

LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR RAPID AND SENSITIVE DETECTION OF PESTE DES PETITS RUMINANTS VIRUS IN FIELD CONDITIONS

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ABSTRACT

Eradication of Peste des petits ruminants (PPR) is currently the center of attention of the Food and Agriculture Organization (FAO). The key to success of efforts put in eradication of PPR are hidden in efficient and sensitive diagnosis of the PPR. For the detection of PPR virus (PPRV), molecular methods such as reverse-transcription PCR (RT-PCR) and loop-mediated isothermal amplification (LAMP) are preferred over serological assays because to their higher sensitivity and specificity. However, molecular techniques are time consuming and require sophisticated laboratories for viral RNA extraction, reverse transcription, amplification and analysis steps. In the present study, single tube, LAMP was successfully established by incubating virus cultures or clinical samples with a special LAMP buffer and an amplification reaction system at 63°C for sixty minutes under isothermal conditions. In the test, the ESE-Quant tube scanner was used as heating block and fluorescence analyzer that can easily be operated in the field conditions by using rechargeable batteries. The new PPRV-LAMP setup excludes the need for pre-requisite steps of total RNA extraction and reverse transcription prior to the amplification of target viral genome and post amplification lengthy analysis by gel electrophoresis, thereby, providing with the option of field based sensitive, reliable and efficient molecular diagnosis of PPRV. The sensitivity and reliability of the LAMP was compared with conventional RT-PCR. The amplified LAMP products were confirmed by both naked eye visualization and agarose gel electrophoresis. This power independent simplistic technology would result in the ease of PPRV diagnosis especially in the far from areas of disease endemic countries like Pakistan. In conclusion, this study provides an important field based molecular diagnostic tool for the detection and surveillance of PPRV.

Keywords: Peste des petits ruminants virus; PPRV, Loop mediated isothermal amplification, LAMP, Latest diagnostic methods for PPR

INTRODUCTION

Peste des petits ruminants (PPR) is an acute, extremely infectious viral disease of goats and sheep that incurs significant losses to the livestock economy (Kwiatek *et al.*, 2011). Clinically, the disease is characterized by serous ocular and nasal discharge that gradually may become muco-purulent, high fever up to 106°F, pneumonia and diarrhea that often result into 80-100% morbidity and 20-80% mortality rates (Abu-Elzein *et al.*, 1990; Zahur *et al.*, 2008; OIE 2013). The causative PPR virus (PPRV) of the devastating disease is a negative sense RNA virus; member of the genus *Morbillivirus* (Taylor *et al.*, 1990). Viral genome of PPRV is ~16kb in length, composed of 15,948 nucleotides that are characterized into contiguous, non-overlapping transcription units that encode six structural proteins (Bailey *et al.*, 2005). These units are arranged from 3 to 5

as nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin (H) and large polymerase protein (L). PPR is considered to be the main transboundary infection that continues to affect a large proportion of the sheep and goat populations around the globe especially in countries with poor economic conditions (Cohen, 2000). In Pakistan, PPR is an evolving disease of small ruminants infecting considerable portion of livestock population (Abubakar *et al.*, 2011).

Based on the genetic differences among the virus strains belonging to various geographical regions, there are four well characterized lineages of the PPRV; out of those lineages, lineage-IV is the most wide-spread lineage present in the Southern Asia, Middle East and many countries of Africa (Banyard *et al.*, 2010). More than 50% of the sheep and goat population is seropositive for PPRV antibodies in Pakistan and the lineage-IV viruses detected so far (Abubakar *et al.*, 2009; Zahur *et al.*, 2011; Munir *et al.*, 2012; Ashraf *et al.*, 2105).

Considering the transboundary nature of PPR, Food and Agriculture Organization (FAO) has currently focused on its eradication by 2030 (Semedo, 2015). The control of PPR would mainly rely on the availability of effective vaccination and cost-effective diagnostic tools for laboratory assessment of suspected cases (Couacy-Hymann *et al.*, 2005). Efficient and confirmed diagnosis is the basis of any disease intervention that not only helps in the deciding for effective drugs but also the prevention of evolution of resistant microorganisms that cause infectious diseases (Urdea *et al.*, 2006). Providentially, effective vaccine, Nigeria-75/1 a lineage-II PPRV, is available for the development of lifelong immunity against PPR, however, sensitive, efficient and cost-effective diagnosis can be improved through the incorporation of molecular methods. Previously, conventional methods of serology in addition to virus isolation had been used to detect PPRV infection in suspected animals (Barrett, 1999). However, these methods require long working hours and often insensitive in detecting PPRV during the initial stage of disease (Singh *et al.*, 2004). Molecular techniques have improved the diagnosis of important transboundary animal diseases, for example, standard reverse transcription-polymerase chain reaction (RT-PCR) was applied to diagnose and differentiate PPRV from Rinderpest virus (RPV) (Nanda *et al.*, 1996); another morbillivirus that caused PPRV like disease in cattle and buffalo. Other forms of PCR, including a multiplex RT-PCR, Enzyme linked immunosorbant assay (ELISA) and real-time PCR has also been used for the specific detection of infectious diseases of animals. Fluorogenic-based detection methods utilizing TaqMan probes are routinely being used for the diagnosis as well as prognosis of viral diseases (Rodriguez-Sanchez *et al.*, 2008). Including benefits of sensitivity and specificity, molecular techniques require sophisticated instruments to reduce the likelihood of cross contamination and training which make them highly expensive and labor intensive (Parida *et al.*, 2008).

Loop-mediated isothermal amplification (LAMP) is a new, cost effective method that ensures high sensitivity. It is based on the amplification of genomic ribose or deoxyribose nucleic acids. It involves the strategy of amplifying target genome by utilizing a set of four to six primers (outer, inner and loop primers) (Misawa *et al.*, 2007; Njiru *et al.*, 2008). The addition of reverse transcriptase in the LAMP reaction mixture makes it possible to convert RNA into complementary DNA (cDNA) sequence forming stem-loop structures that appear as a pattern of DNA bands of varying lengths on electrophoresis in agarose gel (Nagamine *et al.*, 2002). During the LAMP, the amplification process is accomplished at a constant temperature in a single-tube requiring a simple water bath and can be detected by incorporating the fluorescent dyes like SYBR green or Eva green into the reaction (Nemoto *et al.*, 2011; Mairet

et al., 2013). For the utilization of LAMP technology in the field, the major challenge is associated with RNA and cDNA synthesis as these steps require special protocols and containment that cannot be maintained in open field. The present study is an advanced modification of LAMP for field based efficient molecular diagnosis of PPRV for the first time in Pakistan.

Novelty statement: The detection of the causative PPR virus (PPRV) genome is an essential criterion for confirmed diagnosis of PPR infection. As absence or presence of clinical signs is not sufficient proof in the current scenario of PPR eradication, the disease must be diagnosed on the genetic basis. Generally in case of PPRV, RT-PCR is recommended. However, challenges associated with RNA isolation and costly thermal cycling cannot allow this confirmatory test to be applied in field conditions where there is no electricity and sophisticated clean laboratory environment is scarce. In such a situation, the detection of PPRV genome in a single tube reaction facilitated by real-time amplification analysis in temporo-spatial manner, without facing the problems of costly sample transportation to the reference laboratory and subsequent relatively lengthy process of RNA extraction, cDNA synthesis and PCR using expensive thermal cyclers, is quite novel diagnostic application of a molecular tool such as Loop mediated isothermal amplification (LAMP). Previous protocols have used purified RNA as template while in present study, viral genome is detected directly in clinical sample without any tedious processing. This ability of our test enables its application in the field especially in far from areas where the veterinarians or practitioners have no access to sophisticated laboratory conditions. The access of trained manpower to sensitive, reliable and cheap diagnostic tools is key to success for any disease eradication campaign to save time and money. By using the LAMP test mentioned in our study, the local veterinarians can get the opportunity to actively participate for quick and reliable disease reporting and response system ultimately helping out FAO global PPR eradication campaign.

MATERIALS AND METHODS

Sample collection : Clinical samples (n=50) from 29 clinically suspected goats were collected from three districts (Chakwal, Mianwali and Arifwala) of the Punjab province, Pakistan. All the samples were collected from apparently sick goats suffering clinically from pyrexia, ocular and/or nasal discharges, stomatitis, diarrhea and pneumonia. Oral, ocular or nasal swabs were stored in phosphate buffer saline (PBS) [pH 7.2 to 7.6] and blood was collected in vacutainers with anti-coagulant EDTA. Tissue samples were collected from dead animals during post mortem examination and include lymph nodes

(mediastinal and mesenteric), spleen and the lungs and stored at -80°C till subsequent processing.

PPRV-LAMP reaction: For initial standardization and isolation of PPRV-LAMP assay, these clinical samples were inoculated into CHS-20 cell lines provided by Animal Production and Health Laboratories, International Atomic Energy Agency, (APHS/IAEA) Vienna, Austria (Adombi *et al.*, 2011). The LAMP reaction was carried out at 63°C to amplify target viral genome by employing outer primers (F3/B3, 5 pmol each), inner primers (FIP/BIP, 50 pmol each), and loop primers (LF/LB, 20 pmol each). Details of the M-gene PPRV primers are given in Table 1. For sample preparation, 14 µl of 5x sample buffer was mixed with 1 µl of virus culture or clinical sample. Lyophilized LAMP master mix [dNTP (0.4mM), trehalose (460mM), Bsm polymerase (8 units), 0.4X EvaGreen® dye] was used after 5-10 minutes' incubation at room temperature. A total volume of 25 µl reaction was carried out containing 8 µl LAMP master mixes, 15 µl sample buffer mixed with sample, 2 µl of primer mix. Then, the final mixture was incubated at constant temperature of 63°C in ESE Quant tube scanner (Qiagen, Germany). The fluorescent signal corresponding to the amount of amplified product was measured once per minute for one hour.

Optimization of LAMP for PPRV: A number of strategies were applied using reaction mixture of different concentrations and dilutions keeping at constant temperature 63°C. Following the first experiment, the positive reaction mixture composition was adjusted in second experiment to acquire the detection of PPR virus within 20-40 minutes and subsequent analysis by agarose gel electrophoresis to confirm the positive sample.

Detection of PPRV-LAMP products: For the detection of amplification, ESE tube scanner was set at the FAM channel for the detection of fluorescence signal. For end product evaluation, SYBR green (2 µl) was added to the LAMP reaction for the endpoint visualization under UV light. Positive reaction was observed for change of color from faint orange to green while the negative one remained colorless. Ladder-like bands for the PPRV LAMP assay was analyzed using 2.5 µl of the LAMP product on 2% agarose gel (0.5x TBE) with ethidium bromide staining. For the confirmation of positive reaction, a threshold value of 30 mVolts per minute was set for the evaluation of amplification curves generated by the serial increase in fluorescent signal during the progress of LAMP reaction.

Comparative Sensitivity of PPRV-LAMP assay: The reliability of results generated through RT-LAMP assay was assessed by battery-driven device, for comparison, RT-PCR was carried out in a total volume of 50 µl reaction mixture using SuperScript™ One-Step RT-PCR

with platinum® Taq (Invitrogen, Carlsbad, CA, USA) contained 5 pmol of each of F3 and B3 (outer primers pair) in addition to 5 µl of total RNA template. The thermal cycling profile included cDNA synthesis step at 50°C for 30 minutes, denaturation at 94°C for 2 minutes. The repeating amplification, 40 cycles of three consecutive steps included 1) 94°C for 20 seconds, 2) 55°C for 30 seconds and 3) 72°C for 30 seconds. Finally, extension step was continued at 72°C for 10 minutes. The electrophoretic analysis of amplified 217bp long PCR products was performed on 2% agarose gel (0.5x TBE).

RESULTS

Clinical signs and symptoms: The samples were collected from infected goats suffering from high fever (40-41°C) had severe depression and staggering gait (see materials and methods section) (Fig. 1). The affected animals, especially in the district of Arifwal showed reddened eyes with one-sided blindness and specific stomatitis during the later stage of infection

Optimization of PPRV-LAMP assay: With the optimal primer concentration and reactions conditions (see materials and methods) isothermal amplification was attained at 63°C at which positive response showing real time curve in graph (Fig. 2) and required ladder-like DNA bands on agarose gel electrophoresis (Fig. 4).

Confirmation of PPRV-LAMP end-point products: The end-point LAMP products were evaluated by visual examination and agarose gel electrophoresis. Positive reaction turned from orange into green, however, negative remained colourless (Fig. 3). The graphical observations remained steady with those of gel electrophoresis. The ladder-like pattern of amplified DNA was observed for positive reactions (Fig. 4).

Comparative Analysis with one-step RT-PCR: The specificity of the amplified products from LAMP assay was confirmed by using forward and backward outer primers of LAMP assay in one-step RT-PCR that amplified 217bp DNA fragment corresponding to the expected length of PPRV-M gene target region (Fig. 5). The LAMP detected PPRV genome in the ten virus cultures; obtained by inoculating the cultured CHS-20 cells with clinical samples, within first thirty minutes of LAMP reaction. In the clinical samples tested, 34.5% were positive both by RT-LAMP and RT-PCR (Table 2). Both these tests had 100% agreement with each other. RT-PCR, however, took longer working hours of around (4-5 hours) for end-point gel electrophoresis based detection than RT-LAMP (1 hour). In comparison to virus cultures requiring 20-30 minutes, the time of PPRV detection in clinical samples ranged from 30-50 minutes in case of RT-LAMP (Figure 2).

Table 1. Primers detail used for the PPRV-LAMP assay*

Primer description	Sequence (5' to 3')	Position	Length (no. of nucleotide bases)
Forward Outer F3	TTGCAATGCAGTCAACCT	420-437	18
Backward Outer B3	ATTCTCCCATGAGCCGA	620-636	17
F1P	GCACACTATAGTAACCATGTGTCTGA- TGATACTCCCCAGAGTT	496-520	25
B1P	GGAGTTCCGCTCAGCCAATG- TTCTAGGGTTTGTGCCATT	447-464 534-553	18 20
Loop Forward LF	TCTAGTTATGCTCATGTACACAACC	592-610	18
Loop Backward LB	GTAGCCTTCAACATCTTGGTTACAC	468-492 556-580	25 25

* Reference (Li *et al.*, 2010)**Table.2. PPRV samples tested for PPRV-LAMP assay**

Regions	Total no. of samples	No. of isolates		RT-LAMP		RT-PCR	
		Positive	Negative	Positive	Negative	Positive	Negative
Arifwala	12	3	9	3	9	3	9
Chakwal	9	4	5	4	5	4	5
Mianwali	8	3	5	3	5	3	5
Total	29	10	19	10	19	10	19

**Fig.1. Sample collection of peste des petits ruminants virus in goats. Nasal discharge (A), Oral lesions (B), Blood collection from jugular vein (C) and Dehydrated goat that died of PPR (D)**

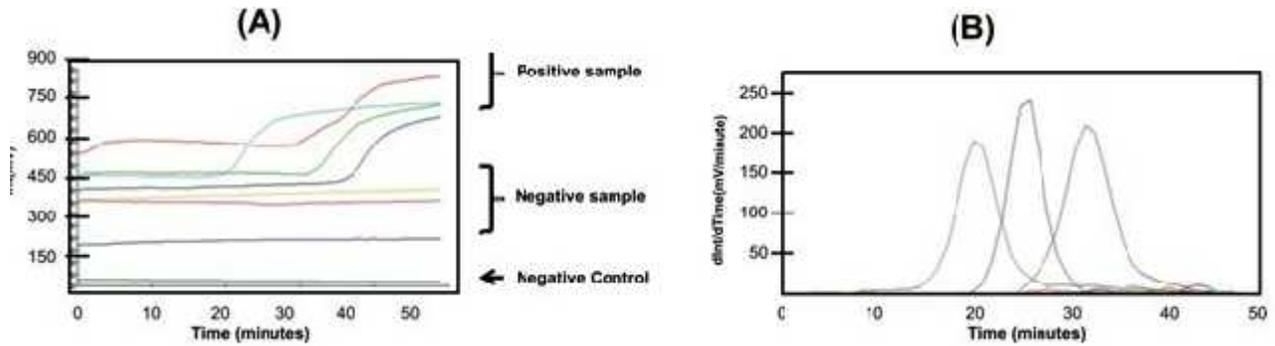


Fig.2. Snapshots of the data in the ESE-Quant tube scanner.(A) Real-time curve was obtained for PPRV samples while negative samples produced no amplification curve.(B) Typical positive reaction amplifications are shown in terms of increase in fluorescence signal per minute.

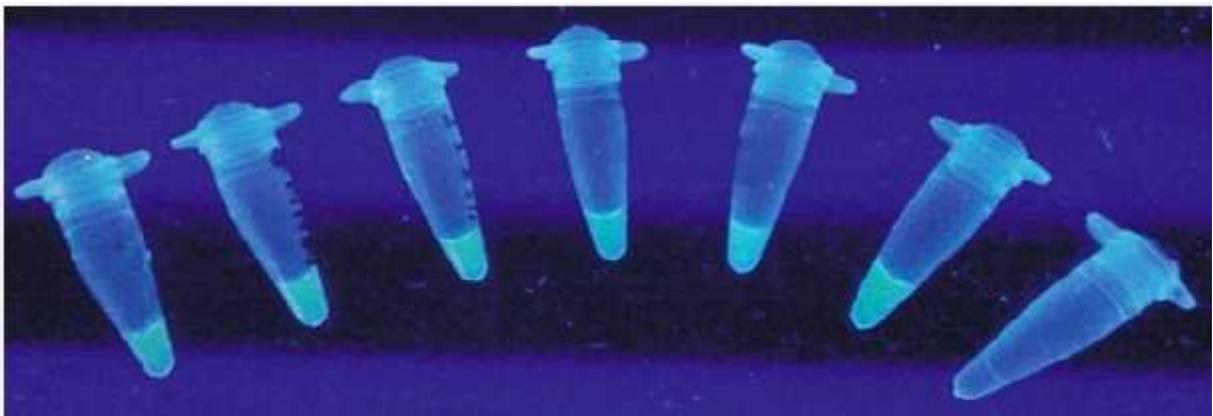


Fig.3. Determination of LAMP assay under UV light. From left to right: 1-6 tube turned green showing positive results and 7 tube remained colorless showing negative result.

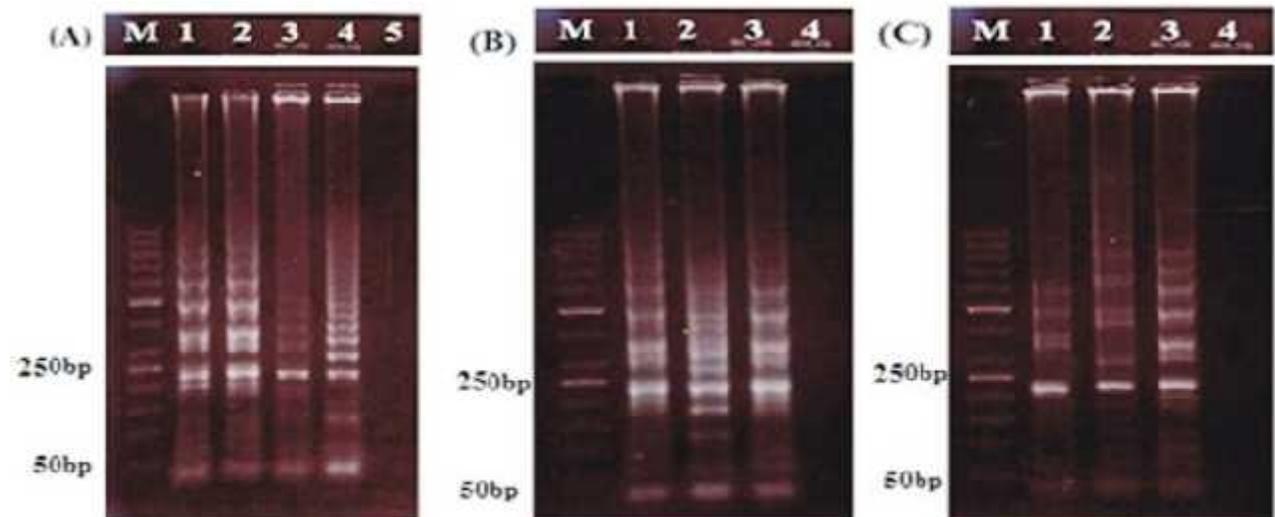


Fig.4. Agarose gel electrophoresis of LAMP products of PPRV. DNA products amplified by LAMP from PPRV infected field samples were analyzed by agarose gel electrophoresis. (A) LAMP assay of Arifwala samples. Lane M: 50bp ladder. Lane 1-4: Positive samples. Lane 5: Negative control. (B) Chakwal samples. Lane M: 50bp ladder. Lane 1-3: Positive samples. Lane 4: Negative control. (C) Mianwali samples. Lane M: 50bp ladder. Lane 1-3: Positive samples. Lane 4: Negative control.

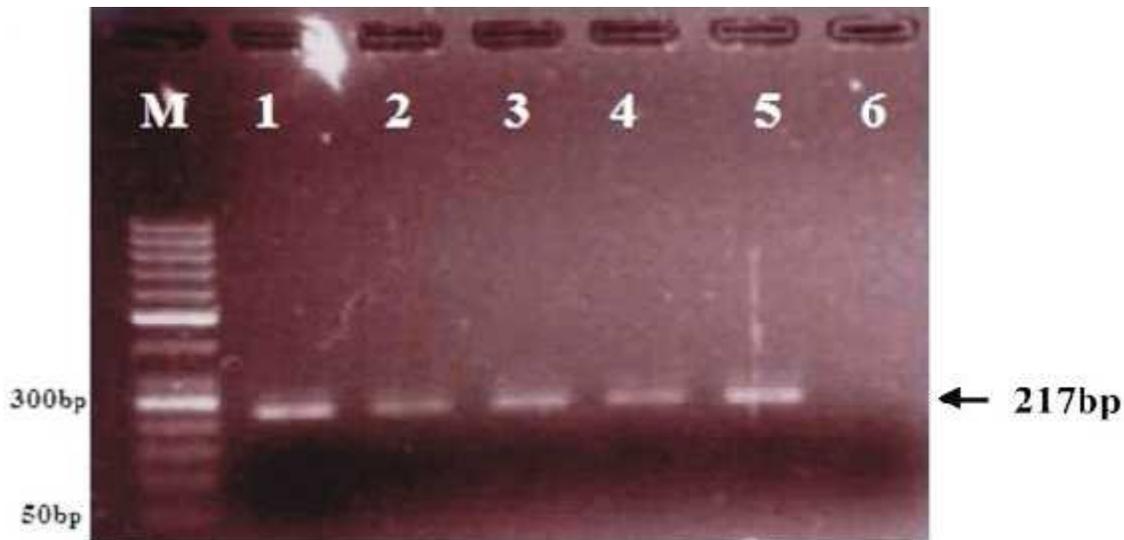


Fig.5. One-step RT-PCR based analysis of PPRV infected clinical samples. Agarose gel electrophoresis of amplified PCR products revealed a ~217bp PCR fragment in positive samples. No amplification observed in negative control well.

DISCUSSION

The current study is the successful pairing of two goals in veterinary diagnostics—molecular detection and simplicity—a combination that allows efficient on-field disease diagnosis. For optimization of the test, different strategies were used based on gradient combination of molar concentrations of LAMP primers, LAMP master mix and template. The fluorescent signal generated at 59°C was too weak for reliable visual detection and DNA banding pattern on agarose gel electrophoresis. However, the detection was significantly improved at 63°C. PPRV positive culture supernatants of viral isolates were detected within twenty minutes in ESE-Quant tube scanner that is indicative of the robustness of the test. Comparative to regular RT-PCR that requires approximately 4-5 hours, the time of detection for these isolates was reduced by 3-4 hours in case of LAMP as it was completed within 60 minutes. RT-PCR with LAMP outer primers also ensured the specific detection of M gene of PPRV by these primers.

LAMP was used to amplify target viral genome within one hour by using isothermal nucleic acid amplification chemistry based on Bsm polymerase strand displacement activity employing a set of six primers. LAMP technology uses special Bst or Bsm polymerase enzyme that harbor strand displacement activity from 5' to 3' end of the primer annealed to the template thereby opening up the zipper of double stranded DNA immediately followed by the synthesis of the template copy. The exponential cycling reaction of LAMP is so sensitive that it can even amplify less than ten copies of DNA to a detectable amount within an hour under isothermal conditions with great accuracy (Kumar

et al., 2012). To demonstrate the effectiveness of this evolving technology for efficient diagnosis of PPR, highly conserved region of the matrix protein gene of the causative virus was selected as a suitable target. M gene encodes the matrix protein that occupies the internal lining of virus envelope (derived from host cell membrane), acts as a cementing material between two external immunogenic proteins, the haemagglutinin (H) and fusion protein (F) and the internal capsid. It also ensures complete formation of progeny virions during multiplication by associating nucleocapsids with them. M gene is highly conserved among PPRVs and the primers used in this study are highly specific to PPRV. According to Li *et al.* (2010) M gene primers had no cross reactivity with RPV, measles virus and canine distemper virus and the test was found as sensitive as real-time RT-PCR with a 10-fold higher detection limit than that of regular RT-PCR. As the RT-LAMP assay was as sensitive as the real-time RT-PCR, with a detection limit ranging from 14.1 ng to 1.41×10^{-5} ng per assay while RT-LAMP had a detection limit of 1.41×10^{-3} ng total RNA. In this study, these primers were incorporated into a special protocol to detect PPRV directly in given sample without a need for isolation of purified RNA—that essentially requires clean environment and sophisticated lab conditions, allowing the test to be carried out in the field on spot. RT-LAMP with this modified approach was found as sensitive as RT-PCR and virus culture.

The idea to use this quite novel diagnostic application of the LAMP assay for the genome based detection of PPRV in suspected clinical samples came from the fact that the standard RT-PCR requires relatively lengthy process of RNA extraction, cDNA

synthesis and PCR using expensive thermal cycler that limits its application in the field. These factors cause an increase in the cost and time of genetic detection. Due to economic issues in developing countries like Pakistan, there is a dire need of a technique which could replace the highly expensive thermocycling equipment (Lau *et al.*, 2008) and be used in the field conditions being independent of constant power to overcome energy crises. In this regard, electricity free technologies for instance simple water incubator maintained at constant temperature with thermal energy and rechargeable batteries based on solar energy have been developed to perform molecular test applicable in field conditions (Njiru, 2012). To utilize LAMP in the field conditions, the PPRV genome should be amplified directly from the clinical sample because RNA extraction requires clean and sophisticated laboratory environment. Previous protocols of LAMP involve the use of purified RNA as template while in our case; viral genome is detectable directly in the clinical samples without any lengthy processing steps prior to amplification reaction. The buffer system used in this study precipitates cellular and viral capsid proteins present in the sample and exposes the intact viral genomes to the efficient LAMP amplification system containing specific primers and Bsm polymerase. The specificity of the test was confirmed by RT-PCR. Three analysis methods including amplification curve, ladder-like display of amplified DNA on agarose gel electrophoresis and color changes were used to confirm the specificity of LAMP results for all PPRV samples. Furthermore, to reduce the time required for analysis of amplification, the test was carried out in ESE-Quant tube scanner, a portable fluorescence reader that serves as heating block as well as generates graphical real-time curve data for analysis (Njiru, 2012; Lucchi *et al.*, 2010). By using DNA intercalating fluorescent Eva green dye in the LAMP reaction mixture, even the quantities amplified by each minute produced enough light signals for detection. The tube scanner offered a very precise and handy setup to conduct LAMP due its ability to work even on power supply of a rechargeable battery. Therefore, it excluded the need of a costly thermal cycler and pre-requisite steps of total RNA extraction, its reverse transcription prior to the amplification of target viral genome and post amplification lengthy analysis by gel electrophoresis. This new LAMP setup, ultimately, provides with the option to detect PPRV by LAMP in the field conditions where on-site availability of electricity is scarce. The mathematical algorithm in tube-scanner software for the confirmation of a positive reaction and the intrinsic property of LAMP to detect eight small sequences of the viral genome within the target region assures highly specific diagnosis. By using special buffer system, intercalating dye and tube scanner based fluorescent signal detection along with the process of LAMP, the

robustness of the test was multiplied which lead to speedy diagnosis with simplicity- a factor which is very important for the efficient application of strategies for disease control ultimately linked to the disease eradication.

Currently, the detection of the causative virus (PPRV) genome is an essential criterion for confirmed diagnosis of the transboundary disease of PPR. WHO and FAO have declared PPR as potential candidate for eradication. The achievement of this goal would mainly rely on the effective implementation of the disease control strategies which essentially requires monitoring of the disease dispersal in the concerned population through timely, sensitive and efficient diagnosis. In the current scenario of PPR eradication, diagnosis simply based on the absence or presence of clinical signs or antibodies in the host blood is not sufficient. Many a time, the clinical symptoms of PPR are not so evident especially in cases of co-infection with other associated bacterial or viral infections and sub-clinical form of disease present in immune-competent hosts. Even different animals respond differently to same epidemic or experimental infection (Diop *et al.*, 2005). Poor diagnosis often allows an un-noticed circulation and spread of PPRV to susceptible population as reported by Diop *et al.* (2005) and Bidjeh *et al.* (1995). Therefore, the disease must be diagnosed on the genetic basis using sensitive and robust molecular tools. Previously, routine diagnosis of PPR was made by serological tests (agar gel immune-diffusion and counter immune-electrophoresis), antigen and antibody detection methods but these techniques are unable to detect the low quantity of the virus (Diallo *et al.*, 1995; Poxton 2005). Although the serological tests like ELISA can detect the antibodies against PPRV, that persist for weeks and months in the blood of recovered animals, those cannot confirm the presence of active viral infections, that are indicative of potential disease foci in a population. Generally in case of PPRV, RT-PCR is recommended. However, challenges associated with RNA isolation and costly thermal cycling cannot allow this confirmatory test to be applied in field conditions lacking the provision of electricity and sophisticated clean laboratory environment required for molecular detection. Other PCR-based diagnostic methods such as RT-PCR, SYBR green/Taqman real-time RT-PCRs are widely used for the nucleic acid amplification, however, require sophisticated equipment thereby increasing the cost of diagnosis and are labor intensive techniques comparatively (Yoshida *et al.*, 2005). The situation is even worse in the distant areas where sample transportation to the reference laboratory is not a suitable option. In such a situation, LAMP established in this study for the detection of matrix gene of PPRV provides an ideal alternative for the detection of PPRV genome in a single tube reaction facilitated by

real-time amplification analysis by ESE-Quant tube scanner.

As Pakistan is facing poor economic conditions, health problems and electricity shortage, the LAMP set-up established in this study provides very effective solution for disease diagnosis under these circumstances. The test is very simple, rapid, sensitive and suitable for the field diagnosis of PPRV infection. The intrinsic ability of this test to omit costly and cumbersome pre-amplification, amplification and post amplification steps enables its application in the field especially in far from areas where the veterinarians or practitioners have no access to sophisticated laboratory conditions. The access of trained manpower to sensitive, reliable and cheap diagnostic tools is the key to success for any disease eradication campaign to save time and money. By using the LAMP test mentioned here, the local veterinarians can get the opportunity to actively participate for quick and reliable disease reporting and response system ultimately helping out FAO global PPR eradication campaign. It will surely contribute to control of PPRV through better diagnosis especially in the distant areas of Pakistan where un-noticed circulation of PPR causes outbreaks and huge economic losses each year. The LAMP setup used could be tailored for simple, cost-effective and robust diagnosis of other diseases of livestock and poultry in the country, such as Foot and Mouth Disease (FMD), Avian Influenza and Brucellosis. Furthermore, the sensitivity and specificity of the current appliance of LAMP will be validated for the diagnosis of PPR using extensive field samples.

Acknowledgements: Authors would like to acknowledge IAEA for the provision of funding for reagents and resources through CRP project Research Contract No. 14567 entitled: Establishment of PCR based method for early & sensitive diagnosis of PPR.

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