Asian Australas. J. Biosci. Biotechnol. 2019, 4 (2), 109-115

Asian-Australasian Journal of Bioscience and Biotechnology
ISSN 2414-1283 (Print) 2414-6293 (Online)
www.ebupress.com/journal/aajbb

Article

Clinical investigation of PPR outbreak and sero-prevalence of PPR viral antibody in different areas of Bangladesh

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Received: 23 July 2019/Accepted: 18 August 2019/ Published: 31 August 2019

Abstract: Peste des petits ruminants (PPR) is an acute, highly contagious, world organization for animal health (OIE) notifiable and economically important transboundary viral disease of sheep and goats associated with high morbidity and mortality and caused by PPR virus. This research work was done in 2016-2017 by executing, surveillance and clinical investigation studies to determine present status of circulating PPR virus and its detection of antibody level of PPRV in different areas of Bangladesh. cELISA was conducted to detect the PPR antibody and RT-PCR also used for identification of N gene PPRV. The clinical outbreak of PPR, the total 124 samples was collected at the six locations of the country and highest case fatality (morbidity) was recorded at Jhenaidah 93.75% (75 out of 80).The highest morbidity rate and mortality rate was 69.23% and 13.07% respectively. The result of RT-PCR indicates the PPR virus circulating in the different regions of Bangladesh. For sero-prevalence of PPR antibodies of 366 serum samples were collected at different region of Bangladesh such as Chuadanga 47.81% (22 out of 47), Sirajganj 34.21% (13 out of 38), Thakurgaon 48.15% (26 out of 54), Satkhira 56.92% (37out of 65), Jhenaidah 33.33% (28 out of 84) and Chattogram 30.79 % (24 out of 78). It is reflected that the selected areas are highly suspected PPR and need to proper vaccination against PPR vaccine that can protect PPR disease in goat and sheep which helps to meet global PPR control strategy as well as contribute to achieve the two (2) number of Sustainable Development Goals (SDGs).

Keywords: PPRV; goat; Sero-prevalence; vaccine; antibody

1. Introduction

Peste des petits ruminants, also known as goat plague, is an acute highly contagious viral disease that causes serious economic losses due to high morbidity and mortality rates (Dhar *et al.,* 2002). 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 genome of PPR virus encodes 6 structural proteins, including a nucleoprotein (N), a viral RNA-dependent polymerase (L), an RNA-polymerase phosphoprotein co-factor (P), a matrix protein (M), a fusion protein (F) and a hemagglutinin protein (H). According to the sequence analysis of N, F or H genes of PPR virus strains, N gene has been proposed as the most appropriate gene for molecular characterization of closely related isolates (Kwiatek *et al.,* 2007).

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.,* 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 2016 to 2017. The detailed outline of materials and methods are given below:

2.1. Study area

This study was carried out clinical investigation for detection of viral nucleic acid against PPR Virus in goat and sheep. For this purpose, the total 124 (one hundred and twenty four) nasal swabs were collected from different areas of Bangladesh such as Chuadanga (N=5), Sirajganj (N=6), Thakurgaon (N=8), Satkhira (N=6), Jhenaidah (N=80) and Chattogram (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.

The study also conducted on surveillance of Peste des Petits Ruminants (PPR) for specific antibodies in goat and sheep. For this purpose, the total 366 (Three hundred and sixty six) sera was collected from 6 (Six) selected areas of Bangladesh namely Sirajganj (N=38), Chuadanga (N=47), Thakurgaon (N=54), Satkhira (N=65), Jhenaidah (N=84) and Chattogram (N=78).

2.2. Collection and storage of samples

A total of 366 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 PPRV. Initially, blood samples were collected by jugular-vein puncture with (3-5) ml sterile syringe. The suspected nasal swabs 124 collected for molecular study. 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 method and the collected samples were stored at −20°C until processed.

2.3. Active field investigation and questionnaire survey

An epidemiological study on PPR outbreak was employed between 2016 and 2017 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. Molecular detection of the virus nucleic acid (N gene of PPRV)

One hundred and twenty four (124) 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).

2.5. 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.

2.5.1. 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 and then the plate was incubated at 37℃ and waited for 45$\pm$4 minutes.
- The plate was washed 3 times with approximately 300μl of the wash solution and to avoid drying of the wells between washings.
- After washing then the conjugate was prepared 1X by diluting the conjugate 10X to 1/10 in dilution buffer 4 and again 100 μl of the conjugate 1X was added to each well and again the plate was incubated at 21℃ and waited for 30$\pm$3 minutes.
- Then the plate was washed 3 times with approximately 300 μl of the wash solution and to avoid drying of wells between washing.
- 100 μl of the substrate solution was added to each well and then the plate was incubated at 21℃ in the dark place and waiting for 15$\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.

2.5.2. Test validation of cELISA method

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.
  $OD_{PC} / OD_{NC} < 0.3$

2.5.3. 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.

2.6. Data management and analysis

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 sero-prevalence visa-vis fixed factors that included animal species, clinical symptoms, sex and age. 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 sero-positivity.

3. Results and Discussion

3.1. PPR clinical disease investigation

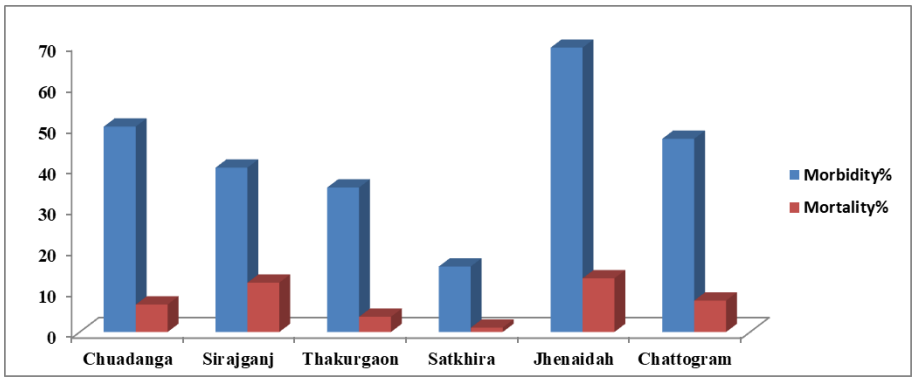
The clinical disease observations and outbreak investigation were done in different district of Bangladesh such as (Chuadanga, Sirajganj, Thakurgaon, Satkhira, Jhenaidah, and Chattogram) were investigated for the presence of the clinical disease (PPR virus), morbidity, and mortality and case fatality rate.

3.2. Identification of virus and confirmation using RT-PCR

For this evaluation, the total One hundred and twenty four (124) 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 124 samples examined with RT-PCR for viral nucleic acid, Among them, the highest presence of PPR virus was recorded at Jhenaidah 93.75% (75 out of 80) samples were positive (Table 1). The highest morbidity rate and mortality rate was 69.23% and 13.07% respectively shown in Graph 1. The photograph of the gel electrophoresis of the PCR products that was analyzed (Figure 1). The fragment size of the amplified products was 351 bp as reported previously by Couacy- Hymann *et al.* (2002).

Table 1. Outbreak investigation of PPR Virus using RT-PCR technique.

Location	No. of sample	Type of sample	Rate of		Result of RT-PCR	
			Morbidity (%)	Mortality (%)	% of positive	% of negative
Chuadanga	5	Nasal Swab	50	6.67	20% (1)	80% (4)
Sirajganj	6	Nasal Swab	40	12	50% (3)	50% (3)
Thakurgaon	8	Nasal Swab	35.18	3.70	25% (2)	75% (6)
Satkhira	6	Nasal swab	15.96	1.06	66.67% 4)	33.34% (2)
Jhenaidah	80	Nasal swab	69.23	13.07	93.75% (75)	6.25% (5)
Chattogram	19	Nasal swab	47.05	7.65	78.95% (15)	21.05% (4)
Total	**124**			**Overall**	**80.65% (100)**	**19.35 (24)**



Graph 1. The percentage of morbidity and mortality rate of PPR Outbreak.

To confirm the detection of PPR viral antigen in 124 suspected nasal swabs samples and RT-PCR protocol was used in this study. The highest presence of PPR virus was recorded at Jhenaidah 75 (93.75%) whereas the lowest presence was observed in Chuadanga 1 (20%) samples was positive. The results showed that overall 80.65% 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 Abu Bakar *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 Jhenaidah district than other districts.

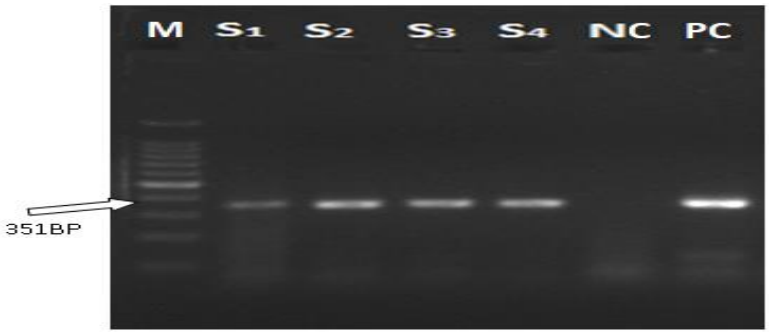
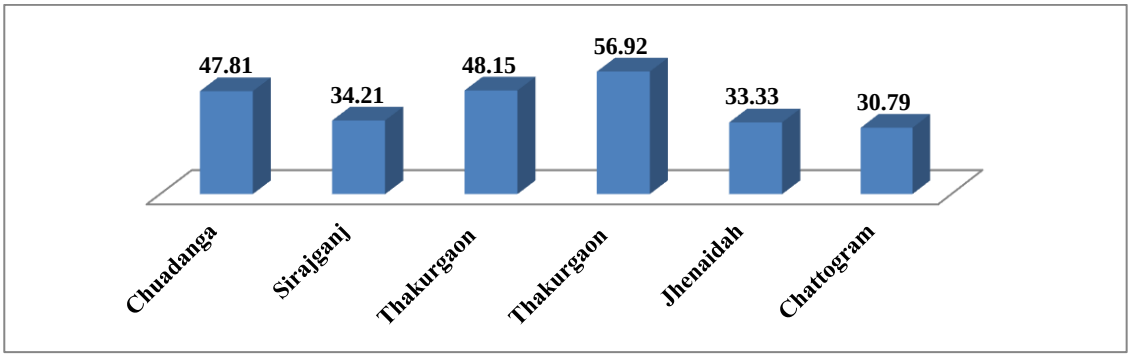


Figure 1. Agarose gel electrophoresis of PCR products (351 bp) amplified with NP3 and NP4, PPR specific primers. Lane M: 100bp DNA molecular weight marker; Lane PC: Positive control; Lane NC: Negative control; Lane S_1-S_{04}: Field samples.

3.3. In case of serological studies

For sero-positive of PPR antibodies of 366 serum samples were collected at different region of Bangladesh such as Chuadanga 47.81% (22 out of 47), Sirajganj 34.21% (13 out of 38), Thakurgaon 48.15% (26 out of 54), Satkhira 56.92% (37out of 65), Jhenaidah 33.33% (28 out of 84) and Chattogram 30.79 % (24 out of 78) that illustrated in the Graph 2. The selected areas (six) were screened for specific antibodies against PPRV using c-ELISA method and shown the test result of cELISA for PPR antibody detection.



Graph 2. During outbreak investigation, the immunity level of PPR virus in goats and sheep.

The overall seroprevalence (44.64%) in the small ruminants of six (6) selected districts of Bangladesh 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).

New entry of goats in the household or village is most important risk factor for PPR virus circulation which was found in several outbreaks in the surrounding villages. Also Purchase of new goats, vaccination during outbreak of PPR Distribution of goats by NGO was one of the risk factor for PPR circulation. 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.

4. Conclusions

The result of RT-PCR indicates the PPR virus circulating in the different regions of Bangladesh. The findings of sero-prevallance of PPR antibodies reflected that, the goat and sheep population in these areas are highly suspected to PPR and need proper PPR vaccination. It seems an opportune time to begin extensive sero-surveillance 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 as well as contribute to achieve the two (2) number of Sustainable Development Goals (SDGs).

Conflict of interest

None to declare.

References

Abraham G, A Sintayehu, G Libeau, E Albina, F Roger, Y Laekemariam, D Abayneh and KM Awoke, 2005. Antibody seroprevalences against peste des petits ruminants (PPR) virus in camels, cattle, goats and sheep in Ethiopia. Prev. Vet. Med., 70: 51–57.

Abu bakar M, Q Ali and HA Khan, 2008. Prevalence and mortality rate of peste des petits ruminant (PPR): possible association with abortion in goat. Trop. Anim. Health Pro., 40: 317–321.

Abu bakar M, S Ashiq, Z Hussain, M Hussain, Sh. Saleha, MJ Arshed and A Bin Za-hoor, 2011. Comparison of antigen detection methods of peste des petits ruminants virus in clinical samples of small ruminants. Bulg. J. Vet. Med., 14: 103−108

Albina E, O Kwiatek, C Minet, R Lancelot, RS de Almeida and G Libeau, 2013. Peste des petits ruminants, the next eradicated animal disease? Vet. Microbiol., 165: 38–44.

Anderson J, JA McKay and RN Butcher, 1991. The use of monoclonal antibodies in competition ELISA for detection of antibodies to rinderpest and peste des petitsruminants viruses. Intern. Atomic Energy Agency, Vienna, Austria, 43-53.

Balamurugan V, P Krishnamoorthy, BM Veeregowda, A Sen, KK Rajak, V Bhanuprakash, MR Gajendragad and K Prabhudas, 2012. Seroprevalence of Peste des petits ruminants in cattle and buffaloes from Southern Peninsular India. Trop. Anim. Health Prod., 44:301–306.

Banik SC, SC Podder, MA Samad and MT Islam, 2008. Sero-surveillance and immunization in sheep and goats against peste des petits ruminants in Bangladesh. Bangladesh Journal of Vet. Med., 6: 185-190.

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

De Nardi M, SM Lamin Saleh, C Batten, C Oura, A Di Nardo and D Rossi, 2012. First evidence of Peste des Petits Ruminants (PPR) virus circulation in Algeria (Sahrawi Territories): outbreak investigation and virus lineage identification. Transbound. Emerg. Dis., 59: 214–222.

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

Diallo A, C Minet, C Le Goff, G Berhe, E Albina, G Libeau and T Barrett, 2007. The threat of peste des petits ruminants: progress in vaccine development for disease control. Vaccine, 25: 5591–5597.

El-Hakim O, 2006. An outbreak of peste des petits ruminants virus at Aswan province, Egypt: evaluation of some novel tools for diagnosis of PPR. Assuit Veterinary Medicine Journal, 52: 146–157.

FAO, 2009. Peste des Petits Ruminants: an increasing threat to small riminants production in Africa and Asia. Empres Transboundary Animal Disease. Bullettin No. 33. Available at ftp://ftp.fao.org/011/i0919e/i0919e00.pdf.

Hamdy FM, AH Dardiri, O Nduaka, SS Breese Jr and EC Ihemelandu, 1976. Etiology of the stomatitis pneumoenteritis complex in Nigerian dwarf goats. Canadian Journal of Comparative Med., 40: 276.

Kivaria FM, O Kwiatek, MK Angolwisye, SS Emmanuel, G Libeau, M Moshy, OM Albano and J Gladson, 2013. The incursion, persistence and spread of peste des petits ruminants in Tanzania: Epidemiological patterns and predictions. Onderstepoort J. Vet. Res., vol. 80 n.1 Pretoria Jan. 2013.

Kwiatek O, C Minet, C Grillet, C Hurard, E Carlsson, B Karimov, E Albina, A Diallo and G Libeau, 2007. Peste des petits ruminants (PPR) outbreak in Tajikistan. J. Comp. Pathol.,136:111–119.

Munir M, M Siddique and Q Ali, 2009. Comparative efficacy of standard AGID and precipitinogen inhibition test with monoclonal antibodies based competitive ELISA for the serology of Peste des Petits Ruminants in sheep and goats. Tropical Animal Health and Production, 41: 413-420.

Munir M, S Zohari and M Berg, 2013. Replication and virulence determinants of Peste des Petits Ruminants virus. In Molecular Biology and Pathogenesis of Peste des Petitis Ruminants Virus. P151. ISBN: 978-3-642-31450-6. http://www.springer.com/978-3-642-31450-6.

Saliki JT, G Libeau, JA House, CA Mebus and EJ Dubovi, 1993. Monoclonal antibody-based blocking enzyme-linked immunosorbent assay for specific detection and titration of peste-des-petits-ruminants virus antibody in caprine and ovine sera. J. Clin. Microbiol. 31: 1075-1082.

Singh RP, P Saravanan, BP Sreenivasa, RK Singh and SK Bandyopadhyay, 2004a. Prevalence and distribution of peste des petits ruminants (PPR) virus infection in small ruminants of India. Scientific and Technical Review of the Office International des Epizooties, 23: 807-819.

Zohari S and K Stalli, 2008. Seroprevalence of antibodies to Peste des Petits ruminants at various governmental livestock farms of Punjab, Pakistan. Asian J. Epidemiol. 1: 82-90.