A simple and rapid diagnostic method to detect new goose astrovirus using reverse-transcription loop-mediated isothermal amplification

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Abstract

In this study, we develop a reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assay for rapid and easy detection of goose astroviruses (GAstVs) in clinical samples. The specific LAMP primer sets were designed targeting the *ORF2* gene of GAstV. The conditions of LAMP amplification were optimized in terms of reaction time and temperature. The optimal conditions are 60 min in a 60 °C water bath. No cross-reactivity was noted with fowl adenovirus serotype 4 (FAdV-4), duck tembusu virus (DTMUV), goose parvovirus (GPV), avian infectious bronchitis virus (IBV), or chicken anemia virus (CAV). The proposed RT-LAMP method was compared with conventional RT polymerase chain reaction (RT-PCR) and with nested RT-PCR. The results showed that the sensitivity of the proposed method was comparable to that of nested RT-PCR and tenfold higher than that of the conventional RT-PCR. Clinical samples ($N=129$) of the liver and kidney from sick geese collected from six commercial goose farms were tested. The positive rate was 39.5% (51/129), 38.8% (50/129), and 34.9% (45/129) using RT-LAMP, nested RT-PCR and conventional RT-PCR, respectively. The developed RT-LAMP diagnostic method is not only simple, rapid, and highly specific, but also portable for use on the field. It may be used in epidemiological investigation to detect GAstVs.

Keywords GAstVs · Reverse-transcription loop-mediated isothermal amplification (RT-LAMP) · Rapid diagnosis

Introduction

Astroviruses (AstVs) are non-enveloped, single-strand, positive-sense RNA viruses that belong to the Astrovirus family and have a genome length of approximately 6.2–7.7 Kb (Cortez et al. 2017; De Benedictis et al. 2011). According to the hosts of infection, the Astrovirus family is classified into two genera—Mamastrovirus, which includes human, pig, cattle, cat, dog, rodent, rabbit, mouse, and bat AstVs, and Avastrovirus, which includes guineafowl, chicken, duck, pigeon, and turkey AstVs (Donato and Vijaykrishna 2017;

Krishnan 2014). Their viral genomes consist of a short 5'-untranslated region (UTR), three open reading frames (ORFs 1a, 1b, and 2), and a 3'-UTR (Zhang et al. 2017b). It is noteworthy that ORF2 encodes capsid proteins, which are required for virion formation and contain a highly conserved N-terminus and highly mutated C-terminus (Zhang et al. 2017b).

In 2015, an outbreak of gout was reported in 1-week-old goslings on a goose farm in Anhui Province, China. By 2017, the disease spread to Jiangsu, Shandong, Guangdong, and Fujian Provinces (Zhang et al. 2018b), causing significant economic loss within the goose industry. The main clinical symptoms of the disease are visceral urate deposition and swelling on the surfaces of the heart, liver, and kidneys (Zhang et al. 2018a). Several researchers have used high-throughput sequencing technology and the rapid amplification of cDNA ends to prove that the disease is caused by a new virus called the “goose AstV” (GAstV) (Niu et al. 2018; Yang et al. 2018; Zhang et al. 2018b).

The ability to quickly and accurately detect GAstV is essential for controlling the disease. Isolation of the virus from cell cultures (LMH or GEK cell), reverse

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transcription-quantitative polymerase chain reaction (RT-qPCR), and next-generation sequencing have been used to identify GAsVs (Niu et al. 2018; Yuan et al. 2018; Zhang et al. 2018b); however, these experimental methods are time-consuming and require high-quality, expensive equipment. Moreover, they are not suitable for field situations or primitive clinical laboratories.

Loop-mediated isothermal amplification (LAMP) is a diagnostic method that depends on Bst DNA polymerase and requires four to six primers targeting four to six specific sequences. LAMP has been used to detect and identify pathogens (Guo et al. 2018; Mansour et al. 2015) and does not need expensive equipment or lengthy experimental steps. A constant-temperature water bath or heating block for a duration of 60 min is the only equipment needed for the samples to be assayed using the LAMP method. In addition, the experimental results can be observed with the naked eye on adding fluorescent dyes. This method has good practicability, rapidity, and sensitivity (Venkatesan et al. 2012). In this study, a RT-LAMP method targeting the *ORF2* of GAsV is successfully established, compared with conventional RT-PCR and nested RT-PCR, and its detection specificity is evaluated using clinical specimens.

Materials and methods

Virus strains and clinical samples

A new goose AstV strain, AH/2018 (accession number: MN099162), was isolated from a sick goose in Anhui Province, China. FAdV-4, DTMOV, GPV, IBV and CAV were

stored at -80°C and preserved in our laboratory. One hundred twenty-nine tissue samples (livers and kidneys) were collected from sick geese from different locations in Anhui Province of China between 2018 and 2019.

Design of primers for RT-PCR, nested RT-PCR, and RT-LAMP

ORF2 genome sequences of several strains of GAsV were obtained from GenBank (accession numbers: MH052598.1; MH807626.1; MH410610.1; MF772821.1; MG934571.1). The sequences were analyzed using MegAlign (DNASTAR, Inc., Madison, WI, USA). Primers for RT-LAMP were designed with online software, Primer Explorer ver. 4 (<https://primerexplorer.jp/e/>). The primer targeted for the highly conserved *ORF2* gene sequences of the goose Astrovirus strain (accession number: MH052598.1). The primer design of RT-PCR and nested RT-PCR using software Primer Premier version 5.0 (PREMIER Biosoft International, Palo Alto, California, USA) were custom synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) with polyacrylamide gel purification. Detailed information on the primers is provided in Table 1.

Nucleic acid extraction

All tissue samples were homogenized in 1 mL of phosphate-buffered saline and centrifuged for 5 min at 12,000 rpm after being frozen and thawed three times. Viral DNA was extracted using the TIANamp Virus DNA/RNA Kit (TIAN-GEN, Beijing, China) according to the manufacturer's instructions. Viral RNA was extracted from the supernatants

Table 1 Primers and reaction conditions used in this study

Test	Primer name	Type	Length (bp)	Sequence	Optimal reaction conditions
RT-LAMP	GAstV-F3	Forward outer	20	GCTCTCACTCATGACCAGTG	60 °C for 60 min, 80 °C for 10 min to terminate amplification
	GAstV-B3	Reverse outer	20	CCACTTCTGTGGAACAGCC	
	GAstV-FIP	Forward inner	42	TCTGCAATGCGCCACTAACTGT-TTCAGCATCAGGGAAAACGG	
	GAstV-BIP	Reverse inner	40	GAGAAGGAGCAACACAGGCGC-AAAACACCTCACCGACACC	
	GAstV-LF	Forward loop	23	ATCCTGGGTATTTACAAGGGTTG	
RT-PCR	GAstV-LB	Reverse loop	20	GTCTCAAATGCGCAGACCTC	95 °C 5 min; 94 °C 30 s, 56 °C 30 s, 72 °C 35 s, 30 cycles; 72 °C 10 min
	GAstV-NF	Forward	16	AGGAAAATGGGCAAAC	
	GAstV-NR	Reverse	16	AGGAAAATGGGCAAAC	
Nested RT-PCR	GAstV-OF	Forward outer	20	ACAAAATGACTACCACGATA	95 °C 5 min; 94 °C 30 s, 54 °C 30 s, 72 °C 30 s, 30 cycles; 72 °C 10 min
	GAstV-OR	Reverse outer	17	AACACCTCACCGACACC	
	GAstV-IF	Forward inner	16	AGGAAAATGGGCAAAC	
	GAstV-IR	Reverse inner	15	AAGGGTTGCCGTTT	
Standard control	GAstV-ORF2-F	Forward	18	ATGGCAGACAGGGCGGTG	95 °C 5 min; 94 °C 30 s, 56 °C 30 s, 72 °C 40 s, 30 cycles; 72 °C 10 min
	GAstV-ORF2-R	Reverse	19	ATGGCAGACAGGGCGGTG	

using the TIANamp Virus DNA/RNA Kit (TIANGEN) according to the manufacturer's instructions. Reverse transcription of 100 ng viral RNA sample was conducted using the FastQuant cDNA Kit (TIANGEN) according to the manufacturer's instructions. The extracted cDNA was stored in -20°C .

Construction of a standard plasmid containing ORF2

The RNA genome of GAsV was reverse transcribed into cDNA, and ORF2 was amplified using PCR with GAsV-ORF2-F and GAsV-ORF2-R (Table 1). The PCR products were purified using a DNA purification kit (TIANGEN), cloned into the pMD19-T vector (TaKaRa, Dalian, China), and transfected into *Escherichia coli* DH5 α cells for amplification. The recombinant plasmid was extracted using the TIANprep Mini Plasmid Kit (TIANGEN) and sent to Sangon Biotech Shanghai Co, Ltd. for sequencing. The concentration of plasmids was quantified using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The copy number of the recombinant plasmid was calculated using the following formula: amount (copies/ μL) = $(6.02 \times 10^{23}) \times (\text{ng}/\mu\text{L} \times 10^{-9}) / (\text{DNA length} \times 660)$.

Reaction and optimizations of RT-LAMP

Referring to the basic reaction system of LAMP, the concentrations of following ingredients were optimized in a total reaction volume of 25 μL : primers, deoxynucleoside triphosphate (dNTP), and MgSO_4 . After determining the optimal reaction system, the temperature and time were optimized in the experiment. First, the RT-LAMP reactions were observed for 60 min using optimal reaction system at 58, 60, 62, 64, and 66 $^{\circ}\text{C}$, each. After selecting the optimum temperature, the reaction time was optimized. At the optimum temperature, the reaction times were 50, 55, 60, 65, and 70 min. Finally, the reaction was terminated in a water bath at 80 $^{\circ}\text{C}$ for 10 min. The reaction products were analyzed using 1.5% agarose gel electrophoresis. Finally, 1 μL 10,000 $\times$ SYBR Green I dye (Solarbio, Beijing, China) was added to the reaction product, and the result was observed with the naked eye. The optimal reaction conditions and times for the optimization of RT-PCR and nested RT-PCR are provided in Table 1.

Conventional RT-PCR and nested RT-PCR

The total volume of the conventional RT-PCR is 25 μL , and its composition was as follows: 1 $\times$ Premix Taq $^{\text{®}}$ Version 2.0 (TaKaRa), 1 μL of template, 0.4 μM GAsV-NF and GAsV-NR primers. The composition of the nested RT-PCR system in a total of 25 μL was as follows: 1 $\times$ Premix Taq $^{\text{®}}$ Version 2.0 (TaKaRa), 1 μL of template, 0.4 μM

GAsV-OF and GAsV-OR primers. One microliter of initial PCR product was used as a template in PCR reaction with GAsV-IF and GAsV-IR primers. The primers and reaction conditions are shown in Table 1.

Sensitivity and specificity test of the RT-LAMP assay

The recombinant plasmid was serially diluted ten times (1×10^9 to 1×10^1 copies) according to the known concentration and used as a template for RT-LAMP, nested RT-PCR, and conventional RT-PCR. Before the specificity evaluation, the concentration of each virus DNA or cDNA was adjusted and unified at 100 ng/ μL . Genomic RNAs/DNAs of avian pathogenic viruses FAdV-4, DTMUV, GPV, IBV, and CAV were used as templates to verify the specificity of the RT-LAMP assay. GAsV was used as a positive control and the blank template was used as a negative control. All reactions were conducted in triplicate. The amplified products were electrophoresed on 1.5% agarose gel with GoldView II (Solarbio) and visually inspected using SYBR Green I dye under daylight conditions (Fig. 1).

Evaluation of the RT-LAMP assay in clinical samples

The 129 tissue samples (liver and kidney) were tested using RT-LAMP, nested RT-PCR, and conventional RT-PCR assays, and the positive detection rates were simultaneously recorded.

Fig. 1 Analysis of LAMP products. LAMP amplification products were analyzed using agarose gel electrophoresis (a) M, DL2000 DNA marker; lane 1, positive control; lane 2, negative control; and visually inspected with SYBR Green I dye under daylight conditions (b) tube 1, positive control and tube 2, negative control

Fig. 2 Comparative sensitivity of RT-LAMP, nested RT-PCR and conventional RT-PCR for GAsVs. **a** Electrophoretic analysis of LAMP amplified products. **b** Visually inspected with SYBR Green I dye under daylight conditions visual observation of LAMP amplified products. **c** Electrophoretic analysis of RT-PCR amplified products. **d** Electrophoretic analysis of nested RT-PCR amplified products. M, DL2000 DNA marker; lane 1, 1×10^9 copies/tube; lane 2, 1×10^8 copies/tube; lane 3, 1×10^7 copies/tube; lane 4, 1×10^6 copies/tube; lane 5, 1×10^5 copies/tube; lane 6, 1×10^4 copies/tube; lane 7, 1×10^3 copies/tube; lane 8, 1×10^2 copies/tube; lane 9, 1×10^1 copies/tube; lane 10, negative control

Results

Reaction mixtures and optimal conditions

After optimization of various parameters, the final optimized in a total reaction volume of 25 μ L contained 1.6 μ M (each) of forward and backward inner primers, 0.2 μ M (each) of outer primers F3 and B3, 6 mM MgSO_4 , 1.4 mM dNTP mix

(TaKaRa), 1 $\times$ Thermo Pol buffer (20 mM Tris-HCl, 50 mM KCl, 10 mM $(\text{NH}_4)_2 \text{SO}_4$, 2 mM MgSO_4 , 0.1% Tween-20), 8 U Bst DNA polymerase (New England Biolabs, Ipswich, MA, USA), and 1 μ L template. The products of RT-LAMP showed multiple characteristic ladder bands at different temperatures. At 60 $^\circ\text{C}$, the bands of light were strongest. The optimal reaction time was 60 min.

Fig. 3 Analysis of RT-LAMP specificity for GAsV. M, DL2000 DNA marker; specificity of the RT-LAMP assay determined by electrophoresis analysis (a) and SYBR Green I staining (b); lane 1, GAsV; lane 2, FAdV-4; lane 3, DTMUV; lane 4, GPV; lane 5, IBV; lane 6, CAV; lane 7, negative control

Sensitivity and specificity

Using 1×10^9 to 1×10^1 copies of the GAsV-ORF2 recombinant plasmid as templates, RT-LAMP, nested RT-PCR and conventional RT-PCR assays were conducted to determine their detection limits. The results showed that RT-LAMP can detect a minimum of 1×10^2 plasmid copies. Moreover, the nested RT-PCR and conventional RT-PCR have detection limits of 1×10^2 and 1×10^3 copies, respectively (Fig. 2). These results suggested that RT-LAMP was ten times more sensitive than conventional RT-PCR and that its sensitivity was similar to that of nested RT-PCR.

The results of specificity determination showed that only GAsV and positive recombinant plasmid templates exhibited a color reaction. Not surprisingly, the other viruses used in the experiment showed no color reaction, which was consistent with the negative control (Fig. 3). This demonstrated the high specificity of RT-LAMP used in our study.

Evaluation of the RT-LAMP assay

The practicability of the established RT-LAMP method was evaluated using the 129 collected samples. Nested RT-PCR and conventional RT-PCR were also used in the detection

processes. As shown in Table 2, the positive detection rates of RT-LAMP, nested RT-PCR and conventional RT-PCR were 39.5% (51/129), 38.8% (50/129), and 34.9% (45/129), respectively. The comparison of the positive detection rates showed that the RT-LAMP method had a slight advantage over the other two methods and was portable enough to be useful for disease detection in the field.

Discussion

Since it was detected in China in 2017, the disease associated with goose gout has increased gosling mortality and caused heavy losses in the poultry industry (Zhang et al. 2018b). This disease was later confirmed to be caused by GAsV. To effectively monitor and prevent it, a portable detection method was vital. A variety of methods are used to detect viral infections, such as conventional RT-PCR and nested RT-PCR (Yuan et al. 2018); however, these methods require highly accurate and expensive equipment, which make them much less suitable for clinical surveillance of animal epidemics in developing countries, such as China. RT-LAMP was recently developed to detect viruses and has proved to be a simple and reliable method. It has the advantages such as being faster and simpler when compared with conventional RT-PCR and nested RT-PCR (Zhao et al. 2014; Zhang et al. 2017a). Conducting an assay under this method requires only a water bath under isothermal conditions for the samples. In our study, three pairs of primers were designed for the ORF2 of GAsV to establish a simple, rapid, and highly specific RT-LAMP assay. We also concurrently evaluated RT-LAMP, nested RT-PCR, and conventional RT-PCR assay. The results of sensitivity showed that the detection limit of RT-LAMP at 1×10^2 copies was better than that of conventional RT-PCR and comparable to that of nested RT-PCR. According to previous studies, a real-time fluorescence qPCR method based on TaqMan can detect a minimum of 52.5 copies (Yuan et al. 2018). Similar results were observed with RT-LAMP, which is a simpler, faster (i.e., only 60 min), and less expensive method. The test environment was strictly partitioned to avoid false-positive contamination. To evaluate the feasibility of RT-LAMP in clinical applications, 129 clinical samples were tested. The results showed that GAsV was widely distributed in the Anhui Province of China, consistent with the results of previous studies (Zhang et al. 2018b).

In summary, the established RT-LAMP could detect GAsV, and it exhibits simplicity, rapidity, and high specificity. This assay could be used in epidemiological investigation to detect GAsV.

Table 2 Performance of conventional RT-PCR, nested RT-PCR and RT-LAMP assays for detection of GAstVs in clinical samples

Samples	Total	Conventional RT-PCR		Nested RT-PCR		RT-LAMP	
		Positive	Negative	Positive	Negative	Positive	Negative
Liver	72	26	46	29	43	29	43
Kidney	57	19	38	21	36	22	35
Total	129	45	84	50	79	51	78

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Compliance with ethical standards

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

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