Rapid and visual detection of novel astroviruses causing fatal gout in goslings using one-step reverse transcription loop-mediated isothermal amplification

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ABSTRACT To visually and rapidly detect a novel goose astrovirus (**N-GoAstV**) causing fatal gout in goslings, an isothermal detection method based on one-step reverse transcription loop-mediated isothermal amplification (one-step **RT-LAMP**) was established. The one-step RT-LAMP assay for N-GoAstV detection, using *Bst 3.0* DNA polymerase with strong reverse transcription activity and primer sets targeting the opening reading frame 1b (*ORF1b*) of N-GoAstV, could be completed in 30 min using a water bath at 61°C; the detection results could be visually observed by adding a pH-sensitive dye containing phenol red and cresol red. The detection limit of the one-step RT-LAMP assay was 57.8 copies, which was similar to that of reverse transcription–quantitative polymerase chain reaction. The assay specifically detected N-GoAstV without any cross-reaction with other reference viruses, and this was further confirmed using enzyme digestion. These results indicated that the newly established RT-LAMP assay could accomplish reverse transcription, amplification, and visual result determination in one step, and the results obtained via this rapid and cost-effective method could be used to support disease control on farms in terms of N-GoAstV infection.

Key words: N-GoAstV, rapidity, visible detection, one-step RT-LAMP

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INTRODUCTION

In February 2016, some farms raising gooses in the Shandong Province of China experienced an outbreak of disease in goslings aged 1 to 3 wk, particularly those aged 9 to 12 D (Yang et al., 2018; Zhang et al., 2018a,b). This was followed by similar reports in other provinces, which caused substantial economic losses to the poultry industry (Zhang et al., 2017a,b; Jin et al., 2018). This fatal disease is characterized by hyperuricemia, with urate deposition on the visceral surfaces, joint capsule, cartilago articularis, kidney tubules, ureter, and other interstitial tissues. It was confirmed that this gout outbreak in the goslings was caused by a novel goose astrovirus (**N-GoAstV**) (Liu et al., 2018; Chen et al., 2020). However, gout may have other endogenous and exogenous causes, such as long-term use of fodder containing high protein; shortage of V_A or V_D; or the misuse, overdose, and toxicity associated with medicines. Therefore, it is difficult to confirm the actual etiology of gout by autopsy. Moreover, the morbidity and mortality owing to gout caused by N-GoAstV were high and the disease showed rapid progression. Therefore, there was an immediate need for an accurate detection method. The disadvantages of using an immunological method as a diagnostic tool for novel pathogens included a need to prepare and screen for highly specific antibodies in the serum. The primary methods currently used for diagnosing N-GoAstV in clinical samples are conventional reverse transcription–polymerase chain reaction (**RT-PCR**) (Yang et al., 2018; Chen et al., 2020) and reverse transcription–quantitative PCR (**RT-qPCR**) (Yuan et al., 2018; Yin et al., 2020). However, these diagnostic methods cannot be widely applied because they require

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sophisticated procedures and expensive instrumentation (Oh et al., 2016). To avoid the issues accompanying such a detection assay based on PCR technology, Notomi et al. (2000) developed a new nucleic acid amplification method called loop-mediated isothermal amplification (**LAMP**) that can amplify nucleic acids within a short duration (typically ≤1 h) under isothermal conditions (60°C–65°C). Moreover, a positive LAMP result can be easily detected via visual observation of change in turbidity or color (Mori et al., 2001), thereby further simplifying the detection. The LAMP assay and reverse transcription LAMP (**RT-LAMP**) (for pathogens with RNA genomes) have recently been widely used for the identification of bacteria, viruses, and parasites as well as for molecular pathology analysis (Lee et al., 2016; Seok et al., 2017; Zhang et al., 2017a,b). Therefore, it was practical and essential to develop a one-step RT-LAMP method for detecting N-GoAstV.

MATERIALS AND METHODS

Viruses and Clinical Samples

Spleen, liver, and kidney tissues collected from goslings that died from gout on the goose farms were immersed in phosphate-buffered saline, washed several times to eliminate residual blood, and stored in centrifuge tubes at −80°C until use.

Strains of N-GoAstV, goose parvovirus (GPV), goose reovirus (GREOV), goose hemorrhagic polyomavirus (GHPV), and Tembusu virus (TMUV) and clinical samples suspected of N-GoAstV previously described and included in this study were stored in the Henan Provincial Engineering and Technology Center of Health Products for Livestock and Poultry, Nanyang Normal University.

Total DNA and RNA Extraction

Total DNA and RNA of the clinical samples were extracted using a commercial kit (EasyPure Viral DNA/RNA Kit; TransGen Biotechnology, Inc., Beijing, China), according to the manufacturer's instructions. The purity and concentration of the DNA and RNA samples were determined using biological spectrophotometry, and the samples were stored at −80°C until use.

Primer Design for LAMP

Primers used for the LAMP assay were designed using the online primer software Explorer V5 (http://primerexplorer.jp/e/v5_manual/index.html) and were selected based on the conserved regions of *ORF1b* in the N-GoAstV genome (Chen et al., 2020) that was available in GenBank. Six primers were designed, including an external primer pair (F3 and B3), an internal primer pair (FIP and BIP), and a loop primer pair (LP and BP); these primers recognized 8 different regions of the gene target. The sequences of the oligonucleotide primer sets are shown in Table 1.

Optimum Temperature and Time for the One-Step RT-LAMP Reaction

For the one-step RT-LAMP reaction, a reaction mixture containing primer sets (0.5 μmol/L of each outer primer, 2 μmol/L of each inner primer, and 0.5 μmol/L of each loop primer), 2 mmol/L dNTPs, 8 U *Bst* 3.0 DNA polymerase (New England Biolabs, Hitchin, UK), 10 mmol/L $(NH_4)_2SO_4$, 50 mmol/L KCl, 6 mmol/L $MgSO_4$, 0.1% v/v Tween-20, and 1 μL of dye (comprising 0.025 mmol/L phenol red and 0.08 mmol/L cresol red), with nuclease-free water added to achieve a final reaction volume of 25 μL, was used. After the addition of 1 μL total RNA template, the mixture was covered with mineral oil to prevent aerosol cross-contamination. The *Bst* 3.0 DNA polymerase provides improved isothermal amplification performance and strong reverse transcription; therefore, the addition of exogenous reverse transcriptase for reverse transcription was not required. The one-step RT-LAMP was conducted at various temperatures (60°C, 61°C, 62°C, 63°C, 64°C, and 65°C) to determine an optimum reaction temperature. The optimum time for the one-step RT-LAMP was evaluated by conducting the assay for 10, 20, 30, 40, 50, and 60 min at the optimum temperature. The one-step RT-LAMP products were electrophoresed on 2% agarose gel in 1× TAE (Tris-acetate-EDTA) buffer or were directly assessed based on the color change observed (yellow indicated a positive result and purple-red indicated a negative result).

TaqMan Quantitative RT-PCR for Comparative Analysis

TaqMan RT-qPCR was performed for N-GoAstV detection using the CFX96 Touch Real-Time PCR Detection System (BIO-RAD, Hercules, CA) with a 20-μL qPCR mixture containing Premix Ex Taq (Probe qPCR, TaKaRa, Dalian, China), primer sets, and the

Table 1. LAMP primers used to specifically target *ORF1b* genes of N-GoAstV.

Primer name	Primer sequences (5′–3′)
F3	CGACGCTCARTTACT *Y*[1] AGG
B3	ACCATCACTCCTTTTAA *Y*CAA
FIP	TGACGACATTCG *Y*GTCRTAATTA ATGATAATGTCAC-CATGATTTGCT
BIP	TGTGGGTTAAACCAGAAAATGTCA**G AATTC**[2]–CGTAAGACCACAGAAAGTCAT
LF	CCTTCCTTATTGACACAAGCCTAT
LB	AGGTCTCTGATGATATTGAGGGT

Abbreviations: LAMP, loop-mediated isothermal amplification; *ORF1b*, opening reading frame 1b gene.

[1]Italic text indicates degenerate oligonucleotides, Y = C + T.

[2]Bold text represents the *Eco*R I restriction site added between B1c and B2.

template (standard plasmid or cDNAs); the reaction procedure was performed as previously described (Wan et al., 2019).

Sensitivity and Specificity of the One-Step RT-LAMP

The analytical sensitivity of the one-step RT-LAMP was determined by the standard plasmid (pT-ORF1b, strain AstV/HN01/Goose/0103/18), which was preserved in our laboratory. Serial dilutions (10-fold) of pT-ORF1b N-GoAstV in nuclease-free water (diluted from 5.78×10^0–10^6) were used to evaluate the sensitivity of the newly developed one-step RT-LAMP and RT-qPCR.

The one-step RT-LAMP primers were examined for specificity using N-GoAstV, GPV, GREOV, GHPV, and TMUV, which are commonly found in goose, and nuclease-free water was used as a negative control. In addition, *ORF1b* of other goose-derived astroviruses (FLX strain, accession number KY271027; AHDY strain, accession number MH410610) (Zhang et al., 2017a,b) that were synthesized at Hongxun Biotechnologies Ltd. (Suzhou, China) were used for the specificity testing. The specificity of the one-step RT-LAMP assay for N-GoAstV detection was further evaluated using enzyme digestion of amplified products. The primers were designed to contain the *Eco*R I restriction sites sequence, and digestion of the amplified products would yield fragments of the expected sizes.

Clinical Sample Testing

In total, 217 clinical samples of goose with gout from goose farms were collected to further evaluate the one-step RT-LAMP assay. Both the one-step RT-LAMP and RT-qPCR assays were performed to determine the positive rate compared with virus isolation, as previously described (Wang et al., 2019). All products of the one-step RT-LAMP assay were observed by staining with phenol red and cresol red.

RESULTS

Validation of Primers

As shown in Figure 1, the change of color and the result of electrophoresis showed that the primers worked well, and they could be used for follow-up testing.

Optimum Reaction Temperature and Time for the Detection of N-GoAstV by the One-Step RT-LAMP

As shown in Figure 2, the temperature range from 60°C to 65°C showed no evident difference in the gradient bands produced, with an optimum temperature of approximately 61°C. With an increase in amplification time, the bands became brighter, reaching a peak at 30 min. Therefore, the one-step RT-LAMP assay for N-GoAstV detection was optimized for reverse transcription and amplification at 61°C for 30 min.

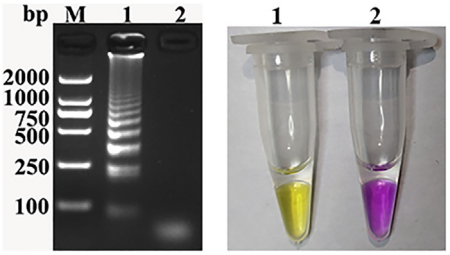

Figure 1. (A) One-step RT-LAMP amplification in the conserved region of *ORF1b* in N-GoAstV genome. (B) Color change of LAMP-amplified products. M, 2000 DNA ladder marker; 1, LAMP-amplified products; 2, negative control.

Specificity of One-Step RT-LAMP

As shown in Figure 3 from the gel electrophoresis results and visualization using a pH-sensitive dye, only N-GoAstV as a template was amplified, and no one-step RT-LAMP amplified products were observed for the other reference goose viral pathogens and nuclease-free water. Furthermore, the one-step RT-LAMP products were confirmed by restriction enzyme *Eco*R I that produced 3 main bands of 234 bp, 293 bp, and 352 bp, which were in accordance with our theoretical calculations.

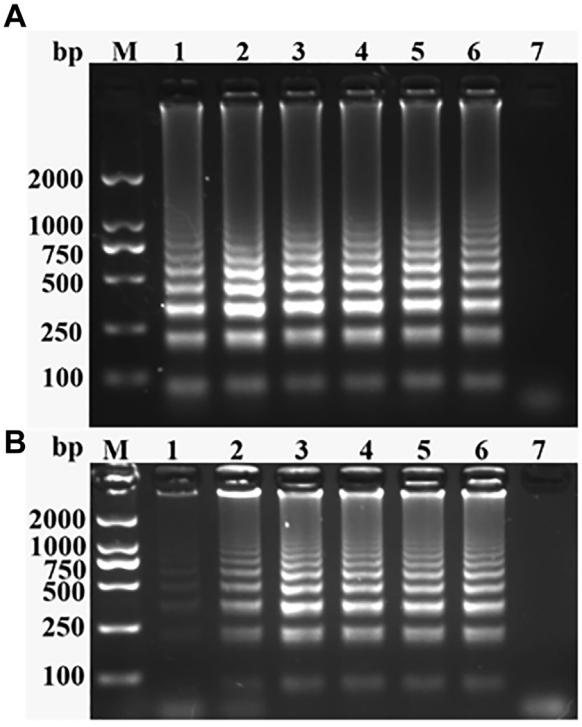

Figure 2. (A) One-step RT-LAMP amplification results at different temperatures. M, 2000 DNA ladder marker; 1: 60°C; 2: 61°C; 3: 62°C; 4: 63°C; 5: 64°C; 6: 65°C; 7: negative control. (B) One-step RT-LAMP amplification results at different reaction times. M, 2000 DNA ladder marker; 1: 10 min; 2: 20 min; 3: 30 min; 4: 40 min; 5: 50 min; 6: 60 min; 7: negative control.

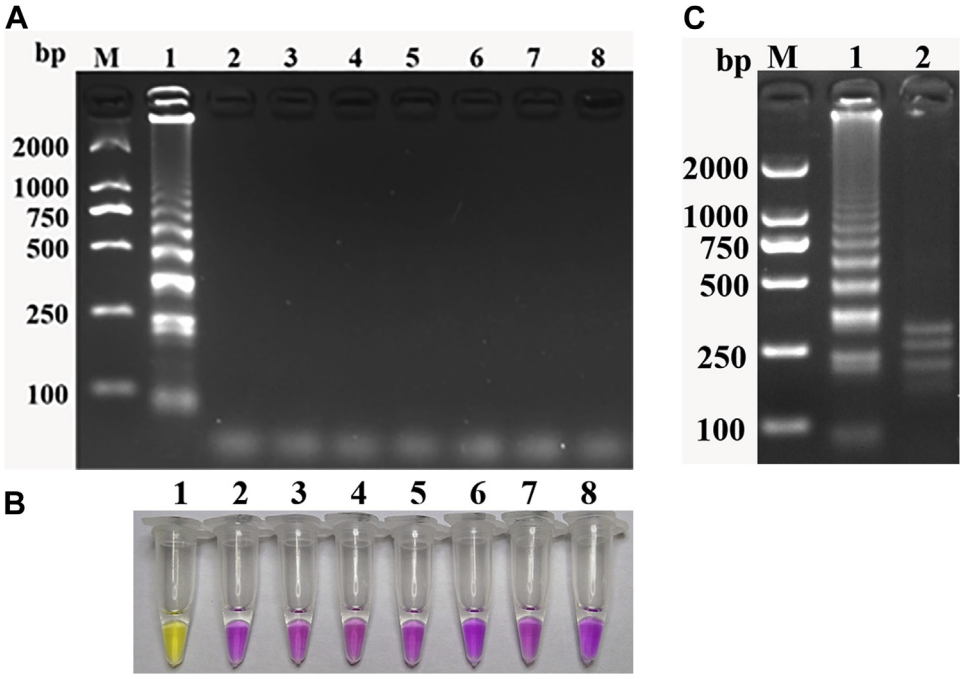

Figure 3. Specificity of the N-GoAstV one-step RT-LAMP amplification: (A) Agarose gel electrophoresis of LAMP amplification products. (B) Visual detection of negative and positive LAMP amplification products. M, 2000 DNA ladder marker; 1, N-GoAstV; 2, GPV; 3, GHPV; 4, GREOV; 5, TMUV; 6, FLX; 7, AHDY; 8, negative control. (C) Enzyme digestion of LAMP amplification products. M, 2000 DNA ladder marker; 1, LAMP amplification products; 2, digested LAMP amplification products.

Sensitivity of the One-Step RT-LAMP

As shown in Figure 4, the color change and electrophoresis of one-step RT-LAMP products showed that the detection limit of one-step RT-LAMP was 57.8 copies, which was similar to the result obtained from RT-qPCR, based on the amplification curve.

Clinical Sample Detection

All the samples ($n = 217$) were examined for the presence of N-GoAstV using both RT-qPCR and one-step RT-LAMP. The positive rates for the conventional RT-qPCR and one-step RT-LAMP assays were 177/217 and 177/217, respectively (Table 2). These results demonstrated that the one-step RT-LAMP assay was as accurate as RT-qPCR for the detection of N-GoAstV in field samples.

DISCUSSION

The outbreak of N-GoAstV has continued since Yang (Yang et al., 2018) reported it in 2018, and this has led to substantial economic losses (Zhang et al., 2017a,b, 2018a,b). Owing to rapid dissemination and clinical complexity associated with this infection, the development of a rapid, highly sensitive, and specific diagnostic method for the detection of N-GoAstV was essential. RT-qPCR methods for the detection of N-GoAstV are sensitive and accurate (Yuan et al., 2018; Wan et al., 2019; Yin et al., 2020). However, these methods are inappropriate for detecting N-GoAstV in field settings and on resource-poor farms, which lack the sophisticated technology and expensive instrumentation required for PCR testing.

In the present study, the one-step RT-LAMP assay was developed and evaluated for N-GoAstV detection. The one-step RT-LAMP assay was able to detect N-GoAstV at 61°C and within 30 min using *Bst 3.0* DNA polymerase for achieving complete reverse transcription, amplification, and detection in a water bath, with a detection limit of 57.8 copies; the results obtained were similar to those obtained using RT-qPCR. The one-step RT-LAMP possessed 6 primer targets for the conserved regions of *ORF1b* of N-GoAstV, which evidently enhanced its specificity. The primer of N-GoAstV for one-step RT-LAMP did not detect the other reference goose viruses (GPV, REOV, GHPV, TMUV, N-GoAstV AHDY, and FLX strains) used, demonstrating that this method was highly specific. The one-step RT-LAMP saved procedural steps and enzyme use, was simple and user-friendly, and only required a water bath with a constant temperature feature. To evaluate the practicability of the one-step RT-LAMP in the field, 217 samples from goslings with gout were examined. The results from the clinical samples demonstrated that the one-step RT-LAMP showed the same positive detection rates as RT-qPCR for N-GoAstV. In addition, rapid detection, which saved further time, was achieved by adding a color indicator containing phenol red and cresol red, which was chosen for product analysis based on a previous report (Tanner et al., 2018). A positive result was determined by a color change caused by a pH change, which in

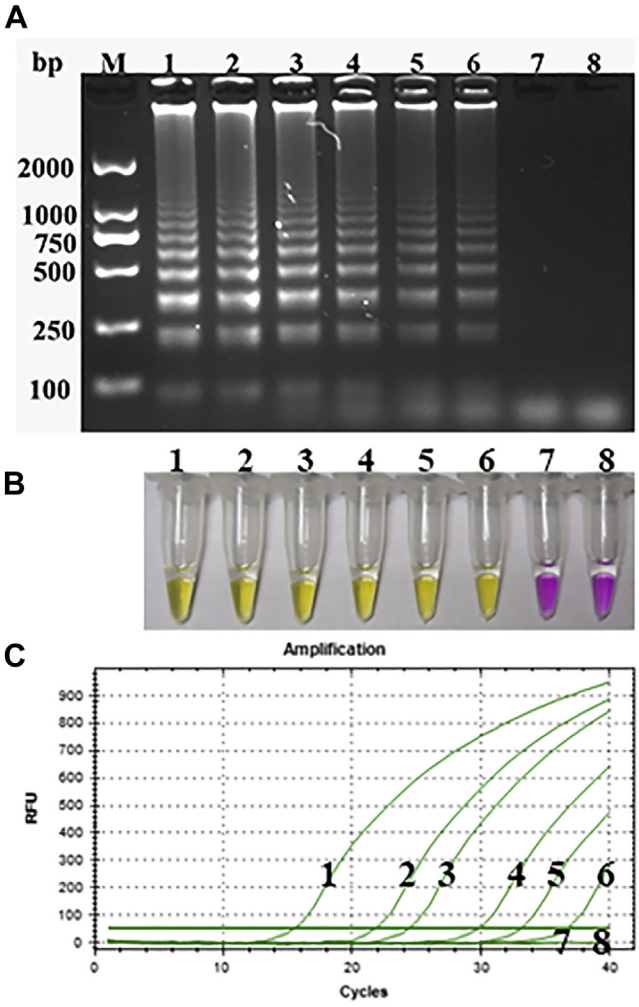

Figure 4. Sensitivity of the N-GoAstV one-step RT-LAMP amplification: (A) Agarose gel electrophoresis of LAMP amplification products. (B) Visual detection of negative and positive LAMP amplification products. (C) RT-qPCR amplification. M, 2000 DNA ladder marker; 1–7, DNA template with 5.78×10^6 to 10^1 copies; 8, negative control.

turn is caused by the formation of byproducts that include a pyrophosphate moiety and hydrogen ions. Using this indicator, for a positive reaction, the color changes from purple-red to yellow, whereas for a negative reaction, the color remains the original purple-red color. Although a visible result can be observed by turbidity or adding SYBR Green I (Badolo et al., 2012; Wang et al., 2013), using the naked eye to assess turbidity was less accurate; therefore, a turbidimeter would be required, increasing both the cost and the operational steps. Moreover, the one-step RT-LAMP assay has excellent amplification efficiency. This aspect is important considering that in a test proposed earlier, the reaction product formed an aerosol that contaminated the surroundings upon opening the tube to add SYBR Green I; in that case, to avoid disturbing the reaction progress, it was essential that SYBR Green I was added after the reaction was completed (Tanner et al., 2018). To avoid such contamination, in the present study, the pH-sensitive reagents phenol red and cresol red were added to the one-step RT-LAMP reaction mixture before amplification, thereby facilitating the direct visualization of the result of the one-step RT-LAMP assay with the naked eye.

These data further suggested that the one-step RT-LAMP is a feasible, cost-effective, naked-eye visible, and rapid method that holds great promise for local on-site detection for N-GoAstV.

CONCLUSIONS

The one-step RT-LAMP method established in the present study provides an effective technique for the rapid detection of the presence of N-GoAstV; this rapid detection will be useful in the control and investigation of the fatal disease that results from this astrovirus.

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Table 2. Comparison of RT-qPCR and one-step RT-LAMP for the detection of N-GoAstV in clinical samples.

Province	Number of samples	Date	Number of positive samples		
			Virus isolation	One-step RT-LAMP	RT-qPCR
Henan	21	2018.01	17	17	17
Anhui	25	2018.05	19	19	19
Anhui	20	2018.07	15	15	15
Henan	24	2018.11	17	17	17
Hubei	20	2019.01	19	19	19
Hubei	25	2019.03	21	21	21
Henan	20	2019.04	16	16	16
Anhui	23	2019.06	18	18	18
Henan	21	2019.08	17	17	17
Hubei	18	2019.09	18	18	18
Total	217		177	177	177

Abbreviations: RT-LAMP, reverse transcription loop-mediated isothermal amplification; RT-qPCR, reverse transcription–quantitative polymerase chain reaction.

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Data Availability Statement: All data generated or analyzed during this study are included in this article. The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this study involved sample collection from goslings.

Conflict of Interest Statement: The authors have no competing interests to declare.

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