloration depended on the quantity of specific antibodies present in the sample to be tested. In the absence of antibodies, a blue solution appeared which becomes yellow after the addition of the stop solution and in the presence of antibodies, no coloration appeared.

2.5. Test procedure of cELISA

All the reagents were allowed to come to room temperature (21℃ ±5℃) before use. Homogenized all reagents by inversion or vortex.

- 25 μl of dilution buffer 13 were added to each well of the ELISA micro plate.
- Then 25μl of the positive control were added to wells (A1 and B1) and 25 μl of the negative control were added to wells (C1 and D1).
- 25 μl of the each sample were added to test to the remaining wells.
- Then the plate was incubated at 37℃ and waited for 45$\pm$4 minutes.
- The plate was washed 3 times with approximately 300 μl of the wash solution and to avoid drying of the wells between washings.
- After washing then the conjugate was prepared 1X by diluting the conjugate 10X to 1/10 in dilution buffer 4 and again 100 μl of the conjugate 1X was added to each well.
- Again the plate was incubated at 21℃ and waited for 30$\pm$3 minutes.
- Then the plate was washed 3 times with approximately 300 μl of the wash solution and to avoid drying of wells between washing.
- 100 μl of the substrate solution was added to each well.
- Then the plate was incubated at 21℃ in the dark place and waiting for 15$\pm$2 minutes.
- 100 μl of the stop solution was added to each well in order to stop the reaction.
- Finally the micro plate was read for OD values with multichannel spectrophotometric ELISA plate reader with interference filters of 450 nm and the reading data was placed into data sheet of Microsoft® Excel program and saved in the computer hard disc with specific identification name.

2.6. Test validation of cELISA

The test was validated if:

- ✓ The mean value of the negative control O.D (OD_{NC}) is greater than 0.7.
  $OD_{NC} > 0.700$
- ✓ The mean value of the positive control (OD_{PC}) is less than 30% of the OD.
  $OD_{PC}/OD_{NC} < 0.3$

2.7. Interpretation of test result

For each sample, the competition percentage was calculated using the following formula

$$S/N\ \% = \frac{OD_{sample}}{OD_{NC}} x\ 100$$

Sample presenting a S/N%:

- Less than or equal to 50% are considered positive
- Greater than 50% and less than or equal to 60% are considered doubtful.
- Greater than 60% are considered negative.

2.8. Molecular detection of the virus nucleic acid (N gene of PPRV)

Fifty nine (59) nasal swabs were collected from infected goats with viral transport media (VTM) for molecular detection using RT-PCR technique. Collected samples were transported to the SAARC Regional Leading Diagnostic Laboratory for PPR, Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka-1341, Bangladesh maintaining cool chain. A reverse transcription polymerase chain reaction (RT-PCR) was adopted for the detection of PPR virus. Total RNA was extracted from the nasal swabs of clinically affected goat and sheep using PureLink^{TM} RNA mini kit (invitrogen by Thermo Fisher Scientific, USA) as per the manufacturer's instruction. The extracted RNA was evaluated both quantitatively and qualitatively using Nanodrop machine. Agpath-ID^{TM} one-step RT-PCR kit (Applied Biosystems by Thermo Fisher Scientific, USA) was used for preparing master mix and 20µl was dispensed to each PCR tube. Then 5µl extracted RNA template was added to the respective tube and the PCR tubes were placed in the thermocycler. The thermal cycler was 35 cycles programmed. As briefly, Reverse transcription at 50ºC for 30 min, initial denaturation at 95ºC for 30 min, denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec, elongation at 72ºC for 30 sec, final elongation at 72ºC for 10 min, held at 4^0C. PCR products were analyzed by electrophoresis on 1.5% agarose gel, stained with ethidium bromide and examined against UV light using an image documentation system and the images were captured. The oligonucleotide primers were selected from published literature to detect of PPR virus and the cDNA was amplified using PPRV specific NP3 (5'- GTC TCG GAA ATC GCC TCA CAG ACT - 3') and NP4 (5' CCT CCT CCT GGT CCT CCA GAA TCT 3') primers as previously described by Couacy- Hymann *et al*. (2002).

2.9. Data management and analysis

The vaccination and non vaccination status of the individual goat population data were stored in Microsoft Excel 2007. The prevalence was determined by dividing the total number of positive samples by the total number of samples, Dohoo *et al.* (2003). Proportions were calculated for seroprevalence visa-vis fixed factors that included animal species, clinical symptoms, sex and age and village. Univariable analysis for the proportions was carried out using Chi-square analysis in Epi Info software version 3.5.1 (Centre for Disease Control and Prevention) to assess association with the herd immunity level, health status, grazing management, introduction of new animals, number of diseased and dead animals. A confidence limit of less than 5% was used to indicate a significant level. All variables with $P<0.05$ (two-sided) in the univariable analysis were further tested by multivariable logistic regression model to assess their effect on PPR seropositivity.

3. Results and Discussion

3.1. PPR clinical disease investigation through participatory approach

The clinical disease observations and outbreak investigation were done in different district of Bangladesh such as (Rajshahi, Sirajganj, Meherpur sadar and Chauadanga). Both vaccinated and unvaccinated flocks were investigated for the presence of the clinical disease (PPR virus), morbidity, and mortality and case fatality rate.

3.2. Community perception of PPR based on questionnaire interview

During the current participatory epidemiological study, different clinical symptoms were reported by livestock keepers in suspected PPR cases. The signs included nasal discharges, diarrhea, respiratory distress, oral ulcers

and nodules, lacrimation and death. For this purpose, the forty five (45) respondents were selected and result was expressed in (Graph 1). The highest clinical symptoms were recorded as nasal discharge (53.33%) and lowest was abortion (1.8%) that shown in (Graph 1). The clinical findings, diagnostic investigation on samples collected from suspected animals as well as virus isolation consolidated the etiology of the disease to be PPRV. The highest clinical symptoms were recorded as nasal discharge (53.33%) and lowest was abortion (1.8%). In general, the clinical features of PPR observed in the study districts are not different from those reported by others, El-Hakim *et al.* (2006); Abu bakar *et al.* (2011).

The flock consisted of 121 goats and could be regarded as homogeneous with respect to the risk of transmission of an infectious disease. Among this group under observation, there was 48 affected goats giving morbidity rates of 39.7% and Nine goats was died of the disease with the mortality rates 7.4%. The case fatality rate (CFR) was 18.8% for goats (Table 1). In affected cases of the disease there was satisfactory response to injectable antibiotics with hyper immunoserum as seen by treating animals during the outbreak. Interestingly, the outbreak in Meherpur District was reported to be associated with the entry of newly purchased animals from a common local market. A complete history of the origin or the source of the animals to the market, whether from an area endemic for PPR disease, was not available.

It seems that the severity of PPR outbreak in Meherpur district is much higher than the other districts since exceptionally an overt clinical signs of PPR were observed. The outbreak involved of newly purchased goat from different local market owned by recently returned youth from different regions. Only few the non vaccinated animals experienced the disease.

The overall morbidity, mortality and case fatality rate in goat meherpur higher than other districts and the outbreak reported as province with 39.7%, 7.4% and 18.8%, respectively (Abd El-Rahim *et al.*, 2010). The highest overall mortality rate of 69% was estimated in a PPR outbreak in Bangladeshi goats. Similarly, El-Hakim *et al.* (2006) reported a higher morbidity of 76% and case fatality of 18% from a respiratory disease outbreak in sheep in central Ethiopia. Mortality in susceptible flocks varies from 10 to 100% and morbidity ranges from 50 to 100%. However, this scenario is likely to change drastically once intensive vaccination programs are implemented for the target species (Banik *et al.*, 2008).

3.3. Serological studies

For this purpose, the total 1860 (one thousand and eight hundred sixty) sera was collected from 8 (eight) selected villages of Meherpur district namely six (6) treatment villages were Chakshamnagar, kola, Amjupi, Amdah, Gopalpur and Chadbill and the control villages (2) seropositive goats were Doforpur and Mayamari. Both the vaccinated and non vaccinated villages were screened for specific antibodies against PPRV using c-ELISA kit and shown the test result of cELISA for PPR antibody detection (Graph 2 and 3).

The overall seroprevalence (45%) in unvaccinated small ruminants (goats) was slightly higher than the finding in previous studies carried out in the country; 30.5% by Megersa *et al.* (2011) but much higher than 6.8% by Abraham *et al.* (2005) and 6.4% by Waret-Szkuta *et al.* (2008). Comparable findings have been documented in other countries with the overall antibody responses to PPRV, 22.4% in Turkey by Özkul *et al.* (2002); 33% in India by Singh *et al.* (2004a); 26% in Bangladesh by Banik *et al.* (2008); 32.8% in India by Balamurugan *et al.* (2012); 22.1% in Tanzania by Kivaria *et al.* (2013) and 34.2% in Pakistan by Munir *et al.* (2013). Few studies had addressed constant risk factors associated with seropositivity to PPR in Bangladesh. In this study, district, age group, sex, communal grazing management and introduction of new animals appears to be a risk factor for seropositive status in the logistic regression analysis. This is in consistent with the findings of Abu baker *et al.* (2011), who reported a progressive increase of seroprevalence with increasing age. Similarly, introduction of new animals purchased from live animal market have been implicated as a source of the disease in India, Singh *et al.* (2004a). In case of unvaccination, the herd immunity has been assessed by using c-ELISA and the antibody seroprevalence result indicated that herd immunity level against PPR was low. So that, increasing the PPR virus antibodies /herd immunity in small ruminants must be vaccinated animals (goats). In case of vaccination, the herd immunity has been assessed by using c-ELISA and the presence of antibody in population result indicated that the vaccine efficacy against PPR was high level. So that, for improving the PPR virus antibodies in small ruminants must be vaccinated animals (goats) and immune status of national herd, which in turn tell about the level of vaccine coverage to better control the disease targeting wide vaccination coverage.

3.4. Virus detection and confirmation using RT-PCR

For this evaluation, the total fifty nine (59) samples from small ruminant in which PPRV RNA had been detected by classical RT-PCR were used. The presence of the virus in the field samples was confirmed by RT-PCR. From the total of 59 samples examined with RT-PCR for viral nucleic acid, Among them, the highest

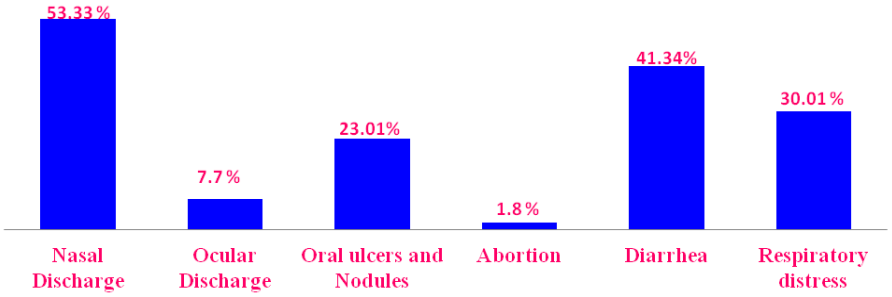
presence of PPR virus was recorded at Meherpur sadar upazila 80.77% (21 out of 26) samples were positive (Table 2). The photograph of the gel electrophoresis of the PCR products that was analyzed (Figure 3). The fragment size of the amplified products was 351 bp as reported previously by Couacy- Hymann *et al.* (2002).
To confirm the detection of PPR viral antigen in 59 suspected nasal swabs samples and RT-PCR protocol was used in this study. The highest presence of PPR virus was recorded at Meherpur sadar 21 (80.77%) whereas the lowest presence was observed in Sirajganj 2 (25%) samples were positive The results showed that overall 69.49% of samples were positive for PPRV antigen. This indicates that PPR virus was the causative agent of the outbreak and that endemic PPR virus is circulating within and between the small ruminant flocks. This can be compared to the findings of 40.98% by Abu bakar *et al.* (2008); 21.4% by Munir *et al.* (2009); 34.3% by Abubakar *et al.* (2011); 25.7% by Munir *et al.* (2013) and 75% by El-Hakim *et al.* (2006) who utilized the same IC-ELISA technique. The current study also revealed a significant higher rate of infection in Meherpur district than other districts.

Table 1. The mortality, morbidity and CFR during PPR outbreak in goat at Meherpur District of Bangladesh.

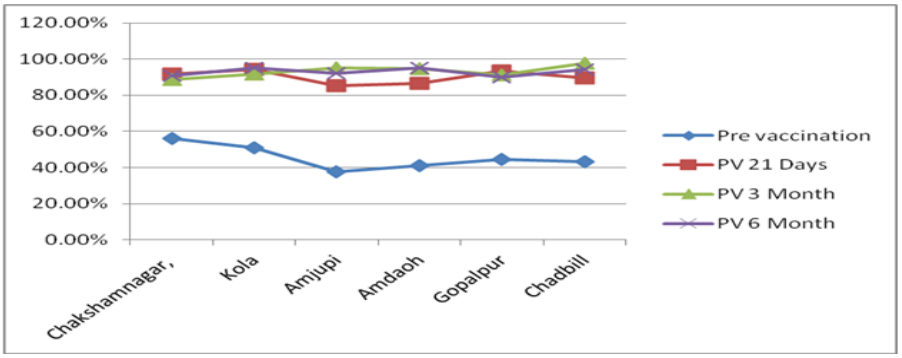
Parameters	Goat
Population investigated	121
Morbidity	48 (39.7%)
Mortality	9 (7.4%)
CFR	18.8%

Table 2. Results of Outbreak investigation of PPR at different regions of Bangladesh.

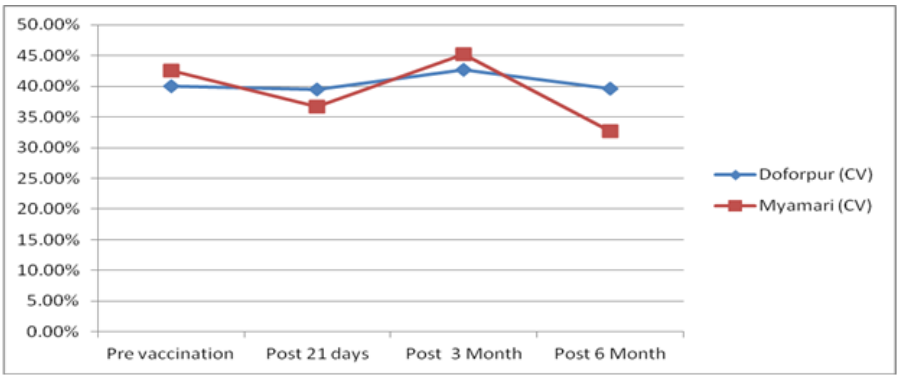
Location	No. of sample	Type of sample	Result of RT-PCR	
			% of positive	% of negative
Rajshahi	6	Nasal Swab	50% (3)	50% (3)
Sirajganj	8	Nasal Swab	25% (2)	75% (6)
Meherpur sadar	26	Nasal swab	80.77% (21)	19.23% (5)
Chuadanga	19	Nasal swab	78.95% (15)	21.05% (4)
Total	59	**Overall**	41 (69.49%)	18 (30.51%)



Graph 1. Suspected clinical symptoms of PPR as reported by respondents (N=45).



Graph 2. The herd immunity against PPR virus at treatment (6 Villages) of Meherpur district.



Graph 3. The immunity status against PPR virus at control (2 Villages) of Meherpur district.

Figure 1. Collection of blood samples (sera).

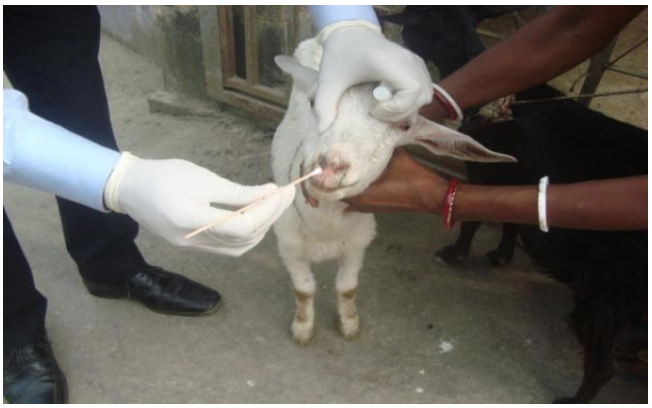


Figure 2. Collection of nasal swab.

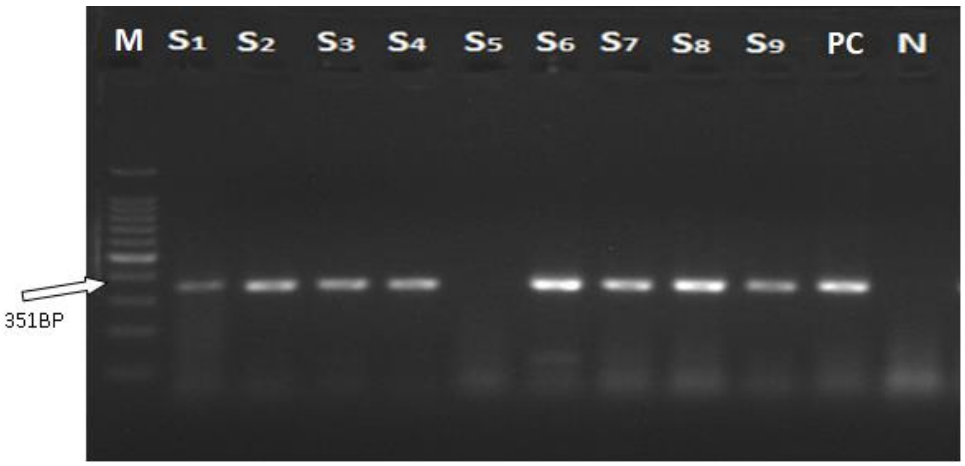


Figure 3. Agarose gel electrophoresis of PCR products (351 bp) amplified with NP3 and NP4, PPR specific primers. Lane M: 100 bp DNA molecular weight marker; Lane PC: Positive control; Lane N: Negative control; Lane S1-S9: Field samples.

4. Conclusions

Bangladesh developed a strategy for the progressive control of PPR that builds upon the lessons learnt from rinderpest eradication. A progressive control campaign based on repeated inoculation of all susceptible small ruminants is unaffordable to be implemented. Hence, an epidemiologically based targeting of endemic populations and high-risk zones will be essential. It seems an opportune time to begin extensive serosurveillance for PPRV in the country along with measurement of clinical survey in the enzootic parts, so that regions can be demarcated into endemic, infected and PPR-free zones. This will help in launching a comprehensive control programme for PPR in the country. Further studies on disease status in the spatial and temporal trends events in the rest of the lowland highland interface of the country are required to define the epidemiology of PPR in these important areas so that to develop effective control strategies for PPR in large area of the country.

Conflict of interest

None to declare.

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