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RESEARCH ARTICLE

Loop-Mediated Isothermal Amplification Assay for Peste des Petits Ruminants Virus Detection and its Comparison with RT-PCR

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ABSTRACT

Peste des petits ruminant (PPR) represents an acute, highly contagious, transboundary viral disease of goats (predominantly) and sheep. The disease is endemic in Pakistan with annual losses of 20.5 billion rupees. The objective of the study was to optimize the rapid, efficient and more sensitive diagnostic tool for disease outbreak investigation and field surveillance. Accordingly, single tube loop mediated isothermal amplification (LAMP) technique was performed by incubating virus and/or suspected clinical samples at 58°C for 60min, and it was compared with conventional reverse transcription polymerase chain reaction (RT-PCR). Based on sequence analysis, 2 primer pairs were used, named as NP3/NP4 and NPx/NPy (this study) for RT-PCR to target the nucleoprotein gene and three sets of primers namely, F3/B3, FIP/BIP, F loop/B loop for RT-LAMP to target the matrix gene. The NP3/NP4 primers performed better compared with our designed NPx/NPy primers in RT-PCR. Twenty-five clinical samples were evaluated by LAMP assay, and the results were found consistent with those of conventional RT-PCR. LAMP primers showed high specificity and had no cross reaction with other animal viruses *i.e.* FMDV and IBDV. The sensitivity of LAMP assay was 10-fold higher than conventional RT-PCR. As a simple, inexpensive and accurate detection method, RT-LAMP assay is well suited for rapid clinical diagnosis of PPR. Therefore, it can easily replace RT-PCR for disease investigation and surveillance of PPRV in field conditions especially in developing countries like Pakistan.

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INTRODUCTION

Peste des petits ruminants (PPR) represents one of the most economically significant, extremely contagious, transboundary disease of small ruminants caused by a Morbillivirus that belongs to the family *Paramyxoviridae* (Fentahun and Woldie, 2012; Anonymous, 2017). It exists in peracute, acute and subacute pathogenic forms and severely affects goats (predominantly) and sheep in about 70 countries worldwide (Munir, 2014; Gitao *et al.*, 2016). The losses were estimated around 1.5-2 billion USD every year with morbidity and mortality rates in susceptible population reaching up to 100% and 90% respectively. Genus morbillivirus has a negative sense ssRNA genome encoding V, C non-structural proteins and six structural proteins: the fusion (F) and haemagglutinin (H) proteins forming the surface glycoproteins, nucleoprotein (N), matrix protein (M) and phosphoprotein (P) forming polymerase complex with large (L) protein (Bailey *et al.*, 2005; Munir *et al.*, 2013; MacLachlan and Dubovi, 2017). Among all these six proteins, nucleocapsid (N) protein is richly transcribed in host cells; it therefore has its significance in the confirmation of PPR virus. Initially ELISA was used for screening and diagnosis of PPRV. However, this method is time taking and also unable to detect PPR virus during early stages of disease (Singh *et al.*, 2004). On the basis of sensitivity and more specificity, RT-PCR has been widely used for diagnosing PPR virus (Toplu *et al.*, 2012). However, it is time taking, laborious, has contamination risks and also costly especially in case of real-time PCR (Bao *et al.*, 2008).

In recent past, a simple, rapid, field applicable, easy to perform diagnostic tool called as LAMP: loop mediated isothermal amplification has been developed for

confirmation of PPR virus targeting N gene (Santhamani *et al.,* 2016). LAMP is considered as the new method of DNA amplification having high sensitivity and specificity (Yamazaki *et al.,* 2013). It is an efficient protocol which yields DNA in higher quantities that is enough for confirmation by visual examination. In case of RNA viruses, the RNA template is used directly as starting material for reverse transcription together with LAMP in a single step, hence making it suitable for diagnostic purpose (Wang *et al.*, 2011; Ashraf *et al.*, 2017). The process is based on strand displacement reaction and the target site is amplified via stem-loop structure rapidly with accuracy, selectivity and high specificity under the isothermal conditions (Parida *et al.*, 2006; Wang *et al.*, 2015). The entire process is simple and amplification can be achieved by incubating all reagents in one tube in less than an hour. Thus lamp assay has been found to be a powerful genetic amplification tool for rapid detection and typing of emerging viruses (Parida *et al.*, 2008). It has also revealed great supremacy over conventional reverse transcription PCR in case of field diagnosis (Dukes *et al.*, 2006; Parida *et al.*, 2006). In Pakistan, LAMP has not been used for rapid diagnosis of PPR under field conditions. Therefore this study was carried out to optimize LAMP assay reaction conditions and compare it with conventional RT-PCR.

MATERIALS AND METHODS

Collection of clinical samples: A total of 25 clinical samples from animals (sheep and goats) suspected of having PPR, which included two samples each from lung and liver, one sample each from spleen, intestine and intestinal lymph nodes, swabs from nasal cavity (14), eyes (2) and oral lesions (2) were collected from various outbreaks occurred in Jhang and Faisalabad region of Pakistan, from animals with clinical signs of PPR. Blood (5ml) was collected from each animals in disposable syringe out of which, 3 ml transferred to serum vacutainer (having no anticoagulant) to let the blood coagulate for serum separation and 2 ml added to vacutainer containing EDTA. After receiving, the samples were stored at -70°C with proper labeling. This study was approved by and carried out under the guidelines of the Ethical Committee and Institutional Animal Care and Use Committee (IACUC) of Animal Sciences Division, NIAB.

Preparation of PPR samples: About 500 µl PBS (Phosphate Buffer Saline) was added to sample collection tube containing nasal swabs from goat. It was mixed properly by vertex mixer. Any cotton was removed and sample was given a short spin to get the sample. From this extracted solution, 150 µl was used for RNA extraction and remaining was placed at -80°C for future use.

RNA extraction: RNA was extracted using FavorPrep viral nucleic acid extraction kit (Favorgen, Taiwan).

Reverse transcription of RNA into complementary DNA (cDNA) synthesis: A 5 µl of extracted RNA, 1 µl random hexamer primer and 6 µl nuclease free water provided by Revert Aid™ First Strand cDNA Kit (Cat # K1622, Fermentas), were denatured at 65°C (5 min) and immediately chilled over ice (2 min). Reverse Transcription was carried out in 20 µl volume having denatured RNA (12 µl), reaction buffer (4 µl), dNTP 10 mM each (2 µl), 1 µl of Ribolock (RNase inhibitor) and 1 µl of Revert Aid (reverse transcriptase). Reaction was performed at 25°C for 5 min, 42°C for 1 hr and 70°C for 5min. 2 µl of cDNA was used in RT-PCR and RT-LAMP reactions.

Polymerase chain reaction: Two PCR reactions were set up using 2 pairs of primers NP3:(TCTCGGAAATCGCC TCACAGACTG); NP4:(CCTCCTCCTGGTCCTCCAGA ATCT) and NPx:(CTCTGGAGCTATGCGATGGGTGT C); NPy:(AGTTGGCTGGGGGTCTCT) and Taq polymerase (Thermo Scientific, Cat # EP0402). The PCR was performed with initial pre-denaturation at 94°C for 5 min and 30 cycles of 94°C for 60 sec, 57°C for 60 sec, 72°C for 60 sec, followed by final extension at 72°C for 10 min. The amplified products were shown on 1% agarose gel as 353 bp DNA band with NP3/NP4 and 407 bp with NPx/NPy primers respectively.

One step RT-PCR: RT-PCR was also performed using verso enzyme one-step Hot-Start RT-PCR kit (Thermo scientific, USA) in a final reaction volume of 25 µl having 0.5 µl Verso enzyme mix, 12.5 µl 1-step Hot-Start PCR Master Mix (2X), 1.25 µl RT enhancer, 5 µl RNA template, 0.5 µl forward and backward primers (10 mM each) and 4.75 µl nuclease free water. Reaction mixture was given incubation at 56°C for 15 min for cDNA synthesis, followed by single cycle at 95°C for 15 minutes, 30 cycles of 95°C for 20 sec, 55°C for 30 sec, 72°C for 60 sec, followed by 72°C for 10 min. The amplified products were observed by using 1% agarose gel.

RT-LAMP reaction: The NP gene specific primers which includes outer F3, B3; inner FIP, BIP and LF, LB loop primers were used as reported by (Li *et al.,* 2010) given in Table 1. The LAMP reaction was performed using RNA amplification kit with *Bsm* DNA polymerase (Thermo scientific, Cat # VO525). Each 25 µl reaction mixture contained 10 µl reconstituted master mixture, 5 µl reaction buffer (5X), 1 µl $MgSO_4$ (25 mM), 1 µl primer mix (5 pmol of outer primers, 40 pmol of inner primers, 20 pmol of loop primers), 1 µl template RNA and 7 µl nuclease free water. After initial optimization of reaction conditions at different temperatures (50-60°C) for various time span (15-60 min), incubation at 58°C for 60 min gave best results, therefore, all the LAMP reactions in this study were done at 58°C for 1 hr and reaction was terminated by heating at 80°C for 5 min. The RT-LAMP product was visually inspected by ESEtube scanner and analyzed using 2.5 µl of LAMP product on 2 % agarose gel with ethidium bromide staining. In ESEQuant tube scanner 95, the assay was considered as positive, if the amplification curve was generated within 40 minutes. At the end Tp Values of positive samples were determined as described by Veronica *et al*. (2016).

Specificity and sensitivity of PPR-LAMP test: PPRV LAMP primer pairs were evaluated for cross reactivity against foot-and-mouth disease virus (FMDV) and infectious bursal disease (IBDV). These FMDV or IBDV RNA containing LAMP reaction mixture also served the

purpose of the negative control. The sensitivity of LAMP for detection of PPRV was done by preparing 10 fold serial dilutions (10^{-1} to 10^{-6}) of RNA standards quantified by using NanoDrop spectrophotometer.

RESULTS

Identification of PPRV genome in clinical samples: Presence of the PPR viral genome was confirmed by RT-PCR using NP3/NP4 primer sets in 13 out of 14 nasal swabs, 2 liver samples and 1 each oral, ocular and spleen samples, while primer pair NPx/NPy could detect only 3 samples (2 nasal and 1 ocular) (Table 2).

Confirmation of PPRV through LAMP assay: The ability of LAMP assay to detect the virus directly from clinical samples was determined by comparing it with conventional RT-PCR. All clinical samples (25 Nos.) were subjected to LAMP analysis as well; 20 samples were found positive in comparison to 18 that were detected positive by RT-PCR (Table 2). Amplification curves that reached beyond threshold (>30 mV/minute for a minimum of 3 measurements) in signal strength were considered positive for PPRV genome.

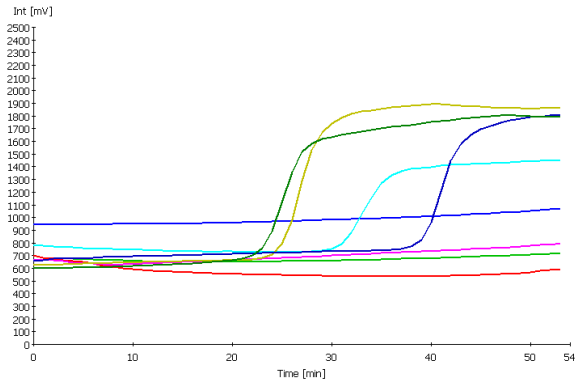

Fig. 1: RT-LAMP assay to detect PPRV in different tissues of an infected goat.

Table 1: List of primers used for detection of PPRV by RT-LAMP assay

Primer name	Sequence (5'-3')
PPRV F3	TTGCAATGCAGTCAACCT
PPRV B3	ATTCTCCCATGAGCCGA
PPRV FIP	CACACTATAGTAACCATTGTCTGATGATACTC CCCAGAGGTT
PPRV BIP	GGAGTTCCGCTCAGCCAATGTTCTAGGGTTT GTGCCATT
PPR FLp	TCTAGTTATGCTCATGTACACAACC
PPR BLp	GTAGCCTTCAACATCTTGGTTACAC

Table 2: The RT-PCR and RT-LAMP detection of clinical samples from animals (sheep and goats) suspected of having PPR

Tissue samples	Number of samples tested	PPRV Positive		
		Via RT-PCR		Via RT-LAMP
		NP3/NP4 Primers	NPx/NPy Primers	LAMP Primers
Lung	2	-	-	-
Liver	2	2	-	2
Spleen	1	1	-	1
Intestine	1	-	-	-
intestinal lymph nodes	1	-	-	-
nasal swab	14	13	2	14
mouth swab	2	1	-	2
eye swab	2	1	1	1
Total	25	18	3	20

Samples from various tissues of an infected goat were subjected to LAMP assay and as shown in Fig. 1, the samples of nasal discharge and liver were positive and gave positive signals. Whereas RT-PCR-negative tissue samples and negative control remained negative for one hour test time signifying that RT-LAMP assay can replace RT-PCR.

Specific and sensitive detection of PPRV genome through LAMP assay: For detection of PPRV, LAMP reaction was performed in a single tube using LAMP primers and PPRV extracted RNA as a template. LAMP viral RNA products were shown as a ladder pattern on agarose gel (2%) given in Fig. 2. The reactions containing samples of FMDV or IBDV as negative control formed no products as indicated in the figure. Results suggest that the LAMP assay with specifically designed primers for PPRV is highly specific and convenient for detecting the PPRV complementary genome.

In terms of sensitivity, when the LAMP assay was performed with a 10-fold dilution series of viral RNA from 10^{-1} to 10^{-6}, the PPRV viral RNA could be detected from 10^{-1} to 10^{-4} (Fig. 3). However, when RT-PCR was done with same dilutions, the viral RNA could be detected form 10^{-1} to 10^{-3} as showed in gel electrophoresis of RT-PCR products given in Fig. 4; indicating that LAMP is more sensitive than RT-PCR.

DISCUSSION

In the present study, out of 25 suspected samples, the PPR antigen was confirmed through RT-PCR in 72% (n=18/25) samples. When our designed NPx/NPy primer pairs were used, only 3 samples were found positive, possible reason for failure of this primer pair was that reverse primer was placed in variable region and it was not complementary to most of isolates. In this way, NP3/NP4 primers performed better as compared with our designed NPx/NPy primers. All those samples that were found positive by NPx/NPy primer pair were also positive with NP3/NP4, So for later analysis NP3/NP4 were adopted. In a similar study, the same gene was amplified by NP3 and NP4 primer using RT-PCR. About 195 samples (nasal swab and tissues) were screened and PPR virus was confirmed in 27.53% of nasal swabs while the detection value from tissues was recorded as 49.12% (Saritha *et al.*, 2015).

Nowadays the diagnosis of PPR infections is based on immunoglobulin detection methods or viral isolation. The nucleic acid based amplification techniques are also used for rapid and sensitive detection of PPR virus (Baron *et al.*, 2014). Recent outbreaks of PPR have also highlighted the importance of the efficient protocols for proper surveillance of this virus especially in developing countries. However for on-site application LAMP has the advantage because of its easiness and rapidity (Nemoto *et al.*, 2011; Tian *et al.*, 2018). In this study, LAMP is compared with RT-PCR as more sensitive and rapid assay for clinical diagnosis of PPRV. LAMP was performed with LAMP primers using the same dilutions of PPRV RNA as described for RT-PCR.

Table 3: Tp values (Time to positivity in minutes) of the RT-LAMP assay for different tissues of infected goats

Sample	Spleen	Lung-1	Liver-1	Lung-2	Liver-2	Nasal swab-1	Nasal swab-2
Tp Value (minutes)	-	-	+30	-	+22	+39	+21

Table 4: Tp values (Time to positivity in minutes) of the RT-LAMP assay for dilutions of PPRV RNA

RNA decimal dilutions	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Tp Value (minutes)	+15	+20	+21	+24	+52	-	-

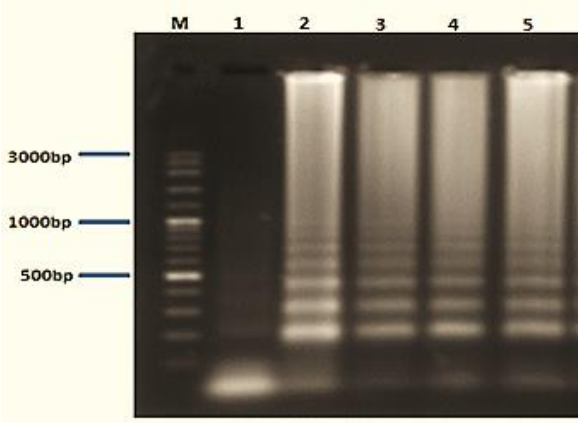

Fig. 2: Agarose gel electrophoresis results of LAMP products. Lane M, 100 bp DNA ladder; Lane 1, Negative control; Lanes 2,3,4,5 showing LAMP products from PPRV RNA sequential 10-fold dilutions.

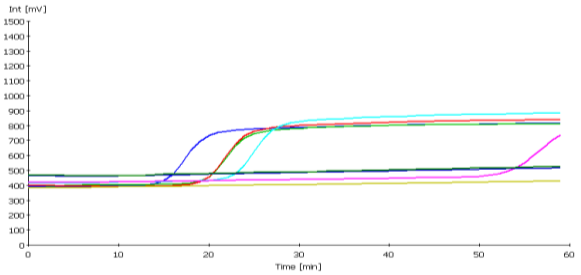

Fig. 3: Pattern of differences in amplification curves obtained during RT-LAMP reaction of 10-fold serial dilutions of isolated RNA samples of PPRV Green, positive control (original isolated PPRV RNA sample); Pink, Red, Light Blue, Light Green, Aqua, 10-Fold serial dilutions of isolated PPRV RNA as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} (Negative dilution); Dark Blue, Negative control (FMDV RNA sample).

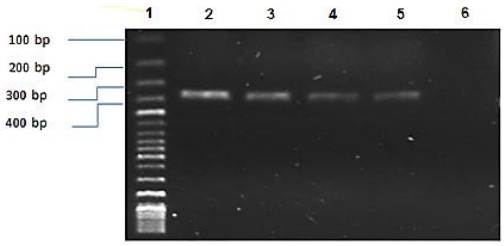

Fig. 4: Gel Electrophoresis (1.5% Agarose) of RT-PCR Products of 10-Fold Serial Dilutions of Isolated RNA Samples of PPRV. Lane 1, 100 bp DNA ladder; Lane 2, Amplified RT-PCR product of PPRV RNA; Lane 3-6, RT-PCR products from 10-Fold serial dilutions of isolated PPRV RNA.

Concentration of extracted RNA was determined as95.7 ng/µl by using NanoDrop Spectrophotometer. Ten fold dilutions were used and it was found that RNA dilutions up to 10^{-4} were detected via LAMP by positive signals and curve on tube scanner screen. In this way, LAMP detected as little as 11.3 ng/µl of RNA which is one serial dilution higher than detected by RT-PCR i-e, 10^{-3} was the last dilution that could be detected; therefore, LAMP is 10-times more sensitive in comparison to RT-PCR. This finding is in-line with Ding *et al.* (2014) and Ashraf *et al.* (2017). These results showed that LAMP assay is useful for detection of PPRV at low levels especially for confirmation at early stages when virus titers are relatively low (Dadas *et al.*, 2012; Balamurugan *et al.*, 2014).

LAMP primers were also tried with IBDV and FMDV RNA samples, and there was no cross activity and primers were specific for PPRV only (Li *et al.*, 2010; Maryam *et al.*, 2017). These results indicate that LAMP assay is a specific technique for PPRV. To evaluate the diagnostic ability of LAMP assay, supernatant isolated form triturated clinical samples was directly processed with this technique rather than RNA extraction from clinical samples as used for RT-PCR. Both LAMP assay and conventional RT-PCR were applied for detection of PPRV in these samples. Out of 25 samples, 20 were detected positive by LAMP assay as compared to RT-PCR which detected 18 samples. These results clearly confirmed the excellent diagnostic ability of LAMP assay.

Conclusions: In summary, RT-LAMP is highly specific, very efficient and more sensitive than RT-PCR for identification of PPRV.

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Authors contribution: YN: performed research, analyzed data, wrote paper. MH: funded research, supervision, analyzed data. SS helped with experiment, analyzed data. MM: analyzed data, wrote paper.

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