



Development of a TaqMan loop-mediated isothermal amplification assay for the rapid detection of pigeon paramyxovirus type 1

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Abstract

Pigeon paramyxovirus-1 (PPMV-1) is a strain of Newcastle disease virus (NDV) that has adapted to infect pigeons and poses a constant threat to the commercial poultry industry. Early detection via rapid and sensitive methods, along with timely preventative and mitigating actions, is important for reducing the spread of PPMV-1. Here, we report the development of a TaqMan loop-mediated isothermal amplification assay (TaqMan-LAMP) for rapid and specific detection of PPMV-1 based on the *F* gene. This system makes use of six novel primers and a TaqMan probe that targets nine distinct regions of the *F* gene that are highly conserved among PPMV-1 isolates. The results showed that the limit of detection was 10 copies μL^{-1} for PPMV-1 cDNA and 0.1 ng for PPMV-1 RNA. The reaction was completed within 25 min and was thus faster than conventional RT-PCR. Moreover, no cross-reactions with similar viruses or with peste des petits ruminants virus (PPRV) or NDV LaSota vaccine strains were observed under the same conditions. To evaluate the applicability of the assay, the TaqMan-LAMP assay and a commercial RT-PCR assay were compared using 108 clinical samples, and the concordance rate between two methods was found to be 96.3%. The newly developed PPMV-1 TaqMan-LAMP assay can therefore be used for simple, efficient, rapid, specific, and sensitive diagnosis of PPMV-1 infections.

Introduction

Newcastle disease virus (NDV), also referred to as avian paramyxovirus 1 (APMV-1), is highly infectious and has devastating effects on commercial poultry and other bird species [2, 11, 22]. The broad circulation of NDV in poultry has led to genetic divergence of virus strains into two groups,

referred to as class I and class II. Class I includes three sub-genotypes, class II contains at least 20 identified genotypes (from I to XXI without XV), and pigeon paramyxovirus 1 (PPMV-1) belongs to class II, genotype VI (VI.1.1) [1]. PPMV-1 can be distinguished as unique antigenic variant of NDV by the hemagglutination inhibition (HI) assay [5]. A member of the family *Paramyxoviridae*, subfamily *Paramyxovirinae*, and genus *Avulavirus*, PPMV-1 has a single-stranded, negative-sense RNA genome that is approximately 15.5 kb in length. This RNA contains six genes encoding the following six structural proteins (from 5' to 3'): RNA-dependent RNA polymerase (L), hemagglutinin-neuraminidase (HN), fusion (F), matrix (M), phosphoprotein (P), and nucleoprotein (NP) [7, 26]. In addition, W and V proteins are produced by RNA editing and are generated during transcription of the *P* gene [25]. The cleavage site of the F protein is ¹¹²G/RRQKRF¹¹⁷ [9], and this feature is generally characteristic of PPMV-1. PPMV-1 was first reported in Italy in 1981 [3] and subsequently reached epidemic levels, spreading via racing and show pigeons to all regions of the world [24]. In China, PPMV-1 was first reported in 1985 in exotic pigeons, and it subsequently spread throughout the country [11, 26]. The mortality rate in pigeons is 70% [10,

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13, 24]. Therefore, an efficient, rapid, and sensitive detection method is needed for early detection of the virus and to reduce economic losses.

Several molecular detection methods have been developed to confirm PPMV-1 infections, with reverse transcription polymerase chain reaction (RT-PCR) being the most commonly used [4, 6, 16, 19, 21]. However, RT-PCR assays are limited in terms of pathogen detection because of the need for specialized laboratory equipment and professional training, as well as their relatively time-consuming nature. As a result, RT-PCR assays are often unavailable in poorly resourced laboratories and under field conditions.

Loop-mediated isothermal amplification (LAMP) was developed by Notomi and is a rapid, simple, and sensitive technique [17] that can be carried out at a constant temperature of 60–65°C for 60 min with four to six primers that recognize six or eight specific regions of the target DNA sequence, as well as a sequence-specific DNA polymerase (*Bst* DNA polymerase) [18, 23]. Recently, LAMP assays have been developed for the rapid detection of important pathogens such as SARS-CoV-2 [14], dengue virus [29], JC polyomavirus (JCV) [12], and porcine senecavirus A [28]. This method is less dependent on complex instruments than RT-PCR and has been widely used to detect viruses and other pathogens [15, 29]. However, due to the high concentrations of primers used in LAMP amplification, it is inevitable that some mismatches and primer dimers occur. This can lead to false positive results during high-speed LAMP amplification [12, 14, 28, 29].

In this study, a method combining a TaqMan probe with loop-mediated isothermal amplification (TaqMan-LAMP) was developed to prevent the false positive results associated with LAMP assays. Simultaneously, we used *Bst* 5.0 DNA polymerase with strong strand displacement ability, efficient DNA synthesis ability, and exonuclease activity. This method takes advantage of the rapid, highly sensitive, and constant-temperature detection that can be achieved by LAMP while mitigating the issue of false positive results. The newly developed TaqMan-LAMP is a simple and rapid method for PPMV-1 surveillance and lays a solid foundation for the wider application of LAMP technology in clinical diagnosis.

Materials and methods

TaqMan-LAMP primer design

Six PPMV-1-specific LAMP primers and a probe targeting the *F* gene were designed based on the genomic sequence of PPMV-1 (GenBank number HM063425.1) using Primer Explorer version 5 (primerexplorer.jp/lampv5e/index.html). The primers included two outer primers (F3 and B3), two

inner primers (FIP and BIP), and two loop primers (LoopF and LoopB), as well as a TaqMan probe (Fig. 1 and Table 1). Primers were synthesized by GENEWIZ (GENEWIZ, Suzhou, China), dissolved in RT-PCR-grade water (Invitrogen) to a final concentration of 100 µM, and stored at -20°C. The sequences of the primer binding sites were compared to the corresponding sites in other related viruses to identify sequence variations, insertions or deletions.

RNA and DNA extraction

Viral RNA was extracted from feces using an RNA extraction kit (QIAGEN, MD, USA). Total DNA was extracted from the DNA viruses porcine circovirus (PCV), Marek's disease virus (MDV), and egg drop syndrome virus (EDSV) using a DNA extraction kit (QIAGEN, MD, USA). The concentration and quality of the RNA and DNA was estimated by measuring the OD₂₆₀ and the OD₂₆₀/OD₂₈₀ ratio using a NanoDrop Lite Spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA and DNA were stored at -80 °C for subsequent analysis.

TaqMan-LAMP assay reaction conditions

A 20 µL TaqMan-LAMP reaction mixture contained the FIP (F1c-F2) and BIP (B1c-B2) internal primers at a concentration of 16 µM, the F3 and B3 outer primers at a concentration of 2 µM, loopF and loopB at a concentration of 8 µM, and 8 µM probe. The mixture also contained 10 µL of LAMP TaqMan reagent (HaiGene, Haibin, China), 1 µL of DNA or cDNA template, and nuclease free water. The reactions were performed at 65°C for 10 s and 65°C for 50 s with 25 cycles. The total time for the reaction was 25 min.

Specificity analysis of the TaqMan-LAMP assay

The specificity of the PPMV-1 TaqMan-LAMP assay was determined using other viruses (PCV, MDV, EDSV, canine parainfluenza virus [CPIV], peste des petits ruminants virus [PPRV] vaccine strain and NDV LaSota vaccine strain), which were maintained at the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences.

Sensitivity and detection limit of the TaqMan-LAMP assay

To prepare a DNA positive plasmid control, a PPMV-1 *F* gene fragment (4550–6211) was inserted into pJET 1.2. The copy number of the DNA standard was calculated using the following formula: DNA copies ml⁻¹ = DNA concentration (g mL⁻¹) × 6.02 × 10²³/DNA length × 660. Tenfold serial dilutions of the DNA positive plasmid standard, from 10⁻¹ to 10⁻⁷, were used to estimate the limit of detection.

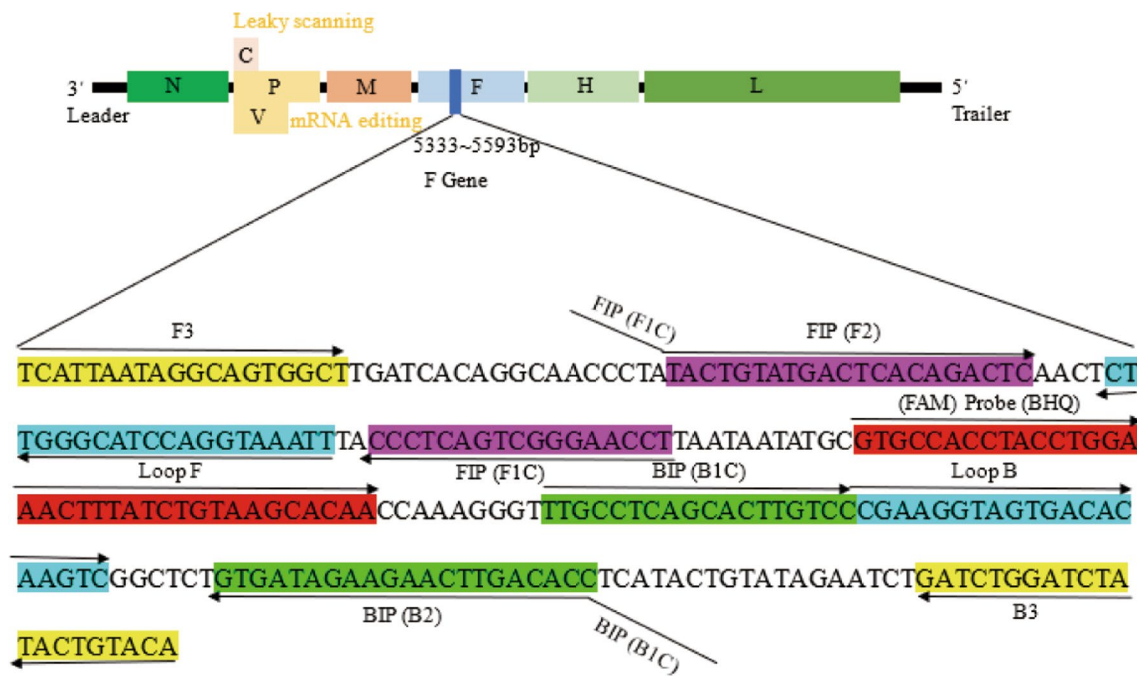


Fig. 1 Primer information. Location and partial sequences of the primer sets targeting the PPMV-1 *F* gene. F3 and B3 are outer primers, FIP (F1c-F2) and BIP (B1c-B2) are inner primers, LoopF and LoopB are loop primers. The primers contained the fluorophore FAM (6-carboxy-fluorescein) and BHQ (Black Hole Quencher). The F1c

and F2 sequences form the FIP primer, and B1c and B2 form the BIP primer. The primers are depicted in different colors, with yellow used for outer primers, indigo for loop primers, magenta for FIP, green for BIP, and red for the probe.

Table 1 Sequences of the primers and probe used in this study

Target	Primer/probe	Sequence (5'-3')	Length	Position (bp)
PPMV-1-F	F3	TCATTAATAGGCAGTGGCT	19	5333-5351
	B3	TGTACAGTATAGATCCAGATC	21	5573-5593
	FIP	AGGTTCCCGACTGAGGGTACTGTATGACTCACAGACTC	38	5370-5390/5417-5433
	BIP	TTGCCTCAGCACTTGTCGGTGTCAAGTTCTTCTATCAC	39	5490-5507/5534-5554
	LoopF	AATTTACCTGGATGCCAAG	20	5395-5414
	LoopB	CGAAGGTAGTGACACAAGTC	20	5508-5522
	Probe	(FAM)GTGCCACCTACCTGGAACTTTATCTGTAAGCACAA(BHQ)	36	5445-5480

Clinical sample testing

In total, 108 fecal samples from different pigeon farms were collected to evaluate the TaqMan-LAMP method. The same samples were also tested by RT-PCR as described previously [16]. RNA extraction and reverse transcription were performed using commercial kits according to the manufacturer’s protocols (QIAGEN, MD, USA). Healthy pigeons were used as healthy controls, and deionized water was used as a negative control.

Results

Primer design

The LAMP primers and probes were designed for the PPMV-1 *F* gene (GenBank accession no. MH807446.1) using LAMP designer software (Premier Biosoft International). All of the primers and probes were checked for sequence conservation by BLAST, available on the NCBI website. Then, a TaqMan-LAMP reaction with nonspecific

amplification in the non-template control set targeting the *F* gene was performed, which was found to have higher amplification efficiency, and was chosen for use in the development of the TaqMan-LAMP detection method. A probe with FAM as the 5' fluorophore and BHQ as the 3' quencher was designed as shown in Fig. 1 and Table 1.

Establishment of the TaqMan-LAMP detection method

The reaction was performed at 65°C for 10 s and at 65°C for 50 s for 25 cycles. Reactions were performed with the newly designed primers and 1.0×10^{-1} ng μL^{-1} of positive plasmid control (pJET-F) or negative control (pJET). No amplification was observed for pJET, and a peak amplification time of 20 min was observed with pJET-F and PPMV-1 RNA from PPMV-1-infected pigeons (Fig. 2). After RNA was extracted from virus-infected tissues, the present method developed on the basis of the specific primer probe was used to detect different amounts of RNA. The results shown in Fig. 3, indicate that this method can detect 0.1 ng of extracted RNA within 25 min, but samples containing 0.01 ng of RNA did not yield a fluorescence signal.

Specificity of the PPMV-1 TaqMan-LAMP assay

Total RNA was extracted from paramyxoviruses (CPIV, PPRV vaccine strain and NDV LaSota vaccine strain), and DNA was extracted from MDV, PCV, and EDSV. The pJET-F plasmid was used as a positive control, and distilled water was used as a negative control. Fifty nanograms of each nucleic acid was used for detection. Specificity tests showed that an amplification curve was obtained with the positive plasmid, but not with any of the other viruses (Fig. 4).

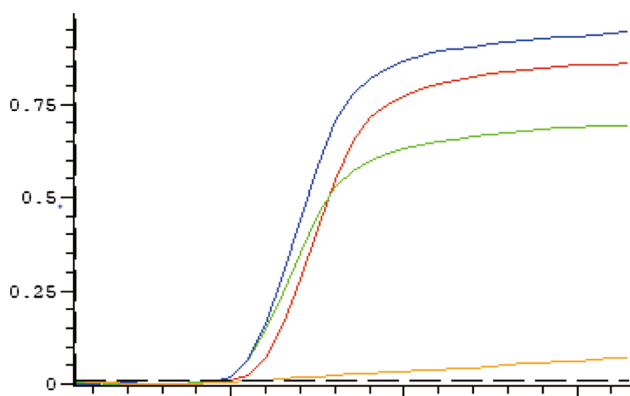


Fig. 2 Detection of PPMV-1 from PPMV-1 infected pigeons, pJET-F, and pJET by TaqMan-LAMP assay. The blue curve represents the positive plasmid control (pJET-F), the red and green curves represent RNA from infected pigeons, the orange line represents the negative control (pJET), and the green line represents the baseline threshold.

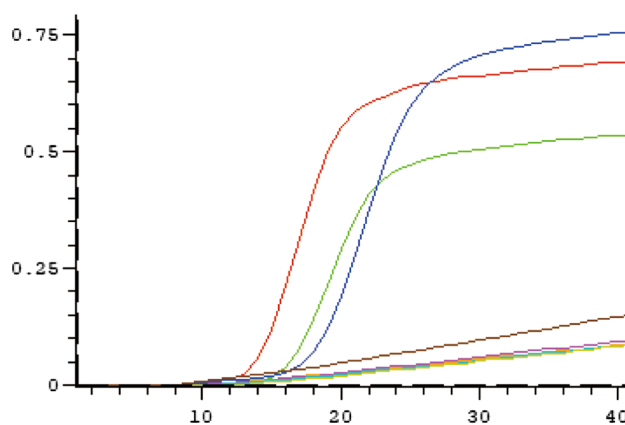


Fig. 3 Sensitivity of PPMV-1 RNA detection at different concentrations. The red, blue, and green curves represent concentrations of 10 ng, 1 ng, and 0.1 ng, respectively. The orange, magenta, turquoise, and light golden lines represent concentrations of 10^{-2} ng, 10^{-3} ng, 10^{-4} ng, and 10^{-5} ng, respectively, and the sienna line represents the pJET negative control.

Sensitivity of the PPMV-1 TaqMan-LAMP assay

The PPMV-1 positive plasmid was serially diluted ten times, with seven dilutions ranging from 10^{-1} to 10^{-7} , and TaqMan-LAMP amplification was performed according to the method established to confirm the validity of the procedure. Sensitivity analysis showed that, with pJET-F plasmid as the detection template, the TaqMan-LAMP method could effectively detect the standard plasmid at a concentration of 10 copies μL^{-1} , and the reaction time was just 25 min (Fig. 5).

Evaluation of the PPMV-1 TaqMan-LAMP method using clinical samples

To evaluate the clinical application of the TaqMan-LAMP assay, it was compared with an RT-PCR assay using 108 clinical samples collected from a pigeon farm. Of those samples, 24 were positive and 84 were negative for PPMV-1 in the TaqMan-LAMP (Table 2). The concordance rate between RT-PCR and TaqMan-LAMP was 96.3%.

Discussion

PPMV-1 infected pigeons can spread viruses via the oral, tracheal or cloacal route and easily infect the sentinel bird in the loft. Early detection and timely diagnosis are crucial in preventing the spread of the virus and further losses. This is especially true for PPMV-1, which is one of the most important contagious viruses affecting pigeon farms. Currently, the preferred methods of confirming the identity of the virus in infected pigeons are RT-PCR, restriction enzyme

Fig. 4 Specificity of the TaqMan-LAMP assay. The common poultry viruses used in this assay include MDV, PCV, and EDSV. Other paramyxoviruses (CPIV, PPRV vaccine strain and NDV LaSota vaccine strain) were also tested. pJET-F and PPMV-1 RNA were used as positive controls, and pJET was used as a negative control. The red curve represents pJET-F detection, and the green curve represents PPMV-1 RNA detection.

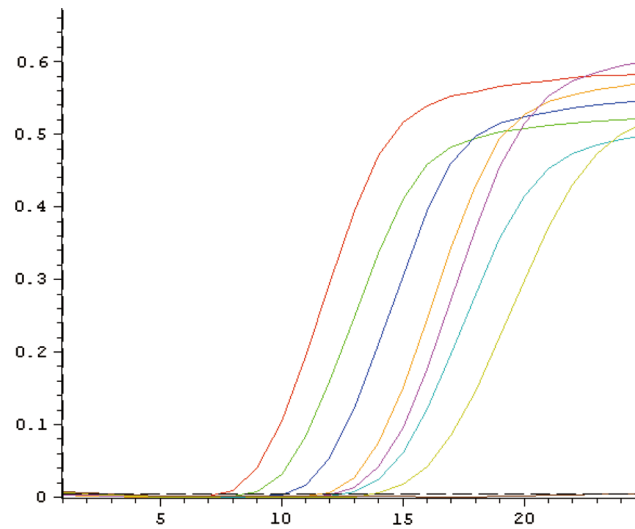
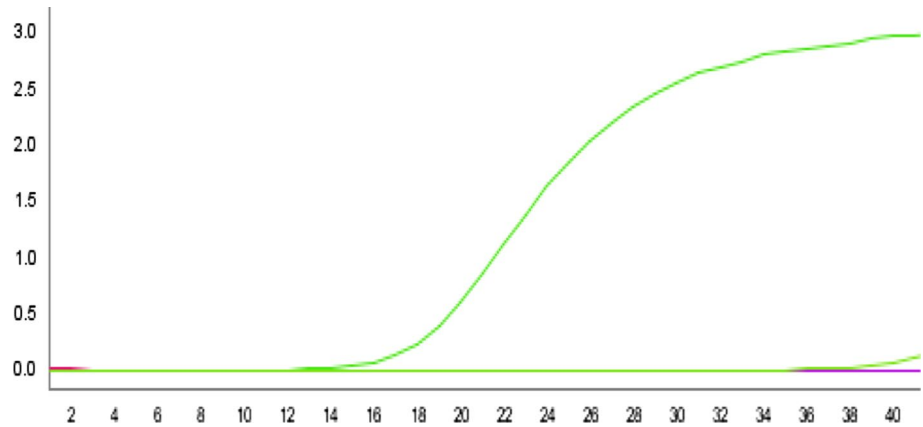


Fig. 5 Sensitivity of the TaqMan-LAMP assay determined using ten-fold serial dilutions of pJET-F. Red, green, blue, orange, magenta, cyan, and light golden lines represent different dilutions containing of 10^7 to 10 copies of the plasmid.

analysis, and RT-nested PCR [13, 23]. However, these methods require specialized facilities and highly trained workers and are time consuming, which renders them unsuitable for detecting pathogens under field conditions. Several studies on the development of diagnostic methods for other pathogens have shown that LAMP assays exhibit equal or even higher sensitivity when compared with qPCR assays [8, 27].

Probe based LAMP techniques are among the most specific and sensitive diagnostic methods available [20]. Our results indicate that the TaqMan-LAMP is a rapid, simple, accurate, and sensitive detection method that can be utilized under both laboratory and field conditions for the early diagnosis of PPMV-1 infections [20].

This is the first report of a TaqMan-LAMP assay used to detect PPMV-1. In this study, a TaqMan-LAMP diagnostic method was established for the detection of PPMV-1 in infected pigeons, using a set of six primers and a probe targeting the *F* gene. The TaqMan-LAMP assay was optimized using RNA extracted from PPMV-1-infected pigeon faeces as a template. PPMV-1 RNA in samples was detected in as little as 25 min, whereas PCR assays require at least 2 h. The method was shown to be highly sensitive, with a detection limit of 10 copies per reaction, and there was no cross-reactivity with other paramyxoviruses (CPIV, PPRV, vaccine strain and NDV LaSota) or other poultry viruses (MDV, PCV, and EDSV). RT-PCR and restriction enzyme analysis are not able to distinguish PPMV-1 from other APMV-1 genotypes [16], whereas TaqMan-LAMP was found to specifically differentiate PPMV-1 from the NDV LaSota vaccine strain. The specificity of this assay derives from the use of a set of six primers and a probe targeting nine regions of the PPMV-1 *F* gene. The diagnostic specificity of the TaqMan-LAMP assay was demonstrated by comparison with RT-PCR. The concordance rate between two methods was 96.3% (Table 2), indicating higher specificity of the TaqMan-LAMP method.

Table 2 Comparison of TaqMan-LAMP and RT-PCR assays

TaqMan-LAMP	RT-PCR		Total	Positive rate (%)	Concordance rate (%)
	Positive	Negative			
Positive	20	4	24	22.22%	96.3%
Negative	0	84	84		
Total	20	88	108		
Positive rate (%)	18.52%				

In conclusion, a sensitive and time saving TaqMan-LAMP assay was developed for the rapid and accurate detection of PPMV-1. This method shows excellent potential as a diagnostic tool that could be used to monitor disease in infected pigeons, to detect PPMV-1 infections when visible symptoms emerge, and to identify latent infections to prevent virus transmission and further disease outbreaks.

Author contributions RL developed the TaqMan-LAMP method and prepared the original draft; LL and KH treated samples; XR collected samples; YJ edited the manuscript; CS collected samples and performed investigations; and SC acquired funding. All authors have read and agreed to the published version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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