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Rapid and visual detection of an isolated and identified goose parvovirus (GPV) strain by a loop-mediated isothermal amplification assay

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Abstract

Gosling plague caused by goose parvovirus (GPV), a highly infectious septic disease with high mortality, has caused substantial loss in the waterfowl industry. A method for the rapid detection of GPV is needed. In this study, we isolated the virus strain of GPV in May 2020 and applied it to the loop-mediated isothermal amplification (LAMP) assay. We designed five sets of primers for the goose parvovirus VP3 gene by LAMP. The GV-1 primer set was selected to detect GPV sensitively and rapidly. LAMP was more sensitive compared to PCR. In addition, the LAMP method could complete detection within 60 min which was faster than the PCR assay. The LAMP provided a convenient and effective experimental method for detection of GPV for inspection and quarantine departments and health care units in China, and it is expected to become a simple and routine detection method, especially suitable for goose farms.

Introduction

Gosling plague is caused by goose parvovirus (GPV), a highly infectious septic disease with high mortality.[1] At present the waterfowl of some areas has been invaded by GPV and new goose parvovirus (N-GPV), such as Europe, Asia and America which have caused considerable economic loss and with a severe hidden danger.[2] After the infection of goslings less than 10 days of age, the morbidity and mortality are above 90.00% and even up to 100%.[3]

The GPV is a DNA virus that includes a coding region and a noncoding region where the noncoding region is located on both sides of the coding region and forms a hairpin structure.[4,5] The coding region contains two open reading frames: the left open reading frame and the right open reading frame.[6] The left open reading frame encodes the nonstructural proteins NS1 and NS2 which play major roles in the replication of the virus genome and in transcriptional regulation.[7] The right open reading frame encodes the structural proteins VP1, VP2, and VP3 which are important components of the viral capsid proteins of GPV.[8]

The detection of virus plays an important role in GPV research and disease diagnosis.[9] The disease could be diagnosed only by epidemiological characteristics and clinical symptoms.[10] At present, many scholars at home and abroad have developed tests based on latex agglutination, colloidal gold immunochromatography, immunofluorescence technology, indirect enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and other methods.[11-13] However, the widespread use of these methods was limited because it was necessary that precision instruments and a more elaborate method were detected the amplification products, although the target nucleic acids could be amplified within one hour.[14,15]

The loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification technique established by the Japanese scholar Notomi *et al*.[16] This new method has the advantages of simple operation, rapid response, low cost and visualization.[17] It has been widely used in the detection of pathogenic bacteria, fungi, viruses and parasites.[18] In this study, the virus strain of GPV was isolated and it was applied to the LAMP assay to analyse the sensitivity and specificity of the LAMP assay GPV. The primer working concentrations, reaction times, temperatures and other conditions contributing to an optimal LAMP reaction environment were determined. To optimize the assay, five sets of primers that targeted the VP3 gene were designed.

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Materials and Methods

Sampling. In May 2020, the samples were collected from Lishu districts of Jilin Province in China. The GPV-CH, *Escherichia coli* and *Salmonella* were stored by the College of Life Sciences, Jilin Agricultural University, Changchun, China. The GPV-CH was used as the positive control in the virus identification experiments. *E. coli* and *Salmonella* were used as specificity tests for the LAMP assay.

Virus isolation. Under aseptic conditions, part of the liver and small intestine of a gosling suspected to be infected with GPV was cut into pieces. Four times the volume of Phosphate Buffer Saline (PBS) containing penicillin and streptomycin (Shanghai Sangon Co., Ltd, Shanghai, China) was added to make a suspension. To ensure that the virus in the cells was released extracellularly, the suspension was repeatedly frozen and thawed. The cells were centrifuged at 10,000 *g* for 5 min and the supernatant was used for the experiment. Thirteen-day-old unimmunized goose embryos were selected and viral supernatants were inoculated in the allantoic cavity. Normal saline was used as a negative control. We placed the embryos in an incubator at 37.00 ˚C and 60.00% humidity to hatch them and checked their development and death regularly. The dead goose embryos within 24 hr were discarded. The allantoic fluids from embryonic eggs died within 5 days were collected and continuously passed to the 5th generation. The 5th generation was used as a template to detect GPV.

Virus identification and sequence of VP3 gene determination and assay. The sequence of identification for forward primer GPV-F was 5'-CCAAGCTACAACAACC ACATCTAC-3', and that for reverse primer GPV-R was 5'-CTGCGGCAGGGCATAGACATCCGAC-3, synthesized by Shanghai Sangon Co., Ltd. The fifth-generation goose embryo allantoic fluid DNA was extracted by a genome extraction kit (Omega Bio-Tek Co., Guangzhou, China) and used as a template for PCR detection. The reaction procedure was: predenaturation at 94.00 ˚C for 5 min, denaturation at 94.00 ˚C for 30 sec, annealing at 54.00 ˚C for 30 sec, extension at 72.00 ˚C for 1 min, 30 cycles and then extension at 72.00 ˚C for 5 min. Finally, 10.00 μL of PCR product was identified by 1.00% agarose gel electrophoresis and the results were analyzed. The VP3 gene of GPV was amplified by PCR and positive results were sent to Shanghai Sangon Co., Ltd. for sequencing. Sequence analysis was performed using DNAMAN biological Software (version 6.0; Lynnon Biosoft, San Ramon, USA). Using the MEGA7.0 Software (version 7.0; Biodesign Institute, Tempe, USA), the VP3 gene sequence of the obtained GPV-CL strain was compared to 15 representative GPV VP3 gene sequences from GenBank and a phylogenetic tree comparison was performed.

Design of LAMP reaction primers. The target gene was identified by bioinformatics analysis of the gene sequence of goose parvovirus (GenBank No. U25749). The target sequence was searched against the US NCBI database (http://blast.ncbi.nlm.nih.gov). The LAMP primer design was performed using the online LAMP primer design Software (http://primerexplorer.jp/e/). The DNA to be amplified was divided into six separate regions and the primers required for the LAMP reaction (forward inner primer and backward inner primer, external primers F3 and B3, loop primers LB, LF) were designed according to the six regions. The LAMP primers were then used to amplify the target genes. Primer synthesis was completed by Shanghai Sangon Co., Ltd. A total number of five LAMP primers were designed to amplify target genes. Information on primer sequences can be found in Table 1.

LAMP assay. The reagents used were provided by Shanghai Sangon Co., Ltd, (Shanghai, China). In a 25.00 μL reaction system, the concentration of each component of the mixture was as follows: 20.00 mM Tris-HCl (pH 8.80), 10.00 mM KCl, 10.00 mM $(NH_4)_2SO_4$, 0.10% Triton X-100, 0.80 M betaine, 8.00 mM $MgSO_4$, 1.40 mM dNTPs, 8.00 U of Bst DNA polymerase, 40.00 pM of FIP and BIP, 5.00 pM of GP-1F3 and GP-1B3, and 20 pM of GP-6LF and GP-6LB. The mixture was placed at a constant temperature of 60.00 - 65.00 ˚C for 60 min. The ddH_2O was used as a negative control. The process was monitored using a LAMP real-time turbidimeter. Turbidity readings at an optical density of 650 nm were obtained every 6 sec and the reaction was considered positive when the turbidity values were > 0.10. For staining, 1.00 μL of fluorescent calcein was added to the mixture and a color change from orange to green observed by the naked eye indicated a positive reaction.

PCR sensitivity test. A 10-fold dilution of GPV genome was used for PCR. The template concentrations were 1.30 × 10^5 pg μL^{-1}, 1.30 × 10^4 pg μL^{-1}, 1.30 × 10^3 pg μL^{-1}, 1.30 × 10^2 pg μL^{-1}, 1.30 × 10^1 pg μL^{-1}, 1.30 pg μL^{-1}, 1.30 × 10^{-1} pg μL^{-1}, 1.30 × 10^{-2} pg μL^{-1}, 1.30 × 10^{-3} pg μL^{-1}, and the negative control. The primers were GV-1F3 and GV-1B3 and the total composition of the 25.00 μL PCR volume was as previously described. The amplification cycle conditions were as follow: predenaturation at 94.00 ˚C for 5 min followed by denaturation at 94.00 ˚C for 30 sec, annealing at 55.00 ˚C for 30 sec, extension at 72.00 ˚C for 30 sec, a total of 35 cycles and a final extension of 7 min. Five μL of the PCR product was taken and electrophoresed at 120 V for 35 min on a 1.00% agarose gel-containing electrophoresis gel. The results were analyzed by photographing under a gel imaging system.

Results

Virus isolation. The strain of GPV was isolated from the 5th generation of allantoic fluids and named GPV-CL. The virus was isolated. Some goose embryos from the experimental group died within 5 days. The allantoic fluid from the dead embryos was passed to the 5th generation.

Table 1. The information of primer sequences.

Primer name		Sequence (5' to 3')
GV-0	F3	GGTTTGGCAGAACAGGGATA
	B3	GCCCGTAGAGTACTGGGTTA
	FIP	GGCCAAATCCTCCGAGATTCGGTTTCAGGGACCTATTGGGGCA
	BIP	CAATCCACCACCGCAGGTGTTTTCCACTTCTGGTGCACGTATT
	LF	TGGAATTTACCATCAGTCTTCGGTA
	LB	ATCAAGAATACACCAGTGCCTGCA
GV-1	F3	TGATGGCAGAGGGAGGAG
	B3	CCCAGGGGGTACTGTATCC
	FIP	TCGCAATGCCAATTTCCCGAGGTTTGGAGCTATGGGCGACTCT
	BIP	GACCACCAGAACCTGGGTCCTTTGCATCTTGAGAGGTTCCGC
	LF	TACCCACTCCATCGGCACCC
	LB	TGCCAAGCTACAACAACCAC
GV-4	F3	GGTTTGGCAGAACAGGGATA
	B3	GCCCGTAGAGTACTGGGTTA
	FIP	GGCCAAATCCTCCGAGATTCGGTTTCAGGGACCTATTGGGGCA
	BIP	CAATCCACCACCGCAGGTGTTTCCACTTCTGGTGCACGTATT
	LF	GGAATTTACCATCAGTCTTCGGTAT
	LB	TCATCAAGAATACACCAGTGCCTGC
GV-5	F3	GGTTTGGCAGAACAGGGATA
	B3	GCCCGTAGAGTACTGGGTTA
	FIP	GGCCAAATCCTCCGAGATTCGGTTTTGGGGCAAAAATACCGAAGA
	BIP	CAATCCACCACCGCAGGTGTTTTCCACTTCTGGTGCACGTATT
	LF	TACCCACTCCATCGGCACCC
	LB	TCATCAAGAATACACCAGTGCCTGC
GV-6	F3	TGGGGCAAAAATACCGAAGA
	B3	TCCCACACCATCTCTACTGT
	FIP	CTTGATGAACACCTGCGGTGGTTTCCATCCTTCTCCGAATCTCG
	BIP	AATACACCAGTGCCTGCAGACCTTTGCCCGTAGAGTACTGGGTTA
	LF	TTGTGCAGGCCAAATCCTC
	LB	CTCCAGTAGAATACGTGCACCAG

Virus identification and phylogenetic analysis of the VP3 gene sequences of the GPV-CL. The allantoic fluid from five generations of dead embryos was used to detect GPV by PCR. The negative control was ddH_2O and the positive control was GPV-CH. The results are shown in Figure 1A. GPV-CL had a specific band at 375 bp which was the same as the positive control. The sequencing results of Shanghai Sangon Co. showed that the isolated strains GPV-CL and GPV had 99.70% nucleotide sequences and were further proven to belong to GPV. The sequencing results showed that the VP3 gene was 1605 bp and encoded 534 amino acids. The nucleotide sequence of the GPV-CL gene was compared to the reference strain GPV-SP and the results showed that six nucleotides were mutated (Fig. 1B). This led to the amino acid at position 31 of the VP3 protein changing from His to Gln, the amino acid at position 32 changing from Arg to Trp and the amino acid at position 145 changing from Ala to Pro.

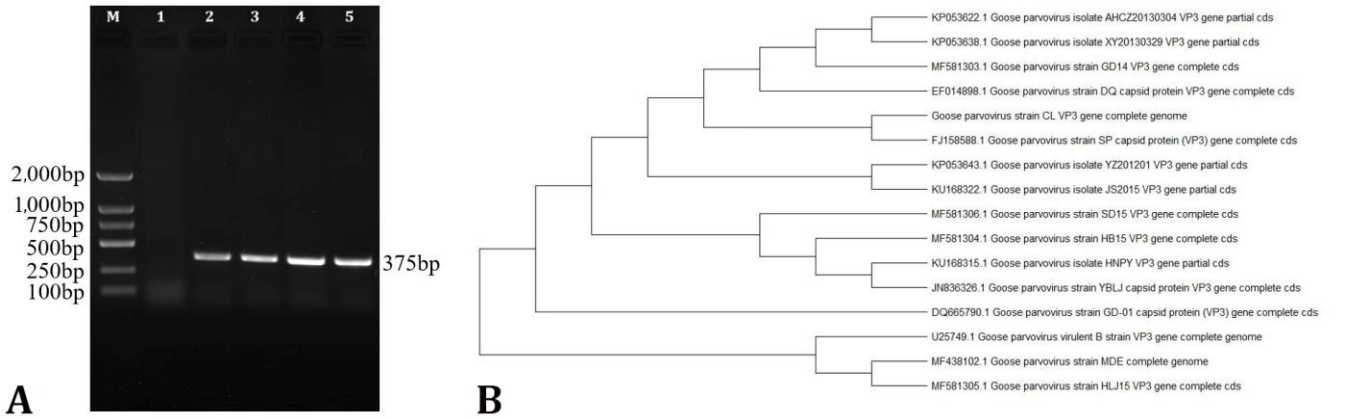

Fig. 1. Virus identification and phylogenetic analysis of the VP3 sequences of the GPV-CL. **A)** PCR results of GPV-CL strains; M: DL100 – 2,000 bp DNA Marker, 1: Negative control, 2: Positive control, 3 - 5: Products of 375 bp of GPV- CL. **B)** Nucleotide phylogenetic tree based on the VP3 sequences of 15 GPV strains constructed in MEGA 7.0 program using the neighbor-joining method.

There were no base insertions or deletions. The nucleotide and amino acid homology analysis of the Jilin GPV-CL VP3 gene and the reference strain VP3 gene domestically and internationally found that the nucleotide homology between the GPV-CL VP3 gene and the reference strain VP3 gene was between 99.30% and 99.90%.

Optimizing the LAMP assay. Five sets of primers designed in this study were used for LAMP amplification of goose parvovirus. As shown in Figure 2A, the GV-1 primer set was selected as the best primer combination because it was the first to cause the LAMP reaction. The optimal reaction temperature for the best primer combination GV-1 was then screened. The temperature range was 59.00 - 66.00 ˚C and the gradient was 1.00 ˚C. Figure 2B shows that the LAMP reaction occurred first at 65.00 ˚C followed by 66.00 ˚C, therefore, the optimal reaction temperature was set at 65.00 ˚C.

Specificity test for LAMP assay and sensitivity test for LAMP assay. The specificity of the LAMP assay using the VP3 primer sets was evaluated using turbidity monitoring and staining. The negative control used sterile deionized water as a template, while the positive control used genomes of the positive strains GPV-CL, GPV-ZL, GPV-LS and GPV-CH as templates, and goose allantoic fluid, *E. coli*, and *Salmonella* as controls to detect specificity. Figure 3A shows that only the curve of the positive control was increased and the other curves showed no change indicating that the designed primer had good specificity. As shown in Figure 3B, only the positive control is shown in green, while the negative results are shown in orange indicating that the designed primers had good specificity. The results of simultaneous staining were consistent with the turbidimetry results.

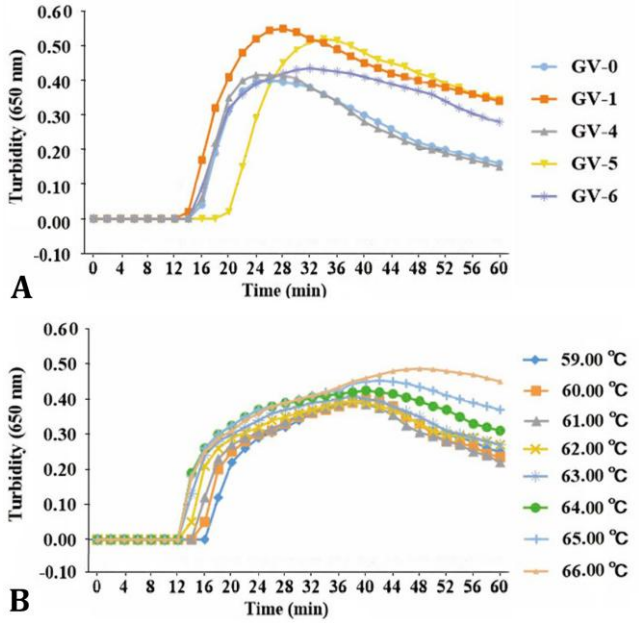

Fig. 2. Optimization of LAMP assay. **A)** The results of optimal primer screening. **B)** The results of optimal temperature screening.

The sensitivity of the LAMP assay using the VP3 primer sets was evaluated using turbidity monitoring and staining. Using the turbidity monitoring method, a 10-fold dilution series of GPV-C was tested and the concentrations were as follow: 1.30×10^5 pg μL^{-1}, 1.30×10^4 pg μL^{-1}, 1.30×10^3 pg μL^{-1}, 1.30×10^2 pg μL^{-1}, 1.30×10^1 pg μL^{-1}, 1.30 pg μL^{-1}, 1.30×10^{-1} pg μL^{-1}, 1.30×10^{-2} pg μL^{-1}, 1.30×10^{-3} pg μL^{-1}. The diluted GPV-C was used as the template for the LAMP reaction. The test results are shown in Figure 4A and the minimum detection concentration of LAMP was 1.30 pg μL^{-1}. Similarly, for the staining method, a 10-fold dilution series of GPV-C was used. The color was green when the concentration was 1.30 pg μL^{-1} and the color was orange at concentrations below 1.30 pg μL^{-1}. Therefore, the minimum detection concentration was 1.30 pg μL^{-1} and the turbidimetric method and the staining method showed consistent experimental results (Fig. 4B). A 10-fold dilution series of the GPV-C genome was also used for PCR. The primers were GV-1F3 and GV-1B3, the target fragment was 209 bp and the DNA marker was DL2000. The test results are shown in Figure 4C and the minimum PCR concentration was 1.30×10^2 pg μL^{-1}.

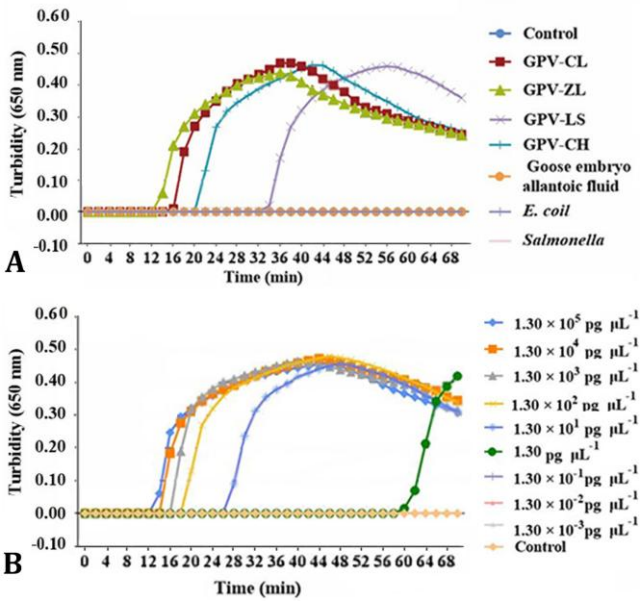

Fig. 3. Specificity test for LAMP assay and sensitivity test for LAMP assay. **A)** The results of primer-specificity testing. **B)** The results of sensitivity testing.

Discussion

Polymerase chain reaction was used to amplify the VP3 gene of GPV-CL. The sequencing analysis showed that the homology of GPV-CL with domestic epidemic strains was higher than that with foreign epidemic strains and the homology with domestic strains was the highest. The GPV mainly infects goslings within 30 days of age. The goslings had neurological symptoms after infection with GPV.

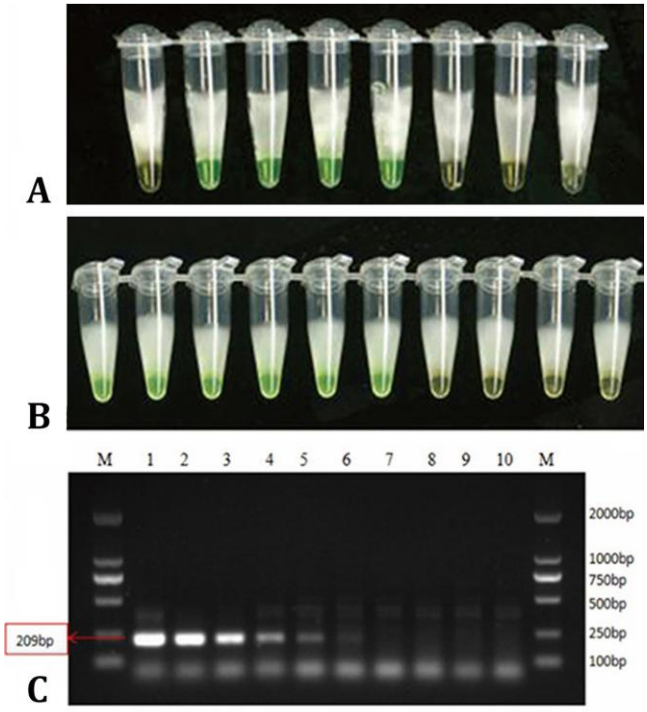

Fig. 3. A) Specificity test for LAMP assay. Staining results. From left to right: Negative control, positive strains GPV-CL, GPV-ZL, GPV-LS, GPV-CH, goose embryo allantoic fluid, *E. coli*, *Salmonella*. **B)** Sensitivity test for LAMP assay. Staining results. From left to right, the concentrations were as follow: 1.30×10^5 pg μL^{-1}, 1.30×10^4 pg μL^{-1}, 1.30×10^3 pg μL^{-1}, 1.30×10^2 pg μL^{-1}, 1.30×10^1 pg μL^{-1}, 1.30 pg μL^{-1}, 1.30×10^{-1} pg μL^{-1}, 1.30×10^{-2} pg μL^{-1}, 1.30×10^{-3} pg μL^{-1} and negative control, **C)** PCR sensitivity results. M: DL100-2,000 bp DNA Marker; 1-10: Different concentrations of GPV: 1.30×10^5 pg μL^{-1}, 1.30×10^4 pg μL^{-1}, 1.30×10^3 pg μL^{-1}, 1.30×10^2 pg μL^{-1}, 1.30×10^1 pg μL^{-1}, 1.30 pg μL^{-1}, 1.30×10^{-1} pg μL^{-1}, 1.30×10^{-2} pg μL^{-1}, 1.30×10^{-3} pg μL^{-1} and negative control.

Anatomical pathology showed swelling of the small intestine and full contents.[19] The small intestine forms an embolus and the intestinal lumen is blocked. In recent years, despite the rapid development of the goose industry, research and development of gosling plague vaccines have been in a relatively mature stage. However, gosling plague still occurs and it has seriously affected the economic development of the waterfowl breeding industry. Reasons why gosling plague still occurs include the mutation of the virus, breeders' lack of biosafety awareness and difficulties with the large-scale detection of GPV. At present, common methods for detecting GPV include traditional reverse transcription polymerase chain reaction (RT-PCR) and nested RT-PCR, however, both require sophisticated and expensive instruments and are not suitable for large-scale testing.

The LAMP technology is a simple and reliable virus detection method that has been developed in recent years. In 2014, a LAMP method for duck circovirus (DuCV) is highly specific and sensitive and can be used as a rapid and direct diagnostic assay for testing clinical samples.[20] In 2017, a quantitative loop-mediated isothermal amplification (qLAMP) assay was developed for the rapid diagnosis of N-GPV.[21] In 2020, a rapid and easy reverse transcription loop-mediated isothermal amplification (RT-LAMP) method was established for the detection of goose astroviruses (GAstVs) in clinical samples.[22] On the basis of these studies, a rapid LAMP assay method was developed for detection of GPV in this study. The best primer for goose parvovirus-specific genes was selected by the LAMP detection method and used to sensitively and quickly detect goose parvovirus. Sensitivity and specificity experiments were performed. The minimum detection concentration of the LAMP method was 1.30 pg μL^{-1}. The minimum detection concentration of PCR was 130 pg μL^{-1}. Therefore, compared to PCR, the sensitivity of LAMP was higher. In addition, the LAMP method could complete detection within 60 min which was faster than the PCR assay.

Presently, most researches in China choose to perform electrophoresis on the LAMP reaction results or add SYBR Green I fluorescent dye after the reaction are completed.[23] However, both methods can easily result in contaminated samples and false-positives and the LAMP reaction detected by electrophoresis does not reflect the LAMP reaction process in real time. In this study, a real-time turbidimeter and fluorescent calcein were used to detect the reaction. The lid was not opened after the reaction was completed which effectively prevented contamination and false-positives.

Although the amplification principle is complicated, the LAMP method is easy to operate, requires less time and shows high sensitivity and specificity. The new method can detect the virus in a single step at a constant temperature of 65.00 ˚C and does not require skilled personnel or specialized instruments. Positive results can be judged by the naked eye: A colour change from orange to green indicates a positive result while negative results remain orange. The LAMP method is suitable for the detection of goose parvovirus by inspection and quarantine departments and health care units in China. It is expected to become a simple, and routine detection method, especially suitable for goose farms, which does not require precision equipment and can detect a large number of samples.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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