Report

Regional Training on Laboratory Diagnosis of peste des petits ruminants (PPR)

7-12 October 2012, Savar, Dhaka, Bangladesh

SAARC

Contents

Acronyms and abbreviations

BAU	Bangladesh Agricultural University
BLRI	Bangladesh Livestock Research Institute
DLS	Department of Livestock Services
ECTAD	Emergency Centre for Transboundary Animal Diseases
ELISA	Enzyme Linked Immunosorbent Assay
cELISA	Competitive Enzyme Linked Immunosorbent Assay
IC-ELISA	Immunocapture Enzyme Linked Immunosorbent Assay
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
HPED	Highly Pathogenic Emerging Diseases
ICDDR, B	International Centre for Diarrhoeal Disease, Bangladesh
MoFL	Ministry of Fisheries and Livestock
OIE	World Organization for Animal Health (Office International des Epizooties)
PCR	Polymerase Chain Reaction
PPR	*Peste des petits ruminants*
RLDL	Regional Leading Diagnostic Laboratory
RNA	Ribonucleic Acid
RSU	Regional Support Unit
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SAARC	South Asian Association for Regional Cooperation
SOPs	Standard Operating Procedures

Summary

Under the overall umbrella of SAARC and FAO, the Regional Support Unit (RSU) funded by European Union (EU) organised a regional training on the laboratory diagnosis of peste des petits ruminants (PPR) at the Regional Leading Diagnostic Laboratory (RLDL) on PPR in Savar, Bangladesh from 7 to 12 October 2012.

The aim of the training was to train staff of National Laboratories of the SAARC member countries in laboratory techniques for the diagnosis of PPR, specifically to harmonize procedures and protocol for PPR diagnosis in the region.

The training was a mix of laboratory lectures and hands-on exercises on various techniques.

Total thirteen laboratory personnel from seven SAARC countries (Bangladesh, Bhutan, India, Maldives, Nepal, Pakistan and Sri Lanka) participated in the training programme.

A Laboratory Manual/Standard Operating Procedures (SOPs), working protocol, working sheet, etc were provided to all the participants. The principal diagnostic techniques included PCR and ELISA for antigen and antibody detection. The participants were also provided background of these tests, results recording and interpretation.

Background

The Food and Agriculture Organization of United Nations (FAO) is implementing an European Union (EU) funded regional project (OSRO/RAS/901/EC) entitled "Regional Cooperation Programme on Highly Pathogenic and Emerging Diseases (HPED) in South Asia" under the umbrella of South Asian Association for Regional Cooperation (SAARC) at FAO Sub-regional ECTAD Unit in Kathmandu, Nepal. The SAARC member counties have identified the Virology Laboratory of Bangladesh Livestock Research Institute (BLRI), Dhaka, Bangladesh as a Regional Leading Diagnostic Laboratory (RLDL) for peste des petits ruminants (PPR). The mandate of this laboratory is to form, coordinate and lead regional network of laboratories and harmonize diagnostic protocols with support from FAO and OIE reference laboratories. The Regional Support Unit (RSU), based within the FAO's Sub-regional ECTAD Unit in Kathmandu, Nepal conducted a Regional Training on "Laboratory Diagnosis of peste des petits ruminants (PPR)" at the RLDL on PPR, Bangladesh Livestock Research Institute, Savar, Dhaka, Bangladesh from 7-12 October 2012 with support from the Government of People's Republic of Bangladesh, SAARC and European Union.

Objective

The aim of the training was to train staff of National Laboratories of the SAARC member countries in laboratory techniques for the diagnosis of PPR. The specific objective was to harmonize procedures and protocol for PPR diagnosis in the region.

Activities undertaken

Day-1

The training programme was inaugurated by Mr. Abdul Latif Biswas, Honourable Minister, Ministry of Fisheries and Livestock (MoFL), People's Republic of Bangladesh. Besides, State Minister, Secretary, MoFL, EU representative, representatives from FAO Bangladesh Office, RSU, Nepal, were present during the inaugural session. Officers from Department of Livestock Services (DLS) and BLRI, Professors from different related universities were also present in inaugural session.

The day-to-day training schedule is attached as annex 1. The training was a mix of laboratory lectures and hand-on exercises on various techniques. The trainers were drawn from BLRI; Bangladesh Agricultural University, Mymensingh; Chittagong Veterinary and Animal Sciences University, Chittagong, International Center for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) and FAO.

In all thirteen laboratory personnel from seven SAARC countries (Bangladesh, Bhutan, India, Maldives, Nepal, Pakistan and Sri Lanka) participated in the training programme. The List of participants is attached as annex 2.

Every day the previous day's training session was reviewed and the participants actively participated in the "recap" discussions.

The course was started with pre-evaluation where different aspects of PPR was included which was followed by PPR situation in Bangladesh and introduction of BLRI activities and its success. An Overview of PPR, general guidelines for sample collection, transportation and preservation was presented. For practical session, a Laboratory Manual/Standard Operating Procedures (SOPs), working protocol, working sheet, etc were provided to all the participants. The laboratory manual is attached as annex 3.

Day-2

The day started with a lecture on principles of PCR and guideline for good laboratory practices followed by practical session on PCR. The practical session included RNA extraction, master mix preparation, thermal cycling, gel preparation, electrophoresis and results interpretation. Brief explanation was given before start any activities.

Day-3

A lecture on filter paper method of sample collection for virus detection followed by RT-PCR with filter paper samples, gel electrophoresis and results interpretation was delivered. The filter paper method does not require RNA extraction and The filter paper strips can be used directly for RT-PCR.

Day-4

On fourth day the principles of ELISA were discussed followed by practical session for detection of PPR antibody by competitive ELISA (cELISA) and data analysis, quality control assurance and results interpretation. The results obtained were discussed.

Day-5

On fifth day discussion on laboratory biosafety and biosecurity were discussed. The practical session started with the explanation of immunocapture ELISA (IC-ELISA) which was used for detection of PPR virus in suspect clinical samples.

The data was analysed and discussions were held on quality control/ assurance and results interpretation .

Day-6

On the last and sixth day discussion was held on "Role of laboratory networks in epidemiology and disease surveillance in the SAARC region and global networks."

During the training programme general discussion on outbreak investigation was held and the following topics were also covered as per the need and the request of the participants-

1. Rapid diagnostic test
2. Sample shipment
3. Use of Gas Burners in Biosafety Cabinets

The course was concluded with course evaluation and general discussion. .

The participants evaluated the training course as overall useful but the duration should be at least two weeks as the time was not sufficient to perform the tests individually.

Annex- 1

Training Schedule

Date/Day	9.00-9:.30	9.30 – 10.30	10.30 – 11.00	11.00 – 12.00	12.00 – 01.00	1.00 – 2.00	2.00 – 300	3:00-3:30	3:30-4:30	4:30-5:30
7th October /Sunday	**Registration**	**Pre-evaluation** (Dr. Jahangir Alam and Dr. Shahin Alam, BLRI) **Inauguration**			Status of PPR in Bangladesh (Chief Veterinary Officer, Bangladesh)	Lunch	Introduction about BLRI DG BLRI	Tea	Role of Laboratory networks in Epidemiology & Disease surveillance (Dr Venkata Subbarao Mandava, FAO)	An Overview of PPR, general guideline for sample collection, transportation & preservation(Prof. E. H. Chowdhury, BAU)
8th October /Monday	Recap	Principles of PCR and Guideline for good laboratory practices (Prof. Md. Rafiqul . Islam, BAU)	Tea	**Practical** **RT-PCR: RNA extraction : Master mix/thermal cycling** **Group A:** Dr. Md Giasuddin, Dr. Papia and Mr. Amal Saha **Group B:** Dr. Jahangir Alam, Dr. Ataur Rahman Bhuyian and Mr. Mukles		Lunch	**Practical** **RT-PCR: gel preparation, electrophoresis/result interpretation** **Group A:** Dr. Md Giasuddin, Dr. KBM Saiful and Mr. Amal Saha **Group B:** Dr. Jahangir Alam, Dr. Ataur Rahman Bhuyian and Mr. Mukles			
9th October / Tuesday	Recap	Filter paper method of sample collection for virus detection (Dr. Ataur Rahman Bhuiyan, FAO)	Tea	**Practical** **RT-PCR using Filter paper as source of sample** **Group A:** Dr. Enamul Haque, Dr. KBM Saiful and Mr. Amal Saha **Group B:** Dr. Jahangir Alam, Dr. Ataur Rahman Bhuyian and Mr.		Lunch	**Practical** **RT-PCR: electrophoresis/result interpretation** **Group A:** Dr. Enamul Haque, Dr. Papia and Mr. Amal Saha **Group B:** Dr. Jahangir Alam, Dr. Ataur Rahman Bhuyian and Mr. Mukles			

				Mukles		
10th October / Wednesday	Recap	Principles of ELISA (Prof. Abdul Ahad, Chittagong)	Tea	**<u>Practical</u>** **cELISA for antibody detection** **Group A:** Dr. Md Giasuddin, Dr. Papia and Ms. Suchitra **Group B:** Dr. Jahangir Alam, Dr. Ataur Rahman Bhuyian and Mr. Mukles	Lunch	**<u>Practical</u>** **cELISA for antibody detection** **Group A:** Dr. Md Giasuddin, Dr. Md. Rafiqul Islam and Ms. Suchitra **Group B:** Dr. Jahangir Alam, Dr. Ataur Rahman Bhuyian and Mr. Mukles
11th October / Thursday	Recap	Lab Bio-safety and biosecurity (Dr. Asadul Gani, ICDDR,B)	Tea	**<u>Practical</u>** **Immunocapture ELISA (icELISA)** **Group A:** Dr Md RafiquelIslam, Dr.Papia and Ms. Suchitra **Group B:** Dr. Jahangir Alam, Dr. Ataur Rahman Bhuyian and Mr. Mukles	Lunch	**<u>Practical</u>** **Immunocapture ELISA (icELISA)** **Group A:** Dr. Md Giasuddin, Dr. Papia and Ms. Suchitra **Group B:** Dr. Jahangir Alam, Dr. Ataur Rahman Bhuyian and Mr. Mukles
12th October / Friday	Recap	Post evaluation (Dr. Jahangir Alam and Dr. Md. Nuruzzaman) General Discussion (All members)	Tea	**<u>Outbreak investigation</u>** Dr. Md Giasuddin/Dr. Jahangir Alam/Dr. Ataur Rahman Bhuyian/Dr. Papia/Mr. Amal Saha	Lunch	Closing

Annex- 2

List of participants

BANGLADESH

Dr Jahangir Alam
Senior Scientific Officer (Virology)
SAARC Regional Leading Diagnostic Laboratory for PPR
Bangladesh Livestock Research Institute
Savar, Dhaka
Tel: +88-02-7791670 ext 234
Mobile : +88-01712819098
Fax: +88-02-7791674
Email: alamjahan2003@yahoo.com; pprblrifp@yahoo.com

Dr Md Giasuddin
Senior Scientific Officer
National Reference Laboratory for Avian Influenza
Bangladesh Livestock Research Institute
Savar, Dhaka
Tel: +88-027791670-3 ext 225
Mobile: +8801711055597
Fax: +88-02-7791675
Email: mgias04@yahoo.com

Dr. Bidhan Chandra Das
Principal Scientific Officer
Central Disease Investigation Laboratory
48, Kazi Allauddin Road, Dhaka
Tel: +88-01711207178
Fax: +88-2-7312483
Email: bidhan07@yahoo.com

Dr Md. Belal Hossain,
Senior Scientific Officer,
Field Disease Investigation Laboratory
Joypurhat
Tel: +88 - 057162509
Mobile: +88-01716205381
Email: belalhossain@gmail.com

BHUTAN

Dr Basant Sharma
Animal Health Specialist
Regional Livestock Development Centre
Tsimasham, Chukha
Tel: +975 8 478779
Mobile: +97517702045
Email: sbasant86@yahoo.com

Mr Sangay Tenzin
Laboratory Officer
National Centre for Animal Health
Serbithang, Thimpu
Tel: +975-2-351085
Mobile: +975-17713069
Fax: +975-2-351095
Email: wamrongsangaytenzin@gmail.com

INDIA

Dr Asimabha Batobyal
Joint Commissioner (LP)
Department of Animal Husbandry, Dairying and Fisheries
Ministry of Agriculture, Government of India,
Room No 248C, Krishi Bhavan
New Delhi 110 001
Tel: +91 11 23389620
Mobile: +91 9891222920
Email: jclp@nic.in

Dr. Akhilesh Kamal
Director (I&C)
Department of Animal Husbandry, Dairying and Fisheries
Ministry of Agriculture, Government of India,
Room No 479, Krishi Bhavan
New Delhi 110 001
Tel: Tel: +91 11 23383699
Mobile: +91 9810179085
Email: akhikamal@rediffmail.com

MALDIVES

Dr G G I A Jayawickrama
Consultant Veterinarian
Division of Agriculture & Forestry
Ministry of Fisheries and Agriculture
Velaanaage, Ameer Ahmed Magu
Male
Tel: +960-3322625
Fax: +960-3326558
Mobile: +960-7880299
Email: jayawickrama64@yahoo.com; Jayawickrama@fishagri.gov.mv

NEPAL

Dr (Ms) Sulochana Shrestha
Senior Veterinary Officer
Central Biological Production Laboratory
Directorate of Animal Health
Department of Livestock Services
GPO Box 7957
Tripureshwar, Kathmandu
Tel:+ 977-1-4252348 (W)
+977-1-4215483 (H)
Mobile: +977-9841284927
Email: sulochanashrestha2@yahoo.com

Dr Krishna Raj Pandey
Veterinary Officer
Central Veterinary Laboratory
Directorate of Animal Health
Department of Livestock Services
Tripureshwar, Kathmandu
Tel: +977-1-4212143
Fax: +977-1-4261867
Mobile: +977-9849337320
Email: drpandeykr@gmail.com

PAKISTAN

Dr Aamer Bin Zahur
Principal Scientific Officer
Animal Health
Animal Sciences Institute
NARC, Park Road, Islamabad
Tel: +92-51-9255029
Mobile:+92-3009502369
Email: aamerabz@yahoo.com

Dr Abdul Latif Shahid
Research Officer
Animal Quarantine Department
Sialkot
Tel: +92-512248254
Mobile: +92-3336563365
Email: shahid_862@yahoo.com

SRI LANKA

Dr (Ms) J K H Ubeyrathne
Veterinary Surgeon
Central Veterinary Investigation Centre
Veterinary Research Institute,
Gannoruwa,
Peradeniya
Tel: +94-812-388311 (W)
Mobile: +94-777-148410
Email: harshiniuj@yahoo.com

Dr (Ms) Amutha Prabaharan
Veterinary Investigation Officer
Veterinary Investigation Center,
Dept. of Animal Production & Health, No 54,
Cemetery Road, Fort, Jaffna
Tel: +94-21-2227446 (W)
Mobile: +94-77-0222303
Email: vicjaffna@yahoo.com

REGIONAL SUPPORT UNIT/SUB-REGIONAL ECTAD, FAO, NEPAL

Dr Venkatasubbarao Mandava
Laboratory Coordinator
Regional Support Unit/Sub Regional
ECTAD Unit (SAARC),
FAO Nepal/ UN House
Pulchowk, Kathmandu
Tel: +977-1- 5010313/14/15, Ext. 113
Fax: +977-1-5010312
Mobile: +977-9801020231
E-mail : Venkatasubbarao.Mandava@fao.org

FAO, BANGLADESH

Dr Mat Yamage
Senior Technical Coordinator
Food and Agriculture Organization of the United Nations
Avian Influenza Technical Unit
Department of Livestock Services
3rd Floor, DLS Main Building
Krishi Khamar Sarak, Farmgate
Dhaka 1215, Bangladesh
Mobile: +880-1747619630
Email: mat.yamage@fao.org

Professor Nitish C Debnath
National Consultant, Outbreak Response and Laboratory Expert
Food and Agriculture Organization of the United Nations
Avian Influenza Technical Unit
Department of Livestock Services
3rd Floor, DLS Main Building
Krishi Khamar Sarak, Farmgate
Dhaka 1215, Bangladesh
Mobile: +880-1712079147
Email: nitish.debnath@fao.org;
principalcgvc@yahoo.com

Dr Md. Ataur Rahman Bhuiyan
National Consultant-Senior Scientific Officer
Food and Agriculture Organization of the United Nations
SAARC Regional Leading Diagnostic Laboratory-PPR
Bangladesh Livestock Research Institute
Savar, Dhaka
Mobile: +88-01715-795856
E-mail: ataur.bhuiyan@yahoo.com

Annex- 3

A Manual for Laboratory Diagnosis of Peste des Petits Ruminants (PPR)

Standard Operating Procedures

SAARC Regional Leading Diagnostic Laboratory for PPR
Bangladesh Livestock Research Institute
Ministry of Fisheries and Livestock

Bangladesh

South Asian Association for Regional Cooperation (SAARC)

September 2011

1. PPR sample collection, preservation and transportation guide line

From live animal

Tears/ nasal swab

- Select the animals those are in acute phase of disease, take sample as many as possible (5-6 animals from an outbreak)

- Use a cotton/dacron swabs to collect the specimen. Collect swab from conjunctiva or posterior nasal cavity avoiding external secretion. Keeping swabs moist after collection is most important. Place swab in 1.5-2ml Viral Transport Media (VTM). Any sterile isotonic fluid, like phosphate buffered saline (PBS) or common tissue culture medium like Eagle's MEM or Glycerin-PBS (50:50) with antibiotics (penicillin 200 IU/ml and streptomycin 200µg/ml) can be used. Commercially available kits containing swabs and viral transport media are also acceptable. Swabs may be broken off and shipped with media. Alternatively, swirl/ agitate the swab in the media for several minutes before removal.
- Or place the swab samples in 100% ethanol if other media is not available. Samples with ethanol can be only used in PCR.

Gum debris

- This material can be collected by a spatula or finger rubbed across the gum and inside the upper and lower lips. The material collected is then scraped into a container with 1.50-2 ml of PBS or any VTM as above.
- Or place the materials in 100% ethanol for PCR

Blood sample

- Collect blood from Jugular vein aseptically in heparinized tubes at acute phase of the disease, store at chilled condition.

Sampling with filter paper

- Filter (Whatman filter paper, Grade1, 5 to 6 cm in length and 0.25 cm in width) paper can be soaked with blood (pour the blood dropwise from bottom towards the tip) and smeared with nasal secretions. Air dried the filter paper avoiding direct sun light and put them in eppendorf tube or polyethane pouch.

Serum Samples

- For sero- prevalence study, sample should be collected considering different age group,

  For example- if a herd size is 25 goat, 20 sample should be collected in the order of, 8 from goats age < 1years old, 8 from 1-2 years old and 4 from >2 years old.

- For diagnostic purpose from a PPR suspected herd, paired sample is preferred, one is at beginning of out break (acute phase of the disease) and other is after 10 - 15 days later (Convalescent stage).
- Collect the blood with 5ml sterilized syringe from Jugular vein (after swabbing with 70% alcohol or iodine swab) in a gentle manner.
- Collect 5 ml of blood, hold the blood for few minutes to clot (Please turn piston little further and put the syringe 30^0 angle)
- Put the entire specimen in vaccine carrier/cool box in the field with ice pack.
- You can put the syringe with clotted blood for overnight at 4^0C (Max.12 hour)
- Separate serum from the clot and put them in sterile pre- marked eppendorf tube

Tissues from dead animals

The following tissues should be collected during post mortem examination:

- Perform post- mortem as early as possible and record all post mortem changes
- Lymph nodes found around the lungs (mediastinal) and alimentary tract (mesenteric)
- Part of the lung (Lower part of anterior or cardiac lobe) and spleen can be taken to glass or non-toxic plastic container.

Packaging & Transport

- Primary container depending on sample type
- Secondary container (Air tight Box/Bottle/Tube/ polythene pouch and envelope)
- Cool box/ vaccine carrier
- Wet ice or Ice packs (put ice packs in deep freezer before transport)
- Pen or permanent marker for labeling samples

Precautions for specimen storage and transport

- Specimens for virus isolation should be refrigerated immediately after collection, if facilities does not permit keep them chilled (at 2 to 4^0C) in refrigerator or with wet ice and shipped to the laboratory as soon as possible (within 48 hours)

- Keep swab in chilled condition for short period (Maximum 4 days at 4^0C) and don't store swab sample at -20^0C. For longer storage keep them at -70^0C.
- Filter paper sample can be kept at normal temperature and can be sent to the PPR lab by normal post
- Serum sample can be kept at 2^0C or 4^0C for maximum period of two days and for longer period store the sample at -20^0C
- Specimens for PCR test should be kept in chilled condition in refrigerator or with wet ice for short time (48 hours), if specimens cannot be processed immediately; they should be kept -20°C for short period or below at -70°C for longer period. When refrigeration facilities are unavailable preserve the specimen in Tryzol reagent or 100% ethanol in screw cap container store at 4°C and shipped to the laboratory as soon as possible
- Use cool box with icepacks for transporting the specimen
- Send the specimen preferably through messenger
- Always avoid freeze thaw cycle

Storage of samples in different conditions:

Storage condition	Swab or other specimen in VTM for virus isolation	Swab or other specimen in VTM for PCR	Swab or other specimen in Ethanol for PCR**	Blood serum for virus isolation	Blood serum for PCR	Blood serum for antibodies
-70^0C or liquid N_2 or dry ice	SR	SR	N/A	SR	SR	SR
-20^0C	NR	A	N/A	NR	A	SR
+4^0C	A*	A	A	A***	A	A
Room temperature	NR	A	A	NR	A*	A*
Dried blot spot on filter paper	N/A	N/A	N/A	N/A	A	A

SR=strongly recommended, NR=not recommended, N/A= not applicable, A=recommended method, A*= for upto 7 days storage, **=where refrigerator is not available, A***= storage upto for 4 days

Labelling and Packaging of specimen for transportation and storage (Label each specimen with):

- Specimen type/name e.g. Blood, swab, PM tissue samples
- Put an identification number
- Place of collection
- Date and time of collection

Use same information about specimen

- On the specimen container
- On the field data collection form (PPR outbreak report form)

Receipt at Laboratory

- Follow appropriate accessioning protocol for recording the receipt of any sample. No sample should be brought /kept in the laboratory without record.
- Decontaminate the surface of cool box or container containing infectious materials with a disinfectant before bringing into the laboratory. Open the box only at the designated place, such as inside a cabinet/ biosafety cabinet, as appropriate.

2. Standard operating protocol for RT-PCR technique for PPR virus detection

Purpose

The SOP manual on RT-PCR technique for PPR, outlines the various steps followed in collection and amplification of RNA from tissue or swab samples submitted to Regional Leading Diagnostic Laboratories (RLDL), Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka, Bangladesh.

Safety

Containment Level BSL –II should be used for over all laboratory practice. Handling of PPR suspected samples should be carried out under a Biosafety Cabinet II. PPR is not zoonotic disease but sample must be handling with care using Personal Protection Equipment (PPE) like Lab coat, disposable gloves.

General Good Laboratory Practices (GLP)

The following general rules must be observed in the laboratory to ensure laboratory discipline, biosafety and biosecurity:

- . The laboratory supervisor must enforce the institutional policies that control access to the laboratory
- Persons must wash their hands after working with potentially hazardous materials and before leaving the laboratory.
- Wear protective lab coats during stay in the laboratory.
- Eating, drinking, smoking, handling contact lenses, applying cosmetics, and storing food for human consumption must not be permitted in laboratory areas. Food must be stored outside the laboratory area in cabinets or refrigerators designated and used for this purpose.
- Decontaminate work surfaces after completion of work and after any spill or splash of potentially infectious material with appropriate disinfectant.
- Do not leave any used glassware, gloves, etc. on the bench
- Label all prepared media and reagents with full name of the item, date of preparation / opening / aliquoting, specifications (molarity/ percentage / pH / supplementation, amount, etc) and the name of the person who prepared it
- Take appropriate precaution before handling any chemical or reagent, e.g., use hand gloves, goggles, mask, biosafety cabinet, follow aseptic measures, as required.

- Return all chemicals, reagents and appliances to appropriate storage place after use
- Do not move unnecessarily between laboratory rooms
- Avoid sharing laboratory supplies and appliances (pipettes, tips, tubes, etc.) between laboratories designated for different types of activities

Procedure

Sample preparation

- Tissues (lung, lymph nodes) pooled, weighed and macerated with sterile mortar and pestle while the tissues are still frozen. Add PBS to make 20% (w/v) suspension and collect in a sterile tube. Centrifuge tissue suspension at 3000 rpm for 10 minutes. Collected supernatant in fresh sterile Falcon tubes with antibiotic (add Gentamycin at 50µg/ml), process for testing or store at -20^{0}C or - 70^{0}C.
- Swirl/ agitate the swab stick in the media for several minutes before removal and Centrifuge suspension at 5000 rpm for 5-10 minutes, remove the supernatant in a fresh Eppendorf tube, process the samples or store at -20^{0}C or - 70^{0}C.

Materials required for conventional RT-PCR protocol

- QIAamp Viral RNA Mini Kit or equivalent extraction kit
- QIAGEN One-step RT-PCR kit or other kit
- Primers
- RNase inhibitor
- TAE or TBE buffer
- Agarose
- Ethidium bromide
- Gel loading buffer
- Appropriate DNA marker (100kb)
- Positive control
- Adjustable pipettes 0.5 - 10µl, 2- 100µl and 100-1000µl with disposable filter tips fit with the pipettes
- Micro-centrifuge, adjustable to 12000-15000 rpm
- Microcentrifuge tubes (0.2, 1.5ml)
- Vortex mixer
- Thermocycler
- Micro-weave oven

- Agarose gel casting tray, electrophoresis chamber and power supply
- UV-light box (302nm)

RNA isolation

Viral RNA is isolated from the tissue suspension or swab samples using RNeasy Kit (Qiagen, Germany) as recommended by the manufacturer.

- Take 450µl of RLT buffer (1ml of stock RLT buffer is added with 10µl of 2-mercaptoehthanol) into an Eppendorf tube.
- Add 250µl tissue suspension, vortex and incubate for 15 minutes at room temperature.
- Add 700µl of 70% ethanol, mixed and transfer to an RNeasy spin column placed in a 2 ml collection tube.
- Transfer 700µl mixture into spin column, centrifuge at 10,000 rpm for 30 sec.
- Discard the flow-through at the collection tube and set the spin column again in the collection tube
- Take the rest 700µl mixture and repeat the procedure as above
- Add 700µl of RW1 buffer to the column, centrifuge as above and discard the flow-through.
- Add 500µl of RPE (Stock RPE is added with 4 volumes ethanol) into the spin column
- Centrifuge for 30 sec at 10,000 rpm.
- Discard flow-through, again add 500µl of RPE and centrifuge for 2 min at 10,000 rpm.
- Place the spin column into a 1.5ml Eppendorf tube
- Add 30-50µl of RNAse free water into the centre of the spin column.
- Centrifuged for 1 min at 10,000 rpm and discard the RNeasy spin column
- Label Eppendorf tube containing RNA
- Store RNA at -20ºC or at -70ºC for short term and long term storage, respectively.

Quantification and quality assessment of RNA

- Quantify RNA by spectrophotometer at 260nm and 280nm (analysis using the convention that one absorbance unit at 260nm wavelength equals 40µg RNA per/ml).
- Check the absorbance at 260 and 280nm for determination of RNA concentration and purity. Purity of RNA is judged on the basis of optical density ratio at 260:280nm.

- Select the samples with acceptable purity (*i.e.* ratio 1.7 - 2.0) and quantify RNA using following formula and use for subsequent analysis.
  Concentration of RNA (µg/µl) = OD260 × Dilution factor × 40/1000

Reaction mixture (Using the Qiagen One-step RT–PCR kit)

Master mix	Nuclease free water	12 µl
	5x Qiagen One-step RT-PCR buffer	05 µl
	dNTP Mix (10 mM of each dNTP)	01 µl
	Primer Forward (100 pmol/µl)	0.5 µl
	Primer Reverse (100 pmol/µl)	0.5 µl
	Qiagen One-step RT-PCR Enzyme Mix	1.0 µl
Template RNA		5µl
Total		25µl

RT-PCR specific for F gene

Primer

Gene	**Primer**	**Sequence**	**Position**	**Size**	**References**
F gene	PPRV F1b	5´-AGTACAAAAGATTGCTGATCACAGT-3´	760-784	448 bp	Ozkul,2002
	PPRV F2d	5´-GGGTCTCGAAGGCTAGGCCCGAATA- 3´	1207-1183		

Thermal Profile

	RT Step	Initial denaturation	Denaturation	Annealing	Elongation	No. of cycles	Final elongation
Temp	50^0 C	95^0 C	94^0 C	50^0 C	72^0 C	35	72^0 C
Time	30 min	15 min	1 min	1 min	2 min		7 min

RT-PCR specific for N gene

Primer

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		

Thermal Profile

	RT step	Initial denaturation	Denaturation	Annealing	Elongation	No. of cycles	Final elongation
Temp	50^0 C	95^0 C	94^0 C	55^0 C	72^0 C	35	72^0 C
Time	30 min	15 min	30 sec	30 sec	30 sec		10 min

Analyses of RT-PCR products by agarose gel electrophoresis

- Prepare 1.0-1.5% Agarose gels (w/v) in 1x TAE or TBE buffer.
- Add ethidium bromide to the agarose solution at 0.5 µg/ml.

- Pour the agarose solution containing ethidium bromide into the gel casting tray.
- When the gel sets completely, remove the comb and transfer the gel into the electrophoresis tank containing 1x TAE or TBE buffer.
- Mix PCR product with loading dye (4.0 vol. of PCR product with 1.0 vol. of 5x Bromophenol blue/Xylene cyanol) and load them into individual slot.
- Load the DNA size marker into to one slot.
- Perform electrophoresis at 100 V for 30-40 min.
- After running wash the gel briefly in running tape water and observe under UV transilluminator.
- Observe and document the result.

Trouble shooting of PCR

Observation	**Possible Cause**	**Solution**
	Incorrect annealing temperature	Recalculate primer Tm values Test an annealing temperature gradient, starting at 5°C below the lower Tm of the primer pair
	Poor primer design	Verify that primers are non-complementary, both internally and to each other
	Poor primer specificity	Verify that oligos are complementary to proper target sequence
	Missing reaction component	Repeat reaction setup
	Suboptimal reaction conditions	Optimize annealing temperature by testing an annealing temperature gradient, starting at 5°C below the lower Tm of the primer pair
	Poor template quality	Check 260/280 ratio of DNA template
	Presence of inhibitor in reaction	Further purify starting template by alcohol precipitation, drop dialysis or commercial clean up kit decrease sample volume
	Contamination of reaction	Reaction tubes prior to use to eliminate biological inhibitors. Prepare fresh

No product	tubes or solutions	solutions or use new reagents and new tubes
	Incorrect template concentration	For low complexity templates (i.e. plasmid, lambda, BAC DNA), use 1 pg–10 ng of DNA per 50 µl reaction For higher complexity templates (i.e. genomic DNA), use 1 ng–1 µg of DNA per 50 µl reaction
Multiple or non-specific products	Primer annealing temperature too low	Increase annealing temperature
	Poor primer design	Verify that primers are non-complementary, both internally and to each other
	Excess primer	Primer concentration can range from 0.05–1 µM in the reaction.
	Incorrect template concentration	For higher complexity templates (i.e. genomic DNA), use 1 ng–1 µg of DNA per 50 µl reaction
	Contamination with exogenous DNA	Set-up dedicated work area and pipettor for reaction setup Wear gloves during reaction setup
Incorrect product size	Incorrect annealing temperature	Recalculate primer Tm values
	Mispriming	Verify that primers have no additional complementary regions within the template DNA
	Improper Mg^{++} concentration	Adjust Mg^{++} concentration in 0.2–1 mM increments
	Nuclease contamination	Repeat reactions using fresh solutions

Special practices for PCR lab

- Keep pre-PCR (extraction), PCR (master mix & amplification) and post-PCR (analysis) work as isolated as possible.
- Strictly adhere to the work flow for pre-PCR, PCR and post-PCR activities. Take utmost precaution to avoid carry-over or cross contamination of PCR reagents with (a) previously amplified PCR products, or (b) extracted DNA/RNA

- Use separate sets of equipment, appliances and supplies for different steps of PCR. Do not move pipette, tips, tubes, marker, pen, note books, etc. between pre-PCR, PCR and post-PCR sections
- Store consumables, reagents and water separately for pre-PCR, PCR and post-PCR work. Aliquot reagents and water in convenient volumes.
- Sharing of reagents and supplies among a group of researchers must be based on mutual trust and understanding under strict control of a single leader.
- Always use disposable gloves and change gloves frequently based on logical judgment. Consider all surfaces potentially contaminated and avoid touching them wearing gloves
- Plan and organize work before beginning a test. Do all calculations; fill in worksheets; ensure availability of all reagents and supplies at respective work areas before start
- Never reverse work-flow. Ideally, nothing should come back from the post-PCR section to the pre-PCR or PCR sections without proper decontamination. Never bring cDNA to the extraction and master-mix room
- Clean and wipe all work surfaces with 70% alcohol before and after each session
- Briefly pulse-centrifuge tubes to collect reagents at the bottom before opening
- Do not leave reagents on the bench (even on ice) any longer than necessary
- Use only freshly prepared ice flakes. Do not freeze ice for re-use Archive all information about new clones and newly synthesized primers

Waste disposal

BIOHAZARDOUS WASTE includes any waste that is infectious or, because of its physical and/or biological nature, may be harmful to humans, animals, plants or the environment. Bio-hazardous waste includes:

a. Animal waste known or suspected of being contaminated with a pathogen
b. Bulk blood or blood products
c. Microbiological waste
d. Pathological waste
e. Infectious waste
f. Waste products of recombinant DNA biotechnology and genetic manipulation
g. Sharps

Waste handling and disposal procedure

- Collect all sharp items (needle, blade, broken ampoule) in sharp disposal cans. When full, dispose the can by incineration.
- Collect all micropipette tips and Eppendorf tubes in tip disposal container. When full, empty the container in a poly bag and dispose by incineration.
- Collect the biodegradable solid waste in large volume of freshly prepared disinfectant (sodium hypochlorite, virkon), leave overnight and dispose as garbage.
- Collect used electrophoresis gel in plastic lined open container, allow them to dry out and then dispose by incineration
- Decontaminate infectious liquid waste in large volume of freshly prepared disinfectant (sodium hypochlorite, virkon), leave overnight and dispose in sanitary sewer
- Collect all used glassware and plasticware (except tips and Eppendorf tubes) in a bucket containing disinfectant (sodium hypochlorite, virkon), leave overnight and proceed for cleaning & sterilization for disposal. Cleaning & sterilization of used glassware must be completed in the shortest possible time

3. Detection of PPRV antibody in sera by competitive ELISA (cELISA)

1. Introduction

Competitive ELISA (cELISA) based on monoclonal antibodies specific for N-protein and H-protein were developed for detection of antibodies in animal sera. Goats and Sheep experienced PPRV infection at younger age remained sero-positive for 1-2 years following exposure. The cELISA has been widely used to detect PPR antibodies in many countries. In the N-protein cELISA, the serum antibodies and the MAb compete on specific epitope on nucleoprotein generated through recombinant technology using baculovirus expression vector system. The absorbance in PPR ELISA is converted to percentage of inhibition (PI). Sera showing PI greater than 50% are scored positive

2. Background

Peste des petits ruminants (PPR) is a highly contagious and infectious viral disease of domestic and wild small ruminants. Small ruminant production and therefore the livelihoods of poor farmers are threatened by Transboundary animal diseases (TADs) such as PPR in many areas of Asia and Africa. This disease inflicts severe economic losses of livestock of Bangladesh every year. Because of its high mortality rate, PPR affects food security directly by reducing the availability of meat and milk for family consumption and of funds for purchasing other commodities. So control of this disease is a priority of the area.

3. Purpose of activity

To detect PPR antibody in blood sera of susceptible animals (goats, sheep and others).

4. Safety

Standard microbiological laboratories facilities (Containment level BSL-I facilities and precaution). Personal Protection Equipment (PPE) include: Lab coat, disposable gloves.

The person(s) performing the procedure should be fully trained and competent.

5. Sample:

- For sero- prevalence study sera samples should be collected considering the different age group, for example- if a herd size is 25 goat, 20 sample should be collected in the order of, 8 from goats age < 1years old, 8 from 1-2 years old and 4 from >2 years old.
- For diagnostic purpose, paired sample is preferred from a PPR suspected herd, one is at beginning of out break (acute phase of the disease) and other is after 10-15 days later (Convalescent stage).

6. Requirements for sample collection:

- 70% Alcohol/ iodine swab
- 5ml sterilized disposable syringe
- Eppendorf tube
- Centrifuge Machine
- Pipette (100µl-1000µl capacity with tips)
- wet ice or Ice pack
- Vaccine carrier or suitable carrier

7. Collection of sample:

- Collect blood with 5ml sterilized syringe from Jugular vein after cleaning with 70% alcohol or iodine in a gentle manner.
- Collect 5ml of blood, hold the blood for half an hour for serum separation (Please turn piston little further and put the syringe 30^0 angle), and put the entire specimen in carrier/cool box in the field with ice pack.
- Separate serum from the clot and put them in sterile pre- marked eppendorf tube

8. Sample labeling:

- Specimen type/ name e.g. Serum
- Put unique identification number
- Place of collection
- Date and time of collection

9. Sample storage and Transport:

- If needed keep the sample at 2^0C or 8^0C for maximum period of two days
- Send the sample for testing as early as possible
- For longer period store the sample at -20^0C

10. Requirement for a serology lab

- Flat-bottomed Nunc Maxisorb ELISA plates
- Multichannel spectrophotometric ELISA plate reader with interference filters of 492 nm.
- Incubator with shaking facilities (37^oc to 39^oC)
- Centrifuge with rpm up to 13000 (Rotor fixed with Eppendorf tube)
- Refrigerator any type with 2^oC to 6^oC
- Freezer (-20^oC)
- Pipettes i. 0.5–20µl ii. 20µl-200µl ., 100µl-1000µl, Multichannel pipette 5µl-50µl with appropriate tips
- Measuring cylinder
- Rack, Beaker, Funnel, Reservoir
- Falcon tube (15ml)
- Eppendorf tube
  - Aluminium foil
  - Cotton
  - Elisa kit
  - PBS
  - Tween 20
  - Conc. Sulphuric acid
  - Waste disposal (1. Disinfectant e.g. 10% hypochlorite sol, Virkon, 2. waste container, waste disposal bag etc)
  - Autoclave facilities

Reagents and test protocol

11.1. Preparation of reagents:

11.1.2. N-PPRV antigen:

11.1.3. Preparation of antigen stock: Freeze dried cell culture derived recombinant N-PPRV antigen is usually supplied in 0.5ml glass vials.

- Thawed it once and reconstitute with 0.5ml distilled by the supplied water and mixed gently until completely dissolved.
- Aliquoted the antigen stock in a 100ul volume in cryo-vials and stored the vials at - 20°C until use.

11.1.4. Preparation of 6 ml working solution (Working dilution: 1: 3000):

Antigen Stock solution : 2 µl

PBS 1 X : 5998µl

11.2.1. Anti-PPRV monoclonal antibody:

11.2.2. **Preparation of anti-PPRV monoclonal antibody stock:** Freeze-dried Anti-PPRV monoclonal antibody is usually supplied by the manufacturer in 0.5ml glass vials.

- Thawed it once and reconstitute with 0.5ml distilled water and mixed gently until completely dissolved.
- Aliquoted the antibody stock in a 100µl volume in cryo-vials and stored the aliquoted vials at - 20°C until use.

11.2.3. Preparation of 6 ml working solution (Working dilution: 1: 150):

Monoclonal antibody stock solution : 40µl

Blocking buffer : 5960µl

12.3.Conjugate:

12.3.1. **Preparation of conjugate stock:** Rabbit anti-mouse immunoglobulin HRPO conjugate (Dako, P 260) is supplied by the manufacturer in 1ml glass vials.

- Aliquoted the HRPO conjugate was further subdivided into 100µl in 1ml cryo-vials and stored at -20°C until use.

12.3.2. **Preparation of 5 ml working solution** (Working dilution: 1: 1000)

Conjugate stock solution: 5 µl

Blocking buffer : 4995µl

12.4. Preparation of control serum stock:

Freeze dried C++ anti PPRV antibody positive(strong), C+ anti PPRV antibody positive(weak) and C- anti PPRV negative antibody, control serum stock (and C-) is supplied by the manufacturer in 1 ml glass vials.

- Thawed them once and reconstitute with 1 ml distilled water and mixed

gently until completely dissolved.

- Aliquoted all the controls in a 100µl volume in cryo-vials and stored the aliquoted vials at -20°C until use.

12.5. Chromogen and Substrate:

12.5.1. **Preparation of chromogen stock solution:**

- Dissolve the supplied one 30 mg OPD tablet in 75ml de-ionized water and aliquot them in 10 ml volume in falcon tube.
- Stored aliquoted tubes at -20°C until use.

12.5.2. **Preparation of substrate stock solution:**

- Dissolve supplied one Per-hydride tablet in 10ml de-ionized water (3% solution, 882 mM) in falcon tube and stored at 4^0C until use.

12.5.3. **Preparation of working solution**:

Substrate stock : 40µl

Chromagen stock (OPD solution) : 9960µl

Working solution was prepared immediately before use.

12.6.1. Preparation of PBS (1X):

NaCl	: 8 g
KCl	: 0.2 g
Na_2HPO_4 anhydrous	: 1.44 g
KH_2PO_4	: 0.24 g

- Dissolve above chemicals in 800 ml distilled water to make the volume upto 1 liter and adjust pH to 7.2- 7.4.

12.7. Coating buffer:

12.7.1. Preparation of Coating buffer stock solution:

- Prepared 1x Phosphate buffered saline stock solution (pH 7.4) store at 4^oC temperature after sterilization.

12.8. Preparation of blocking buffer working solution:

- Phosphate buffered saline (1X) (pH 7.4) + 0.05% (v/v) Tween 20 + 0.5 % negative lamb serum.
- Preparation of 20 ml blocking buffer:

PBS 1X : 19890µl

Tween 20 (0.05%) : 10µl

Negative lamb serum (0.5%) : 100µl

Fresh buffer were prepared and used every day.

12.9. Preparation of washing buffer working solution:

- M Phosphate buffered saline (pH 7.4) + 0.05% (v/v) Tween 20.
- Preparation of 1000 ml washing buffer:

PBS (0.2M) : 999.5 ml

Tween 20 (0.05%) : 0.5 ml

12.10. Preparation of stopping solution:

- Use 1 M Sulfuric acid as stopping solution.
  Preparation of 500 ml stopping solution:

Concentrated sulfuric acid : 27.5 ml

Distilled water : 472.5 ml

- Add slowly concentrated sulphuric acid to distilled/deionised water.
- Store prepared solution at room temperature.

13. Plate layout:

i. **Conjugate control (Cc)**: Wells A1 and A2 are for the conjugate control; they contain only N-PPR antigen and conjugate.
ii. **Strong positive control (C++):** Strong positive control includes wells (B1, B2, C1 and C2) which contain N-PPR antigen, strong positive serum, mAb and conjugate.
iii. **Weak positive control (C+)**: Weak positive control includes wells D1, D2 E1 and E2 which consist of N-PPR antigen, weak positive serum, mAb and conjugate.
iv. **MAb control (Cm):** MAb control includes wells F1, F2, G1 and G2 which consist of N-PPR antigen, mAb and conjugate.

v. **Negative control (C-):** Negative control includes wells (H1 and H2) with N-PPR antigen, negative serum, mAb and conjugate.
vi. **Serum samples:** 40 sera were tested in duplicate.

14. Test protocol:

i. Dilute N-PPR antigen in PBS according to a dilution factor (which depends on the antigen batch number of manufacturer). Add 50µl of antigen solution all wells of the plate; tap the side of the microplates to ensure that the antigen is evenly distributed over the bottom of each well. Incubate 1 hour at 37°C on an incubator with shaking (150-160 rpm) facilities.
ii. Wash the plate three times (5min each) in washing buffer and blot dry.

iii. Add 45µl of blocking buffer to all wells of the plate. Then further add:

- 5µl of blocking buffer to the monoclonal control wells (F1, F2, G1 and G2) and then add 5µl MAb to each well ((F1, F2, G1 and G2)
- 55µl of blocking buffer to the conjugate control wells (A1 and A2).
- 5µl of test serum to test wells.
- 5µl of strong positive control to control wells (B1, B2, C1 and C2).
- 5µl of weak positive serum to control wells (D1, D2, E1 and E2).
- 5µl of negative serum to control wells (H1 and H2).
- 50µl of monoclonal antibody (batch 02/09), diluted (as per instruction of the kit) 1/150 in blocking buffer to all wells of the plate except the conjugate control (A1 and A2).
- Incubate 1 hour at 37°C on incubator with shaking facilities.

iv. Wash the plate three times with washing buffer and blot dry.

v. Add 50µl of anti-mouse conjugate (diluted 1/1000 in blocking buffer) and incubate 1 hour at 37°C on incubator with shaking facilities.

vii. Wash the plate three times as before.

viii. Add 50µl of substrate/chromogen mixture in each well (Prepare the OPD solution adding H_2O_2 3% solution just before use. Keep the final substrate/chromogen solution at 4°C in the dark until use, it safe to wrap the tube with aluminum foil).

ix. Stop the colour development after 10 minute by adding 50µl of 1M H_2SO_4. Turn on the microplate reader and allow warming up for at least 15 minutes before reading.

x. Read OD value with an ELISA reader at 492nm.

15. Calculate the percentage inhibition (PI) (for quality assurance acceptance) using the formula:

PI = 100 - [(Replicate OD of each Control/ Mean OD of the Cm) x 100]

16. Acceptance criteria for control data (UCL=Upper control limit, LCL=Lower control limit)

Cm values		UCL	LCL
	OD Values	1.500	0.500
		UCL	UCL
Cc	PI values	+105	+90
Cm	PI values	+20	-19
C++	PI values	95	80

C+	PI values	79	50
C-	PI values	30	5

17. Calculate the percentage inhibition (PI) of samples (inhibition of mAb binding in the presence of serum) using the formula:

PI = 100 - [(OD of the test wells/ OD of the Cm wells) x 100]

Sera showing PI greater than 50% considered to be PPR positive.

18. ELISA troubleshooting tips (also consider the acceptance criteria of-

18.1Positive results in negative control
18.2High background across entire plate
18.3Low absorbance values
18.4 High absorbance values for samples and/or positive control (absorbance does not go down as the sample is diluted down the plate)
18.5Inconsistent absorbance's across the plate
18.6Color developing slowly

18.1 Positive results in negative control

Contamination of reagents/samples
May be contamination of reagents or samples, or cross contamination from splashing between wells. Use fresh reagents and pipette carefully.

Insufficient washing of plates
Ensure well areas are washed adequately by filling the wells with wash buffer. Ensure all residual antibody solutions are removed before washing.

Too much antibody used leading to non-specific binding
Check the recommended amount of antibody suggested. Try using fewer antibodies.

18.2 High background across entire plate

Conjugate to strong or left on too long
Check dilution of conjugate; use it at the recommended dilution. Stop the reaction using stop buffer as soon as the plate has developed enough for absorbance readings.

Substrate solution or stop solution is not fresh
Use fresh substrate solution. Stop solution should be clear (if it has gone yellow, this is a sign of contamination and it should be replaced).

Reaction not stopped
Color will keep developing if the substrate reaction is not stopped.

Plate left too long before reading on the plate reader
Color will keep developing (though at a slower rate if stop solution has been added).

Contaminants from laboratory glassware
Ensure reagents are fresh and prepared in clean glassware. Sterilize glassware beforehand if possible.

Substrate incubation carried out in the light
Substrate incubation should be carried out in the dark.

Incubation temperature too high

Antibodies will have optimum binding activity at the correct temperature. Ensure the incubations are carried out at the correct temperature and that incubators are set at the correct temperature and working. Incubation temperature may require some optimization.

Non-specific binding of antibody

Ensure a block step is included and a suitable blocking buffer is being used. We recommend using 5-10 % serum from the same species of the secondary antibody, or bovine serum. Ensure wells are pre-processed to prevent non specific attachment. Use an affinity purified antibody, preferably pre-absorbed.

Also check suggestions listed under 'Positive results in negative control'

18.3. Low absorbance values

Target protein not expressed in sample used/ Low level of target protein expression in sample used

Check the expression profile of the target protein to ensure it will be expressed in your samples. If there is low level of target protein expression, increase the amount of sample used, or you may need to change to a more sensitive assay. Ensure you are using a positive control within the detection range of the assay.

Insufficient antibody

Check the recommended amount of antibody is being used. The concentration of antibody may require increasing for optimization of results.

Substrate solutions not fresh or combined incorrectly

prepare the substrate solutions immediately before use. Ensure the stock solutions are in date and have been stored correctly, and are being used at the correct concentration. Ensure the reagents are used as directed at the correct concentration.

Reagents not fresh or not at the correct pH
Ensure reagents have been prepared correctly and are in date.

Incubation time not long enough

Ensure you are incubating the antibody for the recommended amount of time, if an incubation time is suggested. The incubation time may require increasing for optimization of results.

Incubation temperature too low

Antibodies will have optimum binding activity at the correct temperature. Ensure the incubations are carried out at the correct temperature and that incubators are set at the correct temperature and working. Incubation temperature may require some optimization. Ensure all reagents are at room temperature before proceeding.

Stop solution not added
Addition of stop solution increases the intensity of color reaction and stabilizes the final color reaction.

18.4 High absorbance values for samples and/or positive control (absorbance does not go down as the sample is diluted down the plate)

The concentration of samples or positive control is too high and out of range for the sensitivity of the assay. Re-assess the assay you are using OR reduce the concentration of samples and control by dilution before adding to the plate. Take the dilution into account when calculating the resulting concentrations.

18. 5 Inconsistent absorbance's across the plate

Plates stacked during incubations

Stacking of plates does not allow even distribution of temperature across the wells of the plates. Avoid stacking.

Pipetting inconsistent

Ensure pipettes are working correctly and are calibrated. Ensure pipette tips are pushed on far enough to create a good seal. Take particular care when diluting down the plate and watch to make sure the pipette tips are all picking up and releasing the correct amount of liquid. This will greatly affect consistency of results between duplicates.

Antibody dilutions/reagents not well mixed

To ensure a consistent concentration across all wells, ensure all reagents and samples are mixed before Pipetting onto the plate.

Wells allowed to dry out
Ensure lids are left on the plates at all times when incubating. Place a humidifying water tray (bottled clean/sterile water) in the bottom of the incubator.

Inadequate washing
This will lead to some wells not being washed as well as others, leaving different amounts of unbound antibody behind which will give inconsistent results.

Bottom of the plate is dirty affecting absorbance readings
Clean the bottom of the plate carefully before re-reading the plate.

18. 6 Color developing slowly

Plates are not at the correct temperature
Ensure plates are at room temperature and that the reagents are at room temperature before use.

Conjugate too weak

Prepare the substrate solutions immediately before use. Ensure the stock solutions are in date and have been stored correctly, and are being used at the correct concentration. Ensure the reagents are used as directed, at the correct concentration.

Contamination of solutions
Presence of contaminants, such as sodium azide and peroxidase can affect the substrate reaction. Avoid using reagents containing these preservatives.

19. Waste disposal:

- Solid waste such as multiwell plate, micropipette tips, Eppendorf tubes, falcon tube, contaminated gloves etc., should be placed inside heavy-duty Poly bag designated for contaminated waste and incinerated.
- Decontaminate infectious liquid waste in large volume of freshly prepared disinfectant (sodium hypochlorite, virkon), leave overnight and dispose in sanitary sewer

4. Immunocapture enzyme immunoassay for PPR virus detection

1. Introduction

This ELISA assay has been developed to aid in the detection of peste des petits ruminants (PPR) viral antigens. It is a solid-phase immunocapture ELISA (ICE) based on the technique of Libeau et al. (1994). A mouse monoclonal antibody (anti-PPR), which is attached to the microplate, captures the virus present in supernatant of infected cells or from prepared tissue samples of sick animals. A second mouse monoclonal antibody (anti-PPR), which is biotinylated and is directed against the nucleocapsid (N) protein of the respective virus, is used in conjunction with a streptavidin-horseradish-peroxidase conjugate to detect captured antigen. The assay is designed such that it can be performed in one hour on pre-coated plates.

The kit has been specifically designed for use in the conditions prevailing in the majority of laboratories in countries where PPR is endemic.

2. Equipment required

2.1 Photometer: Microplate reader with an interference filter of 492 nm.

2.2 Orbital Shaker: Microplate Shaker.

2.3 Washer: Microplate washer. Alternatively, plastic wash bottles

2.4 Pipettes: Multichannel Pipettes, variable range from 5-50 µl and 50-300 µl, quality tips and reagent troughs.

2.5 Water Purification System: Minimum: glass-distilled or deionised water.

2.6 Microplates: NUNC Immuno I flat bottom, 96 well microplates. Plates should be stored at temperatures between + 4°C and +15°C.

2.7 Refrigerator: Any type in the range of +2 °C to +6°C.

2.8 Freezer: Any type in the range of -15 °C to -20°C.

2.9 Incubator: Any type of radiant, warm wall incubator or hot room in the range of +35°C to +39°C.

2.10 pH Meter or pH strips

2.11 Glassware/Plastic ware: Beakers (20-4000 ml), flasks (50-1000 ml), graduated cylinders (10-2000 ml), graduated pipettes (1-20 ml) with suitable safety bulbs, storage bottles with closures (1-100 ml), dilution tubes (2-4 ml) and suitable racks, wash fluid container with tap (5-10 L) and tubing for connection of this reservoir to plate washer or alternatively, wash bottles (250 ml).

2.12 Bottles

2.13 Vortex mixer

2.14 Timer

2.15 Absorbent towels

2.16 Marker pens (water proof) and adhesive labels.

3. REAGENT AND SAMPLE PREPARATION

3.1 Coating buffer: For 10X PBS stock solution. Dissolve one PBS sachet in 1 L of locally produced distilled/deionised water. Label and store at +4 °C. Working Dilution **1:10**.

3.2 Blocking buffer: 1X PBS + 0.05% (v/v) Tween 20 + 0.5% (v/v) negative lamb serum. Store at +4°C. Use fresh buffer each day.

3.3 Wash buffer: Dilute 1X PBS 1:5 in distilled/deionised water; add 0.05% (v/v) Tween 20.

3.4 Capture antibody: Reconstitute the freeze dried antibody precisely 0.5 ml of sterile water (reconstitution diluent) supplied with the kit and ***mix gently until completely dissolved***. The stock may be stored in its original vial at -20°C as it can be thawed and refrozen several times without a significant loss of activity.

3.5 PPRV detection antibody stocks: Anti-PPRV biotinylated monoclonal antibodies in glycerol. Stock solutions supplied in 0.5 ml vials; store at -20°C. The stock may be stored in its original vial at -20°C as it can be thawed and refrozen several times without a significant loss of activity.

3.6 Conjugate stock: Streptavidin HRPO conjugate (Boehringer Mannheim). Liquid, store at -20 °C.

3.7 PPRV reference antigens: Culture supernatant from cells infected PPRV vaccine strain. Reconstitute the freeze antigen with precisely 1 ml of sterile water (reconstitution diluent) supplied with the kit and ***mix gently until completely dissolved. Store at +4℃ for short term and at -20℃ for long term storage.***

3.8 Negative sample: Culture supernatant from non-infected cells. Reconstitute the freeze dried contents of a vial with precisely 1 ml of sterile water (reconstitution diluent) supplied with the kit and ***mix gently until completely dissolved. Store at +4℃ for short term and at -20℃ for long term storage. Can be thawed and refrozen several times without significant loss of activity.***

3.9 Negative serum: Freeze dried, store at +4°C. Reconstitute the freeze dried content of a vial with precisely 1.0 ml distilled/deionised water. Store at -20 °C.

3.10 Chromogen stock: OPD tablets; store at +4 °C in the dark. Dissolve one tablet in 75 ml locally produced distilled/deionised water (0.04%, 3.7mM) *just before the substrate/ chromogen incubation step* and store in the ***dark at +4℃***. If the whole volume is not required for a days' test, prepare aliquots of 12 ml and ***store at - 20℃ in the dark until use.***

3.11 Substrate stock: Per-hydride tablets 3% (w/v) H_2O_2 (882 mM); store at +4°C. Dissolve one hydrogen peroxide tablet in 10 ml distilled/deionised water. This will give a 3% solution. Store at +4°C until depleted at which point a fresh stock should be prepared in the same manner. ***Immediately before use add 50 µl of this solution to every 12 ml of OPD solution.***

3.12 Stopping solution (not supplied): Sulphuric acid (1M). Slowly add 55 ml of concentrated sulphuric acid to 945 ml of distilled/deionised water, store at room temperature. **Add concentrated Sulphuric Acid to Water. Never add water to concentrated Sulphuric Acid**

4. Assay procedure

4.1 Coating of microplates

- Immediately prepare a working dilution of capture antibody in coating buffer; e.g., 1/600 in 1X PBS.
- Dispense 100µl of diluted capture antibody into all 96 wells of the microplates.
- Tap the sides of the microplates to ensure that the antigen is evenly distributed over the bottom of each well.
- Cover or seal the microplates and incubate overnight at +4°C.
- Alternatively, the microplates can be placed on an orbital plate shaker in a +37°C warm air incubator or hot room and incubated for 45 minutes with continuous shaking.

4.2 Blocking buffer

1X PBS + 0.05% (v/v) Tween 20 + 0.5% (v/v) negative lamb serum. Store at +4°C. Use fresh buffer each day.

4.3 Addition of Test Samples, Control Samples, Detection Monoclonal Antibody and Conjugate

Discard the contents of coated microplates and fill with wash buffer. Again discard the contents. Repeat with two more wash cycles.

One step incubation

Immediately prepare a working dilution of the detecting monoclonal antibody; e.g., 1/300 in blocking buffer for all the plates (3ml of working dilution per plate).

Prepare a working dilution of the conjugate; e.g. 1/100 in blocking buffer in a volume sufficient for all the microplates (3ml of working dilution per microplate

According to the plate layout, add 50µl of test and control samples to the appropriate wells.

- Add 50µl of samples in duplicate to wells G1, G2, H1, H2, A3, A4, B3, B4 etc.

- Add 50 µl of PPR reference antigens in quadruple to wells C1, C2, D1, D2,

- Add 50 µl of the negative control sample in quadruple to wells E1, E2, F1, F2,

- **Add 50 µl of PBS, blocking buffer to the wells of the conjugate control to wells A1, A2, B1, B2.**

- Immediately add 25µl of the working dilution of the detecting PPR antibody in blocking buffer to the appropriate wells (i.e., add 25µl of working strength PPR detection antibody to columns 1 to 12).
- Immediately add 25 µl of working strength conjugate to each well.
- Cover or seal the microplates and place on an orbital plate shaker housed in a +37°C warm air incubator or hot room and incubate for 45 minutes with continuous shaking.

4.4 Addition of substrate/chromogen and stopping solutions

- Immediately before the end of the sample/conjugate incubation, prepare a working dilution of the substrate/chromogen [e.g. for 1 plate, dilute 50µl of substrate stock (H_2O_2) in 12ml of chromogen (OPD) solution.
- A clean microplate (not coated) will be used as the 'blanking plate' for the photometric reading.
- After 45 minutes of sample/conjugate incubation, wash the wells vigorously (using a pipette because samples are usually sticky) in PBS 1/5 + 0.05% Tween 20 and blot dry.
- Immediately after washing, add 100µl of the substrate/chromogen solution to the wells of the microplates, starting with the first column of the 'blanking plate' followed by all 96 wells of the microplates in the test run.
- Immediately begin timing the substrate/chromogen development and incubate for 10 minutes at room temperature in the dark.
- After 10 minutes of substrate/chromogen incubation, immediately add 100µl of the stopping solution to the wells of the microplates, starting with the first column of the 'blanking plate', followed by all 96 wells of the microplates in the test run. Briefly shake the microplates using the orbital shaker to ensure thorough mixing. All wells should now contain 100µl of substrate/ chromogen solution plus 100µl of stopping solution.

4.5 Measurement of substrate development

Place the 'blanking plate' in the microplate reader and initiate the blanking sequence.

Place the microplate in the carriage of the blanked reader and initiate reading sequence. Repeat for each microplate.

5. Assay performance and interpretation

5.1 Data expression

Optical density readings are used in two types of data analysis in relation to this assay:

A) Percent Positivity (PP) values which are used for Quality Assurance (QA) acceptance. These PP values are calculated as follows:

$$PP = \frac{Replicate\ OD\ of\ \ each\ Control}{Median\ OD\ of\ \ PPR\ ref.\ Ag}\ x\ 100$$

B) Percent Positivity (PP) values which are used for acceptance of replicate values for test samples and diagnostic interpretation. These PP values are calculated as follows:

$$PP = \frac{Replicate\ OD\ of\ \ each\ Test\ serum}{Median\ OD\ of\ \ PPR\ ref.\ Ag}\ x\ 100$$

5.2 Calculation and acceptance of control data

The data expressed in OD values and PP values for the PPR (Cr_{ppr}) and the data expressed in PP values for the conjugate control (Cc) and the negative control (C-) are used to determine whether or not the test has performed within acceptable limits of variability and therefore, whether or not the test samples data may be accepted for any given microplate.

The Controls included with the kit have been repeatedly tested at the CIRAD. Given that the variation in OD values and PP values should be normally distributed, Upper Control Limits (UCL) and Lower Control Limits (LCL) for these Controls have been established.

The Controls should be examined in the following order:

I. First level of microplate acceptance

<u>Reference antigen control (Cr)</u>

At least three of the four OD values must fall within the control limits for each reference antigen. If not, the plate **must** be rejected.

When the first level of microplate acceptance has been achieved and the median OD value for each reference antigen control is calculated from the two intermediate values and used in subsequent PP calculations.

Table 1. Acceptance criteria for control data Upper control limit (UCL) and Lower control Limit (LCL)

	UCL	LCL
Cr_{PPR} (OD values)	2.8	0.5
	UCL	LCL
Cr_{PPR} (PP values)	115	85
C- (PP values)	8	0
Cc (PP values)	15	0

II. Second level of microplate acceptance

Reference antigen control (Cr), conjugate control (Cc), and negative (C-) control samples

For the reference antigen controls (Cr_{ppr}), conjugate control (Cc) and the negative control (C-), the replicate PP values should be calculated and recorded on the ELISA DATA SHEET.

i. Reference antigen controls (Cr_{ppr})

Compare the Replicate PP values of the three or four accepted Cr controls to the UCL and LCL for the Cr and use the criteria in Table 2 A. to accept or reject each ***individual*** microplate.

ii. Conjugate control (Cc)

The conjugate control (Cc) is run to determine whether or not the Streptavidin HRPO conjugate is reacting non specifically with components found in the antibody - coated wells. Compare the Replicate PP values to the UCL and LCL for the conjugate control (Cc) and use the criteria in

Table 2 B. to accept or reject each ***individual*** microplate.

iii. Negative control sample (C-)

Compare the Replicate PP values to the UCL and LCL for the negative control sample (C-) and use the criteria in Table 2 B. to accept or reject each ***individual*** microplate.

<u>Microplate rejected if more than one of the above controls fails to meet PP acceptance criteria.</u>

Table 2.A Cr_{PPR}, Control Data

Replicate PP Values(4)*		Status
In*	Out**	
4	0	Accept
3	1	Accept
2	2	Reject
1	3	Reject
0	4	Reject

Replicate PP Values(3)*		Status
In*	Out**	
3	0	Accept
2	1	Reject
1	2	Reject
0	3	Reject

* :refers to the number of Cr_{ppr} replicate OD values within the UCL and

LCL (see Section 6.2. - First Level of Microplate Acceptance).

In* - Within UCL and LCL range

Out** - Outside UCL and LCL range

Microplate rejected if the Cr_{ppr} controls fail to meet the above PP acceptance criteria.

Table 2.B Cc, C- control data

Replicate PP Values		Status
In*	Out**	
4	0	Accept
3	1	Accept
2	2	Reject
1	3	Reject
0	4	Reject

In* - Within UCL and LCL range

Out** - Outside UCL and LCL range

Microplate rejected if more than one of the Cc, C-, controls fails to meet PP acceptance criteria.

5.3 Acceptance of individual test sample data

The diagnostic threshold for this assay has been set at 18 % positivity (18 PP) of the PPR reference antigen control (Cr_{PPR}).

To accept individual test sera, the duplicate PP values of a test sample must both be either greater than or equal to the appropriate threshold value (i.e., 18 PP for PPR) or both be below the threshold value.

Test samples should be retested if their duplicate PP values lie one on either side of the threshold.

5.4 **Diagnostic interpretation of test sample data**

If accepted according to the Criteria in 5.3, the mean PP value for the two replicates should be calculated. Test samples demonstrating mean PP values equal to or greater than 18 (PPR) are considered to be positive. Test samples demonstrating mean PP values less than 18 (PPR) are considered to be negative.

PPR, Immunocapture Assay - Work Sheet

Plate Layout

Controls / Samples	Samples (in duplicate)										
PPR Detecting Antibody											
	1	2	3	4	5	6	7	8	9	10	11	12
A	Cc	Cc										
B	Cc	Cc										
C	Cr_p	Cr_p										
D	Cr_p	Cr_p										
E	C-	C-										
F	C-	C-										
G												
H												

Note:

ELISA DATA SHEET - PPR, Immunocapture Assay

Disease:	Plate No.:	Date:
Run ID.:	Operator:	Filter:

Plate Reader Blank Value:	Comment:

IQC Data	OD1	OD2	OD3	OD4	Median OD
Cr_{PPR}					

IQC Data	PP1	PP2	PP3	PP4	
Cr_{PPR}					
Cc					
C-					

P(PPR) threshold =
18

PP - Value PPR Detecting Antibody									
ID	OD1	OD2	Avg. OD	Avg.PP		OD1	OD2	Avg. OD	Avg.PP
1					22				
2					23				
3					24				
4					25				

5					26				
6					27				
7					28				
8					29				
9					30				
10					31				
11					32				
12					33				
13					34				
14					35				
15					36				
16					37				
17					38				
18					39				
19					40				
2					4				

0					1				
21					42				

The sample is negative if PP (OD mean test sample/OD median ref.
Cc: Conjugate Control
Cr_{PPR}: PPR reference antigen
C-: Negative antigen

5. Virus Isolation and identification in cell culture

1. Introduction

Virus isolation in cell culture is very important for preservation of the isolates and its subsequent use. *Peste des petits ruminants* (PPRV) may be isolated in primary lamb kidney or in African green monkey kidney (Vero) cell tissue cultures. Vero cell was found to yield very high titres and is currently used in many laboratories working on PPRV and RPV. Vero cells are however widely used for their continuity and low liability of contamination. PPRV produces characteristic cytopathic (CPE) effects on Vero cells. However, appearance of cytopathic effects may require at least 8-10 days or several blind passages.

2. Purpose of activity

To isolate PPR virus from field samples and determine biological characteristic of the virus.

3. Safety

Containment Level BSL –II should always be used during laboratory manipulation of the virus. Handling of PPR suspected samples should be carried out under a Biosafety Cabinet II. Use laminar flow cabinet for preparation/handling of fresh and uninfected cell culture, Personal Protection Equipment (PPE) which include, Lab coat, and disposable gloves.

4. Samples

4.1 Swab samples

- Use a cotton/dacron swabs to collect the specimen. Collect swab from posterior nasal cavity or Conjunctiva, avoiding external secretion. Keeping swabs moist after collection is most important. Place swab in 3-4 ml Viral Transport Media (VTM). Any sterile isotonic fluid, like phosphate buffered saline (PBS) with antibiotics (penicillin 200 International Units/ml and streptomycin 200 µg/ml or Gentamycin 500 ug/ml), or common tissue culture medium like Eagle's MEM can be used. Swabs may be broken off to fit within the capped tube containing the VTM and shipped. Alternatively, whirl/ agitate the swab in the media for several minutes before removing it and shipping the suspension
- Commercially available kits containing swabs and viral transport media are acceptable.

4.2 Blood sample

- Collect blood aseptically from Jugular vein in heparini zed tubes when the animal is in an acute febrile stage of the disease and store at chilled condition.

4.3 PM tissue samples

- Collect lung, mesenteric and bronchial lymph nodes aseptically from PPR suspected dead goats during post-mortem.

5. Transportation of the samples

Samples should be transported in chilled condition with ice packs or with wet ice. They should be received by the testing laboratory within 48 hours of collection. If shipment is delayed and facilities are available, the specimens should be frozen at -70^{o} C and shipped on dry ice. Otherwise, store specimens in refrigerator (freezing at -20 ^{o} C reduces viability of virus).

6. Requirement of a cell culture laboratory

- Biosafety cabinet- class-II
- Laminar flow
- Incubator with CO_2 facilities
- Centrifuge machine –4000 RPM at least and rotor that can hold falcon tube (50 ml)
- Refrigerator 1. One double chambered refrigerator (Facilities with 2 to 4 ^{0}C and -20^{0}C 2. one -20^{0}C freezer and two liquid nitrogen containers (10litre and 40 litre)
- Microscope (Inverted)
- Hot air oven
- Water bath
- Graduated bottle, 500 ml, 250 ml, 100 ml
- Measuring cylinder 500ml, 100 ml , 50 ml
- Pipettes, Pipettor, and tips, falcon tubes, syringe and needles, Filter (aero disc 13)
- Tissue culture vessels (flasks 25cm^2,75cm^2, Petri dishes, Flat bottom multiwell plate
- Mortar and Pestle
- Scissors and forceps
- Spirit lamp
- Tray
- Cell counter
- Media, reagents and chemicals for cell culture
- Distilled or deionised water
- Disinfectant (70% alcohol, Clorox, 10% hypochlorite sol)
- Waste disposal container, Bag, etc
- Consumables such as hand gloves, masks and hair cover, cotton swabs, cotton gauze, indelible markers
- Washing and autoclave facilities
- Vero cells frozen and in suspension if available in near by laboratories
- aluminum foil

7. Preparation of inoculums for virus isolation

7.1 Swab samples

- Draw 0.5 ml of sample suspension into 1 cc syringe. Attach 0.2um syringe filter (such as a Acrodisc 13) and push sample through filter directly onto cells or in a eppendorf tube. The drawback is that some samples will not pass through the filter. To overcome clogging the filter make sure suspension is well homogenized before drawing into the syringe.

7.2 Blood samples

- Centrifuges heparinised blood at 1000 rpm for 5 minute and collect the buffy coat. Wash the white blood cell in Minimal Essential Medium (MEM) containing antibiotics (penicillin 200 International Units/ml and streptomycin 200 µg/ml). Resuspend the pellet cells in 1.0 ml of MEM plus antibiotics. Inoculate 0.5 ml of the cell suspension into confluent Vero cells grown in 25 cm^2 tissue culture flask.

7.3 Tissue samples

- Use individual organ or pool the collected tissues (lung, lymph nodes) collected from the same animal, weigh and macerate them with sterile mortar and pestle while the tissues are still frozen.
- Add PBS to make 20% (w/v) suspension and collect the suspension in a sterile tube.
- Centrifuge tissue suspension at 3000 rpm for 10 minutes. Collect the supernatant in fresh sterile Falcon tubes and add antibiotic (Gentamycin) at 500 µg/ml and store at -70^0C.
- Prior to inoculation, filter the suspension, using Acrodisc® Syringe filter (13 mm diameter, 0.2 µm pore size) [Sigma-Aldrich or similar] and use the filtrate as inoculums

8. Inoculation in Vero cells

- Maintain the Vero cell in media M199 with bovine foetal serum (10% for growth and 5% for maintenance).
- Inoculate the flask when Vero cell monolayer became confluent.
- Discard the spent medium, wash the flask/s with 10 ml sterile pre-warmed PBS for 2 times.
- Inoculate the cells with the prepared sample suspension at 200 µl/25 sq. cm flasks.

- Run positive (with PPR Vaccine virus, 200 ul/ per flask) and negative control (200 µl PBS)
- Spreads the inoculum all over the cell sheet and incubate the flasks at 37°C for 1 hour for adsorption of the virus. Spread the inoculum by tilting the flasks every 15 minutes. After one hour, add 5ml of growth medium containing 10% fetal calf serum to the flasks without removing the excess inoculum.
- Incubate the flasks at 37^0 and examine twice daily for the appearance of cytopathic effects (CPE).

9. Harvesting of virus

- Harvest the culture medium at 5th day of each passage or when the maximum CPE manifested.
- Collect the infected tissue culture by freezing and thawing for 4 times, and centrifuge at 2500 rpm for 5 minutes, Collected the suspension in sterile 50ml Falcon tubes.
- Use the collected suspension for further passage in fresh Vero cells.

10. Special practice for tissue culture lab

- Use laminar flow cabinet for preparation/handling of fresh and uninfected cell culture and the biosafety cabinet for handling infectious materials
- Wipe all work surfaces liberally with a disinfectant (70% alcohol) before and after each session of work
- Wipe hands with 70% alcohol before commencement of the sterile work
- Use only sterilized glassware/plastic ware. Decontaminate the surfaces of all glassware with 70% alcohol before placing into the laminar flow cabinet or biosafety cabinet
- Flame all bottles at around the neck before and after opening and before re-closing
- (Note – Use of spirit lamp or Bunsen burner is not allowed in the biosafety cabinet! Rely on decontamination with 70% alcohol)
- Store stock and working media/reagents in a separate freezer designated for tissue culture reagents only. Divide all reagents into suitable aliquots when they are opened for the first time.
- Store viruses and infected cultures in clearly labeled vials and place them in separate designated freezer
- Collect all used glassware in disinfectant (Sodium hypochlorite / Virkon) and follow the procedure of cleaning and disinfection

- Clean and disinfect all surfaces daily at the end of the day
- Collect all used glassware and plasticware (except tips and Eppendorf tubes) in a bucket containing disinfectant (sodium hypochlorite, virkon), leave overnight and proceed for cleaning & sterilization or disposal. Cleaning & sterilization of used glassware must be completed in the shortest possible time

11. Waste disposal

- Collect all sharp items (needle, blade, broken ampoule) in sharp disposal cans. When full, dispose the can by incineration
- Solid waste such as flasks, micropipette tips, Eppendorf tubes centrifuge tubes, contaminated gloves, tissues, etc., should be placed inside heavy-duty Poly bag for contaminated waste and incinerated.
- Collect the biodegradable solid waste in large volume of freshly prepared disinfectant (sodium hypochlorite, virkon), leave overnight and dispose as garbage
- Decontaminate infectious liquid waste in large volume of freshly prepared disinfectant (sodium hypochlorite, virkon), leave overnight and dispose in sanitary sewer

Government of the People's Republic of Bangladesh

SAARC Regional Leading Diagnostic Laboratory for PPR (PPR RLDL)

Bangladesh Livestock Research Institute, Savar, Dhaka-1341

Tel: +88-02-7791690 Fax:+88-02-7791675, E-mail: pprblrifp@yahoo.com

Website: http//www.blri/gov.bd

Sample Submission Form

1. Details of Submitter

Name & Designation: Address: Country:	Submission Date: Tel/Mobile: Fax: E-mail:

2. Details about sample:

Sample ID No.	Species	Sex	Sample Type	Sample Collection Date

3. Age

0-3 months	4-12 months	13-24 months	> 24 months

4. Details about outbreak:

a. Owner's Name, Address & Phone:

b. Latitude & Longitude: c. Total Goat and/or Sheep:

d. No. animal affected: e. No. animal died: f. Vaccination history: Yes/No (if yes) g. Vaccinated……days ago h. Test done (if any):

i. Clinical signs and symptoms:

j. Tentative diagnosis:

k. Comments/Test suggested:

To be fulfilled by SAARC-PPR RLDL

1. SAARC-PPR RLDL ID No. Received by

2. Reception Date and Time: Name :
Designation:

Government of the People's Republic of Bangladesh

SAARC Regional Leading Diagnostic Laboratory for PPR (PPR RLDL)

Bangladesh Livestock Research Institute, Savar, Dhaka-1341

Tel: +88-02-7791690 Fax:+88-02-7791675, E-mail: pprblrifp@yahoo.com

Website: http//www.blri/gov.bd

Outbreak Investigation Form

General Information

Reference Code		Upazila		District	
Name of farmer					
Village/locality		Latitude		Longitude	
Farming System	☐ Intensive	☐ Semi-intensive	☐ Extensive	☐ Nomadic	☐ Other (define)

Outbreak Information

Date of this report	..……./……../…… …	Estimated date of first clinical case	**……/……/…….**

If this is a first report, please fill in this part

Species	No. cases	No. dead	No. at risk	Special comments
Goat				Age: Sex: Breed:
Sheep				

If this is a follow up report, please fill in this part

Species	No. new cases since last	No. new deaths	No. at risk

	report		

Clinical & other Information

Disease Suspected

Tentative diagnosis		Differential Diagnosis	

Epidemiology Information

Recent introductions?	Yes□	No□	Similar disease nearby?	Yes □	No□	Vaccination for suspected in past 12 month?	Yes□	No□

Laboratory Information (if samples sent)

Type of sample:	Nasal swab/Buccal or Rectal Mucosa/PM	Lab. Samples submitted to:	
Date submitted	..……./……../…… ….	Local	Foreign
Results	Suspected disease?	Result available Yes No	

(pos/neg, doubtful	Other disease?		Local Lab		Foreign Lab
	Name, if positive for other disease:				

Control Measures (Yes or No)

Ring Vaccination		Movement control		No. destroyed		Dip	
Treatment		Quarantine		No. slaughtered		Other	
If other (please mention)							

Feedback to farmer	Information	
Follow-up action		
Name	Signature	Date

Reporting officer

Name	**Designation (profession)**	**Signature**	**Date**
			…….../……../……