A quantitative loop-mediated isothermal amplification assay for detecting a novel goose astrovirus

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ABSTRACT In November 2017, a severe infectious disease that devastated the major goose-producing regions in China was found to be caused by a novel goose astrovirus (**N-AstV**). The objective of this study was to develop a quantitative loop-mediated isothermal amplification (**qLAMP**) assay for the rapid diagnosis of N-AstV characterized with gout, hemorrhage, and swellings of the kidneys. A set of 4 specific primers, 2 inner and 2 outer primers, targeting the *ORF1a* gene of N-AstV were designed for the assay which could be completed within 60 min at 65°C in a water bath or on a real-time PCR instrument for quantitative analysis. The qLAMP assay showed a high sensitivity with a detection limit of 1×10^1 copies of the target DNA/μL. There were no cross-reactions with other viruses, and the reproducibility of the assay was confirmed in intrasensitivity and intersensitivity assay tests with variability ranging from 0.61 to 2.21%. The results indicated that the qLAMP assay for N-AstV was a simple, accurate, rapid, sensitive, and specific, especially useful for field detection.

Key words: Novel goose astrovirus, EvaGreen dye, qLAMP, PCR, ORF1a

2020 Poultry Science 99:6586–6592
https://doi.org/10.1016/j.psj.2020.09.077

INTRODUCTION

Astrovirus (**AstV**) infections in poultry are typically characterized by high morbidity and mortality, poor feed conversion, and growth retardation (Day and Zsak, 2010; Pantin-Jackwood et al., 2011). Most avian species, including chickens, ducklings, goslings, and Turkey poults, are susceptible to AstV, and infections could lead to severe economic losses (Baxendale and Mebatsion, 2004; Chen et al., 2012; Zhang et al., 2017). In 2017, a novel disease of gooselings characterized by severe articular gout, hemorrhage, and swelling of the kidneys was first reported in several provinces in China. The morbidity in gooselings due to the disease was up to 80%, and the mortality was up to 50%, resulting in serious economic losses to the Chinese goose industry (Niu et al., 2018). After isolation, propagation, biological characterization, and genomic analysis, the causative agent was identified as a novel goose astrovirus (designated as **N-AstV**) (Yang et al., 2018).

Members of the family *Astroviridae* are classified into 2 genera, *Mamastrovirus*, whose members infect mammals (mammalian astroviruses) and *Avastrovirus*, whose members infect birds (avian astroviruses). The International Committee on Taxonomy of Viruses recognizes 3 virus species within the *Avastrovirus* genus: *Avastrovirus 1*, including turkey astrovirus 1 and 2; *Avastrovirus 2*, including avian nephritis virus and chicken astrovirus; and *Avastrovirus 3*, including the duck astrovirus (**DAstV**) types 1 and 2 (Smyth, 2017). Based on the amino acid (**aa**) sequences of the ORF2-encoded capsid protein, the N-AstV was found to belong to *Avastrovirus 3* (Zhang et al., 2018). Like other avian astroviruses, N-AstV has a positive-sensed single-stranded RNA genome and a nonenveloped virion particle of approximately 27- to 30-nm diameter (Biđin et al., 2012). The homology of nucleotide (**nt**) and aa sequences homology between the genome of N-AstV and those of other avian astroviruses is low (nt: 44.2–67.7%, aa: 28–72.5%). Although isolated from goose, the nt and aa homology of N-AstV

Received July 21, 2020.
Accepted September 23, 2020.

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compared with those of other goose astroviruses was also quite low (nt: 49–61.1%, aa: 39.1–63.4%) (Yang et al., 2018).

Current assay methods used for detecting avian astroviruses are mainly based on the PCR (Koci et al., 2000; Tang et al., 2005; Day et al., 2007; Zhao et al., 2011). The ELISA is also used but mainly for detecting antibodies against avian astroviruses (Tang et al., 2005; Hewson et al., 2010; Meliopoulos et al., 2014). Among the more recent advances in virological diagnostic techniques, the loop-mediated isothermal amplification (**LAMP**) technique (Notomi et al., 2000) has been applied successfully in various forms for the rapid detection of various families of viruses, including avian astroviruses (Imai et al., 2006; Curtis et al., 2008; Mekata et al., 2009; Zhao et al., 2012; Yu et al., 2020). Unlike PCR, which requires one pair of primers, LAMP assay uses a set of 4 specific primers that recognize 6 distinct sequences in the target DNA to conduct an autocycling strand displacement DNA synthesis reaction by the Bst DNA polymerase large fragment. It is considered to be a low-cost, time-saving, highly specific, and sensitive method (Chotiwan et al., 2017) and can be completed within 1 h at a temperature range of 60°C–65°C without the use of a thermocycler. The amplified DNA products can either be detected as a ladder-like pattern using agarose gel electrophoresis or be visualized under UV light after adding a fluorescent dye (Yang et al., 2017). Alternatively, the reaction can be assessed by following a by-product of the reaction, magnesium pyrophosphate, which can be visualized by the naked eye (Tomita et al., 2008; Mekata et al., 2009).

Recently, an EvaGreen-based quantitative LAMP (**qLAMP**) assay has become popular (Lee et al., 2017). Compared with the SYBR Green I–based qLAMP assay, EvaGreen has the important advantage of being less inhibitory to the PCR and is less likely to cause nonspecific amplification (Ihrig et al., 2006). Another advantage of the EvaGreen method over SYBR Green is that aerosol contamination in the latter can be avoided because the tubes are closed throughout the reaction (Yang et al., 2017). By using a portable measuring device, such as the LA-200 Loopamp Real-Time Turbidimeter (Eiken, Japan) or the Genie III fluorescence detector (OptiGene, China), the assay can be used for on-site diagnosis (Mekata et al., 2009; Kurosaki et al., 2016).

In this study, a rapid, simple, sensitive, and specific qLAMP assay for the detection of N-AstV infection and disease surveillance was developed by amplifying the *ORF1a* gene of N-AstV.

MATERIALS AND METHODS

Ethics Approval

This study was approved by the Committee on the Ethics of Animal of Shandong (permit number 20,156,681). All subjects gave informed consent for their participation in the study.

Viruses and Clinical Samples

The N-AstV isolate used in the study was originally isolated from morbid goose kidneys in Pingyuan city, Shandong province, China (Yang et al., 2018). Purified preparations of Newcastle disease virus (GD14 strain), Tembusu virus (JX14 strain), goose circovirus (Hebei14 strain), DAstV (LC strain), goose-origin H9N2-AIV (GT strain), and goose parvovirus (GD14 strain) were obtained from the Research Institute of Poultry Disease of Shandong Agricultural University. A total of 30 kidney samples from suspected cases of N-AstV–infected goslings, characterized with gout, were collected from different areas of China during disease outbreaks. The viruses and clinical samples were stored at −80°C for subsequent use.

RNA Extraction and cDNA Synthesis

RNA was extracted from the viruses and clinical samples using the TRIzol RNA Extraction Kit (Catalog No EC301-11, TransGen Biotech, Beijing, China) according to the manufacturer's instructions. The first strand of cDNA was generated from the RNA using the reverse-transcriptase M-MLV (Catalog No 2641Q, Takara,

Table 1. Primers used in the study.

Assay	Primer[1]	Sequences (5′-3′)	Fragment (bp)	Annealing temperature
qLAMP	N-AstV-F3	GGTTCAGAAAGAAAACGCAG		
PCR	N-AstV-B3	GAATGGTCTCATTTTTTTGTTCA		
	N-AstV-FIP	GCGTAAGAGGTTGTGCCTTCATTA CGAGATGTTCAAATCTGCC		
	N-AstV-BIP	TCTTCGCCAATGGTGATCAGATCCA TCAACGTGGATAAGCT		
	N-AstV-F	ATTCTTGGCTCGGTTGTC	489	53°C
	N-AstV-R	CCTGTGTTGCTCCTTCTC		

Abbreviations: N-AstV, novel goose astrovirus; qLAMP, quantitative loop-mediated isothermal amplification.

[1]N-AstV-F3 = forward outer primer, N-AstV-B3 = backward outer primer, N-AstV-FIP = forward inner primer, N-AstV-BIP = backward inner primer, N-AstV-F, forward PCR primer, N-AstV-R, reverse PCR primer.

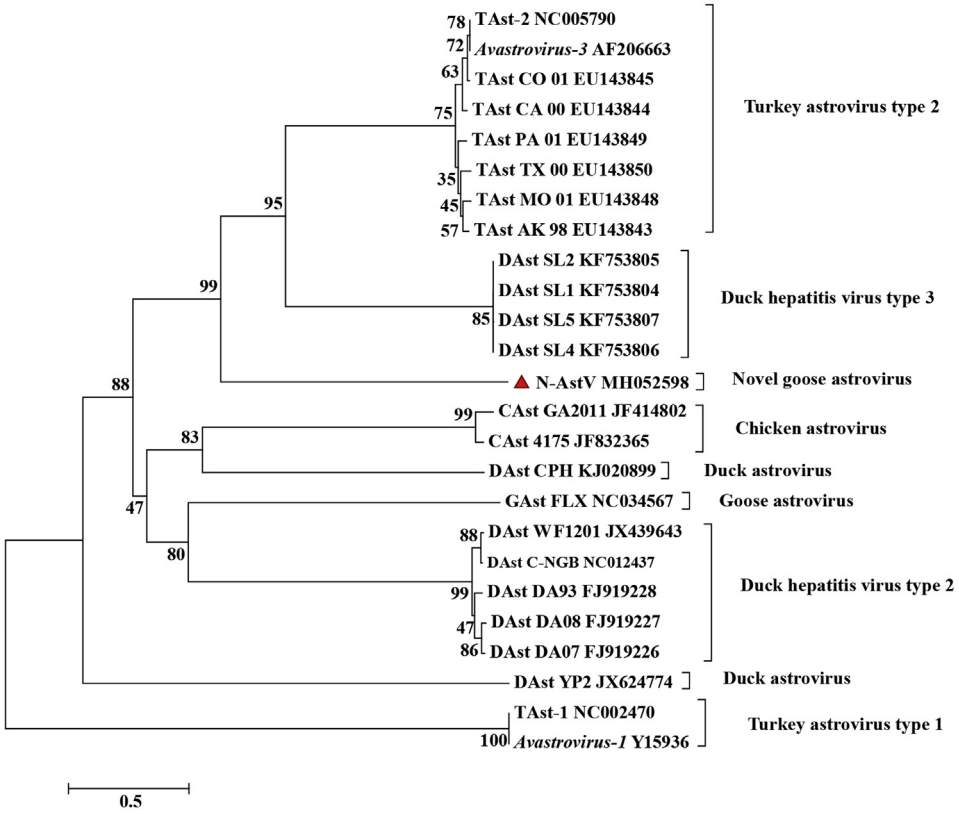

Figure 1. Phylogenetic relationship between the N-AstV and other avian astrovirus strains based on the *ORF1a* gene in the phylogenetic tree built using the neighbor-joining method. Abbreviation: N-AstV, novel goose astrovirus.

Dalian, China) using random primers (Catalog No 3801, Takara, Dalian, China) and used immediately for subsequent experiments.

Phylogenetic Analysis of the ORF1a Genes of Avastroviruses

Phylogenetic analysis of the *ORF1a* genes of the N-AstV strain and other avian astroviruses was carried out using the MEGA version 7.0 program. The neighbor-joining method and the bootstrap validation method with 1,000 replications (Niu et al., 2017) were used. The GenBank accession numbers of the avastroviruses used in the sequence comparison are listed in Supplementary information (Supplementary Table 1).

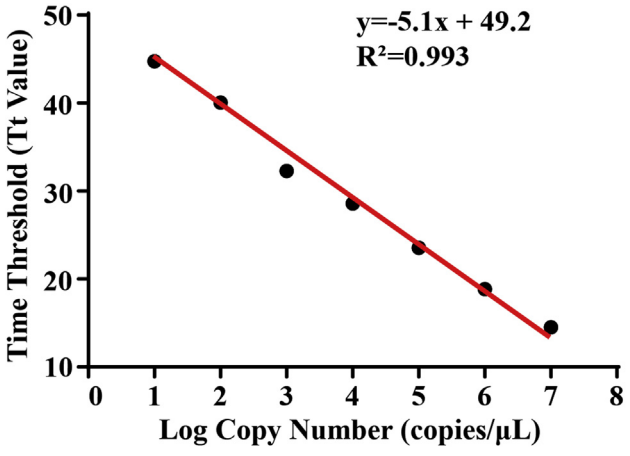

Figure 2. Standard curve for the qLAMP assay using ten-fold serial dilutions of the N-AstV *ORF1a* recombinant plasmid standards in Tris-EDTA buffer (1×10^7 copies/μL to 1×10^1 copies/μL). Abbreviations: N-AstV, novel goose astrovirus; qLAMP, quantitative loop-mediated isothermal amplification.

Primer Design

A set of 4 qLAMP primers and a pair of PCR primers (N-AstV-F and N-AstV-R) were designed based on the *ORF1a* gene of N-AstV using an online program (PrimerExplorer V5, http://primerexplorer.jp/e/) and the Primer 5.0 software, respectively. The two inner primers (N-AstV-FIP and N-AstV-BIP) and 2 outer primers (N-AstV-F3 and N-AstV-B3) for the qLAMP assay and the primers used for PCR assays are shown in Table 1.

Preparation of N-AstV ORF1a Recombinant Plasmid Standard

The outer primers F3 and B3 were used to amplify the *ORF1a* genome of N-AstV by PCR, and the amplified PCR products were purified using a gel extraction kit (Takara, Dalian, China), cloned into the pMD18-T vector (Takara, Dalian, China), and transformed into *Escherichia coli* DH5α competent cells. The cloned PCR fragment in the recombinant plasmid, pMD18-ORF1a, was sequenced by BGI Tech (BGI Tech, Shenzhen, China)

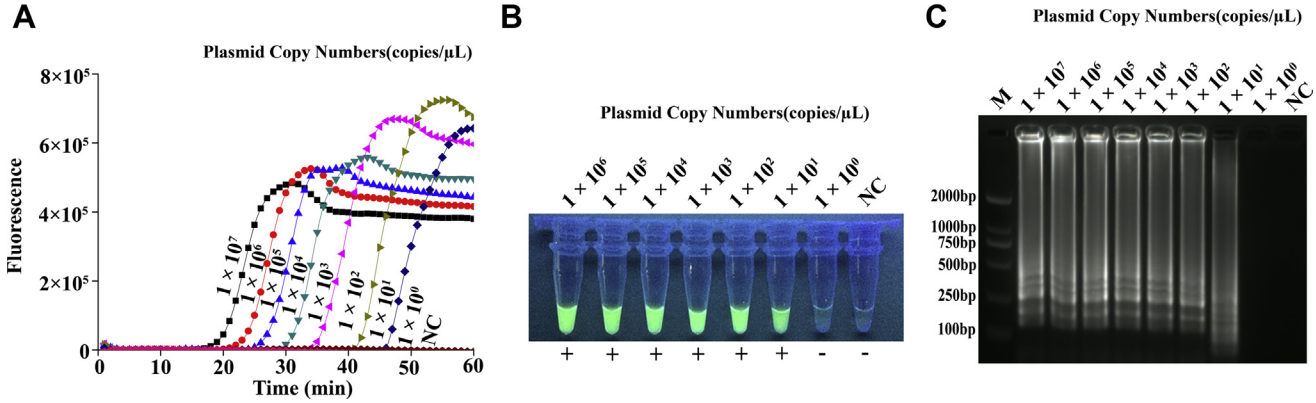

Figure 3. Sensitivity test results of the qLAMP assay using ten-fold serial dilutions of the plasmid standards in TE buffer (1×10^7 copies/μL to 1×10^0 copies/μL). (A) Real-time amplification plots of the different dilutions of the plasmid standards. (B) Fluorescence of the end products in under UV light with EvaGreen dye. (C) Results of agarose gel electrophoresis. Abbreviation: qLAMP, quantitative loop-mediated isothermal amplification.

and verified. The plasmid was extracted using the TIANprep Mini Plasmid Kit (TIANGEN biotech, Beijing, China). The concentration of the plasmid was determined by the DeNovix DS-11 Spectrophotometer (Denovix), and the copy number of the plasmid was calculated using the following formula: amount (copies/μL) = $(6.02 \times 10^{23}) \times (\text{ng}/\mu\text{L} \times 10^{-9})/(\text{DNA length} \times 660)$.

Optimization of the N-AstV qLAMP Assay

The qLAMP assay was conducted in a 25-μL reaction volume using Bst 2.0 WarmStart DNA Polymerase (New England Biolabs, Frankfurt, Germany) according to the manufacturer's instructions, which contained 1 μL of the FIP (5′-GCGTAAGAGGTTGTGCCTTCATTACGAGATGTTCAAATCTGCC-3′) and BIP (5′-TCTTCGCCAATGGTGATCAGATCCATCAACGTGGATAAGCT-3′) primers (80 μM of each stock), 1 μL of the F3 and B3 primers (10 μM of each stock), 2.5 μL of the 10 × isothermal amplification buffer, 1.5 μL of 100 mM $MgSO_4$, 3.5 μL of the 10 mM dNTP mix, 1.25 μL of the 20 × EvaGreen dye, 1 μL of Bst DNA polymerase (8,000 U/mL stock), 2 μL of the plasmid standard, and 11.25 μL of nuclease-free water. The reaction temperature (60°C, 63°C, and 65°C) and reaction time (40, 50 and 60 min) were optimized in a 7300 Real-time PCR system (Applied Biosystems, Foster City, CA). The time threshold (**Tt**) values and standard curve were analyzed using the SDS software program (version 1.4) and visualized using OriginPro 8.5 software (OriginLab, Northampton, MA). The qLAMP results were also detected by fluorescence observation under UV light and 2% agarose gel electrophoresis.

PCR Assays

The PCR assay was conducted in a 20-μL reaction volume, containing 10 μL of 2 × Ex Taq Mix, 6 μL of ddH_2O, 1 μL of the forward primer (N-AstV-F) and 1 μL of the reverse primer (N-AstV-R), and 2 μL of cDNA and performed using the PCR system (Applied Biosystems, Foster City, CA) (Yang et al., 2018). The PCR conditions were as follows: 95°C for 5 min, then 30 cycles, each consisting of denaturation (95°C for 30 s), annealing (53°C for 30 s) and extension (72°C

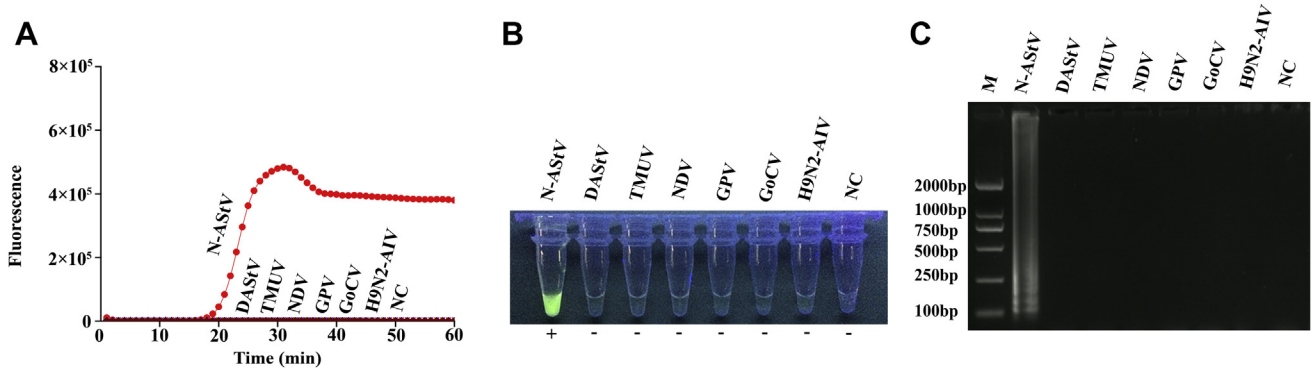

Figure 4. Specificity test results of the qLAMP assay using Newcastle disease virus (NDV), Tembusu virus (TMUV), goose circovirus (GoCV), duck astrovirus (DAStV), goose-origin H9N2-AIV (H9N2-AIV), and goose parvovirus (GPV). (A) Real-time amplification plots of the different virus strains. (B) Fluorescence of the end products under UV light with EvaGreen dye. (C) Results of agarose gel electrophoresis. Abbreviation: qLAMP, quantitative loop-mediated isothermal amplification.

Table 2. Reproducibility analysis of the N-AstV qLAMP assay.

	Replicated assay variability of Tt value			Repeated assay variability of Tt value		
Copy numbers (copies/μL)	Mean	SD	CV (%)	Mean	SD	CV (%)
1.0×10^7	14.53	0.12	0.86	14.41	0.18	1.28
1.0×10^6	18.84	0.12	0.63	18.98	0.36	1.88
1.0×10^5	23.55	0.14	0.61	23.78	0.27	1.11
1.0×10^4	28.59	0.46	1.60	28.30	0.57	2.00
1.0×10^3	32.28	0.27	0.81	32.99	0.30	0.91
1.0×10^2	40.06	0.72	1.79	39.26	0.70	1.77
1.0×10^1	44.76	0.99	2.21	44.56	0.59	1.32

Abbreviations: N-AstV, novel goose astrovirus; qLAMP, quantitative loop-mediated isothermal amplification; Tt, time threshold.

for 15 s), and followed by a final extension at 72°C for 10 min. The PCR results were analyzed by gel electrophoresis with 2% agarose gel.

Sensitivity and Specificity Assessments of the N-AstV qLAMP Assay

Sensitivity and specificity assessments of the N-AstV qLAMP assay were conducted using the 7300 Real-time PCR system. A 10-fold serial dilutions of the pMD18-ORF1a plasmid standard, ranging from 1×10^7 to 1×10^0 copies/μL in 10-μM Tris-EDTA buffer (pH 8.0), was prepared and used as templates in the qLAMP reactions to determine the detection limit of the assay.

The specificity of the qLAMP assay was tested against Newcastle disease virus, Tembusu virus, goose circovirus, DAstV, goose-origin H9N2-AIV, and goose parvovirus using 2 μL of the cDNA of each virus, and water as the negative control.

The sensitivity of the corresponding conventional PCR assay was determined using the qLAMP F3 and B3 primers and the same plasmid dilution series. The PCR were monitored using the Applied Biosystem PCR machine, and the products were electrophoresed in 2% Tris-acetate-EDTA agarose gel.

Assessing the Reproducibility of the N-AstV qLAMP Assay

The reproducibility of the qLAMP assay for N-AstV Diagnosis was tested in terms of replicated assay reactions and repeated assay experiments, using the 10-fold dilution series of plasmid standards, ranging from 1×10^7 copies/μL to 1×10^0 copies/μL. In the replicated assay test, each dilution in the dilution series was assayed triplicates in the same run. For the repeated assay test, the assay of each dilution was repeated in 3 independent runs. The CV of the Tt values obtained were calculated and used to determine the reproducibility of the assay (Yang et al., 2017).

Table 3. Results of N-AstV diagnosis of clinical samples obtained with the conventional PCR and the qLAMP assays.

	N-AstV PCR		
N-AstV qLAMP	+	−	Total
+	17	13	30
−	0	0	0
Total	17	13	30

Abbreviations: N-AstV, novel goose astrovirus; qLAMP, quantitative loop-mediated isothermal amplification.

Diagnosis of Clinical Samples

A total of 30 samples from suspected cases of N-AstV–infected gosling kidneys were used to detect the repeatability of this method and were stored at −80°C for subsequent use. cDNAs were generated from tissue homogenates, and they were used directly in parallel for both the conventional PCR and qLAMP assays.

RESULTS

Phylogenetic Analysis of the ORF1a Genes of Avastroviruses

The phylogenetic tree of the *ORF1a* genes of avastroviruses was generated (Figure 1). Overall, it showed that the N-AstV strain was localized as an independent small branch, divergent strain group, and within the large branch that contained the turkey astrovirus type 2 and duck hepatitis virus type 3 groups.

Establishment of N-AstV ORF1a Recombinant Standard Curve

The concentration of the N-AstV *ORF1a* recombinant plasmid preparation was 499 ng/μL, determined by a DS-11 Spectrophotometer (Denovix), and the copy number was 1.68×10^{11} copies/μL. The standard curve was established between the log of plasmid copy numbers (copies/μL) and the Tt values ($R^2 = 0.992$), with a regression line revealing an average intercept and slope of 49.21 and −5.1, respectively (Figure 2).

Determination of the Conditions of the qLAMP Assay

For best amplification the optimum temperature was 65°C for the activation of Bst DNA polymerase. Although we could get good amplification at every

temperature, the best parameters were found to be 60 min at 65°C. The thermal cycling profile for qLAMP proceeded as follows: 60 cycles of 64°C for 10 s and 65°C for 50 s. Fluorescence signals for each sample were collected at the end of the 65°C step, and the cutoff point for Tt value was determined as 50 as determined previously (Tang et al., 2016).

Sensitivity and Specificity of the N-AstV qLAMP Assay

Performed using 10-fold serial dilutions (1×10^7 to 1×10^0 copies/μL) of the N-AstV *ORF1a* recombinant plasmid standards, the qLAMP assay for N-AstV detection had the lowest detection limit of 1×10^1 copies, and no amplification signals were observed in the negative control (Figure 3A). The results of qLAMP assay could be observed not only by the naked eyes at the dilution end point with the EvaGreen fluorescent dye under UV light (Figure 3B) but also by agarose gel electrophoresis (Figure 3C). However, sensitivity tests for conventional PCR assay indicated that the detection limit of a conventional PCR assay was 1×10^4 copies (Supplementary Figure 1).

The specificity experiments showed that strong fluorescent signals were obtained from reactions with N-AstV, but not from the other viruses and the water control (Figure 4A). In addition, the results of the specificity test could also be confirmed by green fluorescence observed under UV light (Figure 4B), and a typical ladder pattern observed on the agarose gel electrophoresis (Figure 4C). The results indicated that the qLAMP assay had a high specificity for N-AstV detection.

Reproducibility of N-AstV qLAMP Assay

As summarized in Table 2, the CV of the Tt values varied from 0.61 to 2.21% in the replicated assay reactions and from 0.91 to 2.00% in the repeated assay experiments. These results revealed that the qLAMP method has a high reproducibility and excellent stability in detecting N-AstV.

Diagnosis of Clinical Samples

A total of 30 kidney samples were used for the comparative evaluation of the qLAMP and PCR. The results obtained by gel electrophoresis of the reaction products are summarized in Table 3. The positive frequency of N-AstV was determined to be 57% using conventional PCR and 100% using the qLAMP assay.

DISCUSSION

The sudden outbreak and rapid spread of the N-AstV, which mainly causes gosling gout, had led to enormous economic losses to the goose industry of China. The results of the phylogenetic analysis, based on the *ORF1a* gene, revealed that the N-AstV strain was quite distinct from all the other strains in the tree. Further understanding of the evolutionary relationship between the N-AstV strain and other avian astroviruses is warranted to determine whether the novel characteristic symptoms of N-AstV observed in infected goslings are genetically related.

A sensitive and accurate portable detection method is vital for the effective and efficient prevention and control of a disease. The EvaGreen qLAMP assay developed in this study was rapid, simple, sensitive, and specific and can be performed without the need for a thermal cycler. In comparison with a SYBR Green–based LAMP assay, the SYBR Green I would inhibit amplification if added before LAMP reaction (Eischeid, 2011), whereas addition of the fluorescent aye after the reaction could cause aerosol pollution, giving rise to false-positive results. Therefore, the EvaGreen-based LAMP assay has overcome these disadvantages.

To the best of our knowledge, this study was the first to report the EvaGreen-based LAMP assay for detecting N-AstV. This assay for N-AstV had a detection limit of 1×10^1 copies/μL, especially the reproducibility of the assay was confirmed in intrasensitivity and intersensitivity assay tests with variability ranging from 0.61 to 2.21%. In addition, the specificity of this method was 1,000 times higher than the traditional PCR assay.

In conclusion, the qLAMP assay developed for the detection of the N-AstV–specific nucleic acid sequence in the *ORF1a* gene was proven to be a highly sensitive, specific, and reliable diagnostic method that could detect N-AstV in a rapid, simple, and cost-efficient manner. Considering these advantages, this assay could be applied as a practical molecular diagnostic tool for detecting N-AstV infections and disease surveillance in both the laboratory and the field.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program of China (2018YFD0501506); the Higher Education Support Program of Youth Innovation and Technology of Shandong Province, China (2019KJF022); the China Agriculture Research System (CARS-42-19); and funds of the Shandong "Double Tops" Program.

DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.09.077.

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