Genetic detection of Peste des Petits Ruminants Virus by cost-effective Semi-Quantitative Real-Time RT-PCR Assay

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ABSTRACT:

Peste des Petits Ruminant (PPR) caused by PPR virus (PPRV) is an important transboundary disease of small ruminants. Rapid Diagnosis is a basic requirement for the control and eradication of the disease. In the current study, SYBR Green-based qRT-PCR was standardized using newly designed primers for rapid diagnosis of PPR. A total of 46 clinical field samples were collected. All samples were tested using three PCR assays; conventional RT-PCR, real-time one-step reverses transcriptase-PCR (TaqMan® probe), and SYBR Green quantitative PCR. Of the 46 samples, 27 (58.69 %), 35 (76.08 %), and 40 (86.95 %) were positive by conventional RT-PCR, qRT-PCR (TaqMan® Probe), and SYBR Green quantitative PCR respectively. The sensitivity of conventional PCR against qRT-PCR (TaqMan®) was 68.57% and specificity was 72.73% while the sensitivity of SYBR Green quantitative PCR was 94.29% and specificity was 36.36%. The data suggests that the sensitivity of qRT-PCR (SYBR Green) and qRT-PCR (TaqMan®) was higher than that of conventional RT-PCR, while the sensitivity of both types of qRT-PCR (TaqMan® and SYBR Green) was almost equivalent as both detected 0.1 million times dilution of PPRV vaccine strain. However, SYBR Green-based qRT-PCR is more cost-effective than qRT-PCR (TaqMan® Probe) and can be used as a screening test for large-scale studies aimed at eradicating of PPR by 2030.

Key-words: One-step reverse transcriptase-PCR (TaqMan® probe), SYBR Green quantitative PCR, PPRV, Eradication

INTRODUCTION:

Peste des Petits Ruminants (PPR) holds a dominant position among the significant diseases of animals (small ruminants). It is an extremely contagious disease of sheep and goats with high morbidity and mortality rates. PPR disease was detected first time in 1942 in West Africa (Gargadennec & Lalanne, 1942). Initially, PPRV was thought to be prevalent in Africa only, but later on, PPR outbreaks were confirmed indifferent regions of the world including the Middle East, and South Asia.

PPR is declared as an acute, endemic, and widespread disease in Pakistan with a prevalence of 65.37%, mortality of 26.51%, and case fatality rate of 40.40% (Aamer Bin Zahur et al., 2014). The clinical signs of PPR are frequently confused with Contagious Caprine Pleuro Pneumonia (CCPP), bluetongue, Contagious pustular Dermatitis (CPD), and Foot and Mouth Disease (FMD) (Singh et al., 2009). Goats are more prone to this disease as compared to sheep although sheep show a fast recovery rate while cattle display no clinical signs and work as reservoir hosts (Abubakar, Ali, & Khan, 2008).

PPRV infection was also reported in wild animals (Abubakar, Rajput, Arshed, Sarwar, & Ali, 2011; Fentahun & Woldie, 2012; Furley, Taylor, & Obi, 1987; Munir, Zohari, & Berg,

2013; Ogunsanmi, Awe, Obi, & Taiwo, 2003). Four lineages are prevalent in the world while in Pakistan only lineage IV was identified (Abubakar et al., 2008; Aamer Bin Zahur et al., 2014). Clinical signs, symptoms, necropsy lesions, and epidemiology are the basis for PPR field diagnosis but laboratory testing is required for the confirmation. Techniques like immuno-capture ELISA, counter immuno-electrophoresis (CIEP), or agar gel immuno-diffusion (AGID) can be used for the recognition of PPRV antigens in the laboratory (Obi & Patrick, 1984). For instant and definite diagnosis of PPR, the cell culture and molecular methods are prerequisites. All the above-mentioned tests have variable specificity and sensitivity levels.

Numerous RT-PCR procedures are being used for the detection of PPRV. However, conventional RT-PCR has myriad steps, which increase the risk of contamination (Bao et al., 2008). As compared to RT-PCR, the qRT-PCR is a more precise assay as it has high sensitivity and specificity. qRT-PCR (TaqMan probe) was developed using N and M-genes (V Balamurugan et al., 2010; Bao et al., 2008) but quantification and recognition on the basis of the TaqMan probe is costly and requires more expertise to build up the primers and probe (Schmittgen et al., 2000). On the other hand, SYBR green qRT-PCR is more sensitive, cost-effective, and easily implemented. It has proved better results than TaqMan® probe qRT-PCR.

Pakistan is in the phase of controlling PPR, so a panel of diagnostic assays is essential for the control strategy. Up till now a few studies have been conducted on SYBR Green qRT-PCR assay for PPRV diagnosis. There have been few studies regarding the comparison of sensitivity and specificity of PPR quantitative assays i.e. SYBR green-based qRT-PCR and probe-based qRT-PCR. The current study was carried out to standardize SYBR Green qRT-PCR and compare it with conventional RT-PCR and qRT-PCR (TaqMan probe) assays for the detection of PPRV.

MATERIALS AND METHODS:

Samples:

Samples were collected from suspected field outbreaks of PPR in different regions of the country reported. A total of 20 swab samples (Oral, ocular, and nasal) and 26 different tissue samples (Spleen, lungs, and lymph nodes) were collected from sheep and goats during suspected outbreaks. These Samples were processed and stored at -70 °C for further experimentation. For standardization of assays extracted RNA of vaccine strain of PPRV i.e. Nigeria 75/1 was used.

Primer designing:

Primers were designed to target the nucleoprotein (N) gene of PPRV. Genomic data was collected from Gene Bank (accession no. KJ466104, KJ867544, AJ849636, FJ905304, EU267273, KM463083, KM212177, KM091959, KF727981, X74443, KP789375), aligned using Clustal-W and designed with geneious 9.0.4. Primers sequences (Forward: 5'– CCTCGTGAGGCTCAAAGATC– '3; Reverse: 5'– GTTGTCTTCTCCCTCCTCCT– 3) were synthesized by Gene Link.

RNA Extraction and *c*DNA Synthesis:

RNA extraction was performed by "Invitrogen Viral RNA/DNA mini kit" according to the manufacturer's instructions and RNA was stored at -70°C. Synthesis of cDNA was performed by Gena Biosciences SCRIPT cDNA Synthesis Kit.

Standardization of qRT-PCR with SYBR Green for PPRV Detection:

Quantitative Polymerase Chain Reaction (SYBR Green) qRT-PCR with SYBR Green dye was developed and standardized. PCR was done with a final reaction volume of 25µl (Maxima SYBR green Master Mix 12.5µl, 0.75µl of forward and reverse primer (10µM), 2µl cDNA template, and nuclease-free water) using Thermo Scientific Maxima SYBR Green/ROX qPCR kit. Thermal profile for qPCR (SYBR Green): UDG Pretreatment at 50°C for 2 minutes, enzyme activation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds, annealing at 52°C for 20 seconds, extension at 72°C for 20 seconds and final extension at 72°C for 2 minutes. For q-PCR, ABI7500 real-time thermo cycler (Applied Biosystems) was used. During the annealing step of each cycle Ct values for each sample were calculated.

Conventional RT-PCR:

For PPRV detection, NP3 and NP4 primers were used for amplification (Couacy-Hymann et al., 2002). Results of PCR products were evaluated on 1 % agarose gel with the help of the gel Electrophoresis technique (GenoSens 1520, BIO-EQUIP).

qRT-PCR (TaqMan Probe):

qRT-PCR technique was done by using a Thermo Scientific Verso 1-step qRT-PCR kit (Bao et al., 2008). The thermal profile used for qRT-PCR (TaqMan Probe) was: c-DNA synthesis at 50°C for 30 minutes, enzyme activation at 95°C for 5 minutes, 40 cycles of denaturation at 95°C for 20 seconds, and annealing-extension at 60°C for 60 seconds. For qRT-PCR assay ABI7500 real-time PCR system (Applied Biosystem) was used. Ct value for each sample was determined at the annealing step of each cycle, which is the description of the threshold value.

Comparison of PCR-Based Assays:

The diagnostic sensitivity and specificity of newly developed PCR assay and conventional PCR were compared against qRT-PCR (TaqMan®) as the gold standard assuming it has 100% diagnostic sensitivity and specificity. The diagnostic sensitivity (Se) represents the probability of identifying true positive samples using the given diagnostic test. The diagnostic specificity (Sp), on the other hand, is the proportion of truly negative samples that are identified correctly by the diagnostic test (Thrusfield, 2005). This represents the probability of identifying the true negative samples by the diagnostic test.

RESULTS:

Standardization of RT-qPCR (SYBR Green):

PPRV vaccine strain (Nigeria 75/1) c-DNA was used to standardize the newly developed RT-qPCR (SYBR Green) assay. Table I shows the Ct values and detection limit of tenfold serial diluted cDNA.

Table I: Detection limit of RT-qPCR (SYBR Green)

Serial Dilutions	c-DNA Concentration (µg/µL)	Copy Number	Cyclic threshold (Ct) values (SYBR Green)
Log 10	7.5	$3.78x10^{7}$	21.63
Log 10^{-1}	0.75	$3.78x10^{6}$	25.62
Log 10^{-2}	0.075	$3.78x10^{5}$	29.17
Log 10^{-3}	0.0075	$3.78x10^{4}$	31.45

Log 10^{-4}	0.00075	$3.78x10^{3}$	35.01
Log 10^{-5}	0.000075	$3.78x10^{2}$	38.35
Log 10^{-6}	0.0000075	$3.78x10^{1}$	Undetected

For the determination of assay detection limit and validity, the standard curve was developed using a 10-fold serial dilution of c-DNA. This assay can detect 3.78 x 10^2 c-DNA copies in the reaction mixture. The R^2 value was 0.9958, which shows its validity

All PPR-suspected samples were tested by qRT-PCR (SYBR Green). Out of 46 samples, 40 samples were confirmed as positive with distinct Ct values, and 6 samples were confirmed as negative for PPRV with qRT-PCR (SYBR Green). So, 86.95% of samples were found as positive.

qRT-PCR (Probe):

Tenfold serial dilutions of the RNA extracted from the PPRV vaccine were prepared up to six levels. RNA concentration and copy numbers were calculated as above. Table II shows the cyclic threshold values of the amplification of the template.

Table II: Serial dilutions results of qRT-PCR (Probe)

Serial Dilutions	RNA Conc. (µg/µL)	Copy Number	Ct values (Probe)
Log 10	9.0	$5x10^{7}$	19.87
Log 10^{-1}	0.9	$5x10^{6}$	23.33
Log 10^{-2}	0.09	$5x10^{5}$	25.9
Log 10^{-3}	0.009	$5x10^{4}$	30.43
Log 10^{-4}	0.0009	$5x10^{3}$	35.67
Log 10^{-5}	0.00009	$5x10^{2}$	37.54
Log 10^{-6}	0.000009	$5x10^{1}$	Undetected

Ct values are increasing as the copy number decreases. However, as dilution increases, the copy number of RNA decreases up to a certain limit after which no fluorescent signal was detected by the PCR machine. These results show that this assay can detect $5x10^2$ copy numbers at a minimum. The R-value for this assay was 0.9884.

This assay can detect 5.0 x 10^2 RNA copies in a reaction mixture with its Ct-value of 37.54. The assay dynamic range was over the 10-log-unit span of viral RNA concentration, stretching from 5.0 x 10^1 to 5.0 x 10^7 RNA copy number per reaction mixture.

Samples tested by RT-PCR (SYBR Green) were also tested with qRT-PCR (TaqMan). Out of 46 samples, 11 samples were confirmed as negative after performing qRT-PCR (TaqMan) and 35 samples were declared as positive with different Ct values. So, 76.08% of samples were positive by qRT-PCR (TaqMan).

Conventional RT-PCR:

RT-PCR was performed for PPRV detection from outbreak samples. Amplification was done by using N gene primers. Out of 46 samples, 19 samples were negative for PPRV while 27 samples were positive for PPRV by RT-PCR. Fig.1 shows a representation of positive PCR results on agarose gel. So, 58.69% outbreak samples were detected positive for PPRV with the help of RT-PCR.

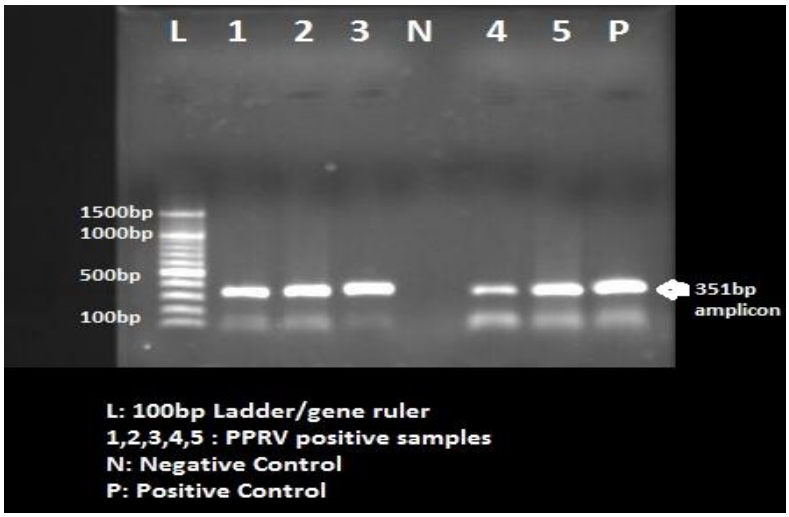

Fig. 1: Agarose gel image showing RT-PCR positive samples.

Comparison of RT-PCR, qRT-PCR (TaqMan) and qRT-PCR (SYBR Green)

Fig.2 shows the comparison of RT-PCR, qRT-PCR with TaqMan probes, and qRT-PCR with SYBR green probes. RT-PCR (58.69%), qRT-PCR (TaqMan) (76.08%), and qRT-PCR (SYBR Green) (86.95%) were compared. The Percentage obtained from the SYBR Green method was higher, as shown in the graph above than RT-PCR and qRT-PCR (TaqMan).

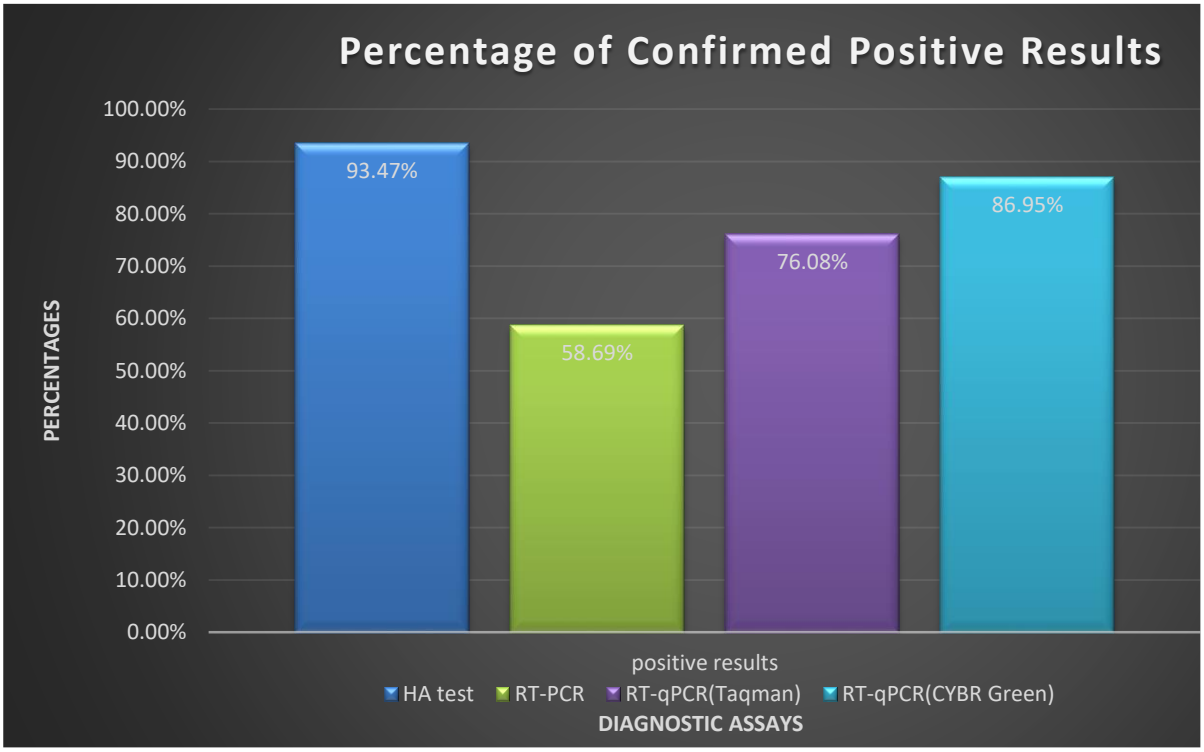

Fig. 2: Comparison of RT-PCR, qRT-PCR (TaqMan) and qRT-PCR (SYBR green)

The diagnostic sensitivity and specificity of each test were compared against qRT-PCR (TaqMan®). The diagnostic sensitivity of conventional PCR against qRT-PCR (TaqMan®) was 68.57% (95% CI; 50.71% - 83.15%) and specificity was 72.73% (39.035-93.98%). Both tests showed weak agreement (Kappa= 0.33). The diagnostic sensitivity of SYBR Green quantitative PCR was 94.29% (95% CL; 80.84%-99.3%) and specificity was 36.36% (95%CI; 10.93% - 69.21%). The kappa value for agreement was 0.36, representing a poor agreement. The data suggests that the sensitivity of qRT-PCR (SYBR Green) was higher than that of conventional RT-PCR, while the sensitivity of both types of qRT-PCR (TaqMan® and SYBR Green) was almost equivalent as both detected 0.1 million times dilution of PPRV vaccinal strain. Though the assays showed poor diagnostic specificity, the newly developed assay has a much better specificity than that of qRT-PCR (SYBR Green). However, the assay developed in this study is more cost-effective than qRT-PCR (TaqMan® Probe) and can be used as a screening test for large-scale studies aimed at eradication of PPR by 2030.

DISCUSSION:

PPR is a highly transmissible disease of small ruminants, with great economic influence, and is also included in the transboundary diseases of animals. PPRV is globally distributed throughout South Asia, the Middle East, and also in Africa (Barrett, Banyard, & Diallo, 2006). Clinical signs that appear in animals during PPR disease include ocular and nasal discharges, fever, pustules (lesions) in the mouth, cough, and diarrhea with a high rate of morbidity and mortality. Economic losses in PPR disease occur due to milk and meat product losses, abortion in female animals, and high death rates (Nawathe & Lamorde, 1984). The main income source of poor farmers especially in the developing countries is small ruminants (goats and sheep). Thus, disease control is very important to reduce poverty in developing countries (Diallo, 2006).

Real-time RT-PCR for PPR diagnosis and quantification has been standardized by various researchers using probe-based technology and SYBR Green dye. The first time Bao and coworkers in 2008 developed 1-step qRT-PCR using N-gene primers and probe and compared its sensitivity with conventional RT-PCR (Bao et al., 2008). Kwaitek et al in 2010 and Baten and his colleagues in 2011 developed another one-step RT-PCR for the diagnosis of all four lineages of PPRV using N-gene primers and probe. They also compared this assay with conventional RT-PCR (Batten et al., 2011; Kwiatek et al., 2010). In 2012, Balamurugan et al 1st time developed real-time one-step RT-PCR using SYBR Green for the detection of PPRV. They used M-gene primers and compared the assay with conventional RT-PCR and real-time RT-PCR (TaqMan probe) (Vinayagamurthy Balamurugan et al., 2012). In 2014, Abera et al developed the SYBR green assay using N-gene primers and compared it with conventional RT-PCR (Abera, Thangavelu, Chandran, & Raja, 2014). However, Abera did not compare it with real-time RT-PCR (TaqMan probe) for estimation of its sensitivity. In this study, we developed qRT-PCR (SYBR Green) by designing new primers using the N –gene of the PPRV sequence and compared it with conventional RT-PCR, qRT-PCR (TaqMan Probe).

Conventional RT-PCR is considered as gold standard test for PPRV detection (A. B. Zahur et al., 2008). Real-time RT-PCR is a rapid, more sensitive technique for the diagnosis of PPRV (Bao et al., 2008; Batten et al., 2011). The detection limit of this assay was 5.0×10^2 RNA copies which were lower than Bao et al results i.e. 8.1 RNA copies (Bao et al., 2008). This was probably due to the difference in reagents and real-time machines.

Further, we selected sequences of the N gene for designing new primers. These newly developed primers were used in real-time RT-PCR analysis by using SYBR Green mixture. This assay was optimized and the standard curve was developed using a 10-fold dilution of c-DNA (PPRV vaccinal strain). The detection limit of this assay was 3.78×10^2 c-DNA copy numbers. Sensitivities of both types of qRT-PCR were almost equivalent to each other but greater than conventional RT-PCR.

After optimization of both assays, PPRV-suspected samples were tested. qRT-PCR (TaqMan Probe) showed 76.08% positive while qRT-PCR (SYBR Green) showed 86.95 % positive results. This shows that qRT-PCR (SYBR Green) is more sensitive than the TaqMan probe as also indicated by Balamurugan for M-gene (Vinayagamurthy Balamurugan et al., 2012). In this study, the sensitivities of both qRT-PCR (TaqMan and SYBR Green) were found to be comparable and other studies have reported that the sensitivity of the SYBR Green method is greater than TaqMan for PPRV detection but it is less expensive than the TaqMan probe. So, this method can be used as an alternative of the TaqMan method (Abera et al., 2014).

Both qRT-PCR methods (SYBR Green and TaqMan) are more rapid, specific, and more sensitive than conventional RT-PCR. A larger number of samples can be tested at one time with more feasibility than conventional RT-PCR. Chances of cross-contamination are also less in these methods. These methods revealed high recommendations for field samples of PPRV, as it was clear from the results of outbreak samples. Thus real-time PCR (SYBR Green) is suggested as a faster technique with greater specificity, sensitivity, and reliability than conventional RT-PCR in PPRV detection. This assay is comparatively less expensive than the qRT-PCR (TaqMan Probe) and it is more sensitive, thus this method can be used as the substitute for conventional RT-PCR for rapid PPR diagnosis in animals.

ACKNOWLEDGEMENT:

We are thankful to the Agriculture Linkages Program (ALP) project on PPR for financial support, the National Reference Laboratory for Poultry Diseases (NRLPD), and the National Veterinary Laboratories (NVL) for providing equipment for qRT-PCR.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES:

Abera, T., Thangavelu, A., Chandran, N. D. J., & Raja, A. (2014). A SYBR Green I based real time RT-PCR assay for specific detection and quantitation of Peste des petits ruminants virus. *BMC veterinary research, 10*(1), 22.

Abubakar, M., Ali, Q., & Khan, H. A. (2008). Prevalence and mortality rate of peste des petitis ruminant (PPR): possible association with abortion in goat. *Tropical Animal Health and Production, 40*(5), 317-321.

Abubakar, M., Rajput, Z. I., Arshed, M. J., Sarwar, G., & Ali, Q. (2011). Evidence of peste des petits ruminants virus (PPRV) infection in Sindh Ibex (Capra aegagrus blythi) in Pakistan as confirmed by detection of antigen and antibody. *Tropical animal health and production, 43*(4), 745-747.

Balamurugan, V., Sen, A., Venkatesan, G., Yadav, V., Bhanot, V., Bhanuprakash, V., & Singh, R. K. (2012). A rapid and sensitive one step-SYBR green based semi quantitative real time RT-PCR for the detection of peste des petits ruminants virus in the clinical samples. *Virologica Sinica, 27*(1), 1-9.

Balamurugan, V., Sen, A., Venkatesan, G., Yadav, V., Bhanuprakash, V., & Singh, R. (2010). Isolation and identification of virulent peste des petits ruminants viruses from PPR outbreaks in India. *Tropical animal health and production, 42*(6), 1043-1046.

Bao, J., Li, L., Wang, Z., Barrett, T., Suo, L., Zhao, W., . . . Li, J. (2008). Development of one-step real-time RT-PCR assay for detection and quantitation of peste des petits ruminants virus. *Journal of virological methods, 148*(1), 232-236.

Barrett, T., Banyard, A. C., & Diallo, A. (2006). Molecular biology of the morbilliviruses *Rinderpest and Peste des Petits Ruminants* (pp. 31-IV): Elsevier.

Batten, C. A., Banyard, A. C., King, D. P., Henstock, M. R., Edwards, L., Sanders, A., . . . Barrett, T. (2011). A real time RT-PCR assay for the specific detection of Peste des petits ruminants virus. *Journal of virological methods, 171*(2), 401-404.

Couacy-Hymann, E., Roger, F., Hurard, C., Guillou, J., Libeau, G., & Diallo, A. (2002). Rapid and sensitive detection of peste des petits ruminants virus by a polymerase chain reaction assay. *Journal of virological methods, 100*(1), 17-25.

Diallo, A. (2006). Control of peste des petits ruminants and poverty alleviation? *Zoonoses and Public Health, 53*(s1), 11-13.

Fentahun, T., & Woldie, M. (2012). Reviewon Peste Des Petits Ruminants (PPR). *European Journal of Applied Sciences, 4*(4), 160-167.

Furley, C., Taylor, W., & Obi, T. (1987). An outbreak of peste des petits ruminants in a zoological collection. *The veterinary record, 121*(19), 443-447.

Gargadennec, L., & Lalanne, A. (1942). La peste des petits ruminants. *Bull. Serv. Zoo. AOF, 5*, 15-21.

Kwiatek, O., Keita, D., Gil, P., Fernández-Pinero, J., Clavero, M. A. J., Albina, E., & Libeau, G. (2010). Quantitative one-step real-time RT-PCR for the fast detection of the four genotypes of PPRV. *Journal of virological methods, 165*(2), 168-177.

Munir, M., Zohari, S., & Berg, M. (2013). Pathophysiology and Clinical Assessment of Peste des Petits Ruminants *Molecular biology and pathogenesis of Peste des Petits Ruminants virus* (pp. 33-48): Springer.

Nawathe, D. R., & Lamorde, A. G. (1984). Socio-economic impact of rinderpest in Nigeria. *REVUE SCIENTIFIQUE ET TECHNIQUE-OFFICE INTERNATIONAL DES EPIZOOTIE, 3*(3), 575-581.

Obi, T., & Patrick, D. (1984). The detection of peste des petits ruminants (PPR) virus antigen by agar gel precipitation test and counter-immunoelectrophoresis. *Journal of hygiene, 93*(03), 579-586.

Ogunsanmi, A., Awe, E., Obi, T., & Taiwo, V. (2003). Peste des petits ruminants (PPR) virus antibodies in African grey duiker (Sylvicapra grimmia). *African Journal of Biomedical Research, 6*(1).

Schmittgen, T. D., Zakrajsek, B. A., Mills, A. G., Gorn, V., Singer, M. J., & Reed, M. W. (2000). Quantitative reverse transcription–polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Analytical biochemistry, 285*(2), 194-204.

Singh, R. K., Balamurugan, V., Bhanuprakash, V., Sen, A., Saravanan, P., & Yadav, M. P. (2009). Possible control and eradication of peste des petits ruminants from India: technical aspects. *Vet Ital, 45*(3), 449-462.

Thrusfield, M. (2005). *Veterinary Epidemiology* (3rd ed.). Oxford, UK: Blackwell Publishing.

Zahur, A. B., Irshad, H., Hussain, M., Ullah, A., Jahangir, M., Khan, M. Q., & Farooq, M. S. (2008). The epidemiology of peste des petits ruminants in Pakistan. *Rev Sci Tech, 27*(3), 877-884.

Zahur, A. B., Irshad, H., Ullah, A., Afzal, M., Latif, A., Ullah, R. W., . . . Jahangir, M. (2014). Peste des Petits Ruminants Vaccine (Nigerian Strain 75/1) Confers Protection for at Least 3 Years in Sheep and Goats. *Journal of Biosciences and Medicines, 2*(06), 27.