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A reverse transcription recombinase-aided amplification assay for the rapid detection of goose astrovirus

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Abstract

China's burgeoning animal husbandry sector has witnessed a notable expansion in goose farming. Among the various health challenges, a novel goose astrovirus (GoAstV) has emerged as a significant threat to the industry, necessitating prompt detection strategies to mitigate its economic impact. This research introduces a novel detection approach using real-time fluorescence-based reverse transcription recombinase-aided amplification (RT-RAA), offering a rapid and reliable method for GoAstV identification. We meticulously designed specific primers and probes, and optimized the RT-RAA reaction conditions. The assay's specificity, sensitivity, repeatability, and clinical efficacy were rigorously assessed. Our method achieves detection within a swift 26-min window at a constant temperature of 39.00 ℃, boasting a detection threshold as low as 1.19×10^2 copies *per* μL. Notably, the assay exhibited no cross-reactivity with closely related viruses, including Newcastle disease virus, avian influenza virus H9 subtype, goose circovirus, goose parvovirus, duck Tembusu virus, and avian adenovirus type 4. Validation through testing of 40 clinical samples confirmed a 100% agreement with pre-existing data. The study's outcomes underscore the high specificity, sensitivity, and operational simplicity of the developed RT-RAA assay, positioning it as an ideal candidate for the rapid and on-site detection of GoAstV.

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Introduction

Goose astrovirus (GoAstV), a member of the Astroviridae family and Avastrovirus genus, poses a significant health challenge to the goose farming industry, particularly affecting goslings aged four to 21 days.[1,2] The virus is known to trigger gout and high mortality rates, with symptoms typically manifesting around day five and a peak in fatalities occurring between days 10 and 15.[3] By the age of 20, the mortality rate can soar to 20.00 - 30.00%, inflicting considerable economic damage on China's goose farming sector.[4] Geese are the natural hosts of GoAstV, and multiple breeds can be infected by this virus. It can also infect various chickens and ducks, posing a significant threat to the poultry farming industry. Li *et al.* have found that infection with GoAstV in chicks can lead to clinical symptoms similar to those observed in gout among goslings.[5] Also, GoAstV has caused similar gout symptoms in Henan Pekin ducks, Shandong ducks, and Shandong cherry valley ducklings, indicating its potential for cross-species transmission.[6-8] Therefore, further research should continue to focus on the pathogenicity and transmission characteristics of GoAstV to effectively assess and mitigate the threats it poses to both the poultry industry and public health. The GoAstV is characterized as a non-enveloped, positive-sense, single-stranded RNA virus with a broad host range and a genome including a 5' untranslated region, a 3' untranslated region, a polyadenylate tail, and three open reading frames (ORFs), including ORF1a, ORF1b, and ORF2.[9,10] The ORF1a and ORF1b are responsible for encoding non-structural proteins involved in replication, whereas ORF2 encodes the capsid protein essential for virus particle formation. The GoAstV is classified into two genotypes, GoAst V-1 and GoAst V-2, both contributing to gout development.[11]

Diagnostics for astroviruses encompass a range of techniques, including electron microscopy, conventional polymerase chain reaction (PCR), real-time quantitative PCR (qPCR), enzyme-linked immunosorbent assay, colloidal gold detection, metagenomic sequencing, and loop-mediated isothermal amplification.[12-18] Despite their utility, these methods often require complex procedures

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and advanced equipment, and may not be amenable to rapid on-site detection or use in resource-constrained settings. Loop-mediated isothermal amplification, while not requiring special equipment, has stringent primer design requirements and potential for non-specific reactions. Moreover, these techniques can be prohibitively time-consuming, potentially delaying critical pathogen confirmation and disease management during outbreaks.

In light of these challenges, there is a pressing demand for more efficient diagnostic technologies facilitating swift and accurate GoAstV detection. Recombinase-aided amplification (RAA) technology offers a promising alternative, akin to recombinase polymerase amplification in specificity, sensitivity, and speed, but utilizing recombinases from bacterial or fungal sources.[19] The RAA is an innovative isothermal nucleic acid amplification technique employing recombinases, single-strand binding proteins, and DNA polymerases to function at a constant temperature.[20] This method has demonstrated its advantages in speed, simplicity, and sensitivity in the rapid detection of a variety of pathogens.[21-27]

Building on the principles of RAA, this study introduces a refined real-time fluorescence-based reverse transcription RAA (RT-RAA) method. This method is designed to enhance reaction efficiency and reduce costs, offering a novel and effective technical solution for the rapid detection of GoAstV.

Materials and Methods

Materials. The GoAstV (Accession No. OP179660) was initially identified through clinical diagnostics and subsequently preserved within our laboratory for further study. Concurrently, we procured positive nucleic acid samples of several other avian pathogens, including Newcastle disease virus (NDV), avian influenza virus H9 subtype (AIV-H9), goose circovirus (GoCV), goose parvovirus (GPV), duck Tembusu virus (DTMUV), and avian adenovirus type 4 (FAdV-4), from the Sichuan Provincial Center for Animal Disease Control and Prevention, Chengdu, China. These samples served as controls and comparators in our experiments. For molecular extraction, we utilized the Viral Genomic DNA/RNA Extraction Kit procured from Tiangen Biotech Company (Beijing, China) to ensure high-fidelity isolation of nucleic acids. Additionally, the OMEGA Gel Extraction Kit (D2500), Cycle Pure Kit (D6492), and Plasmid Mini Kit I (D6943), acquired from Omega Bio-Tek Corp. (Norcross, USA), were employed for purification of nucleic acids from various sources. The RT-RAA and RAA Nucleic Acid Amplification Kits (both fluorescent) were sourced from Hangzhou ZC Bio-Sci & Tech Co. (Hangzhou, China) to facilitate the amplification of target sequences. The MEGAscript™ T7 Transcription Kit, obtained from Thermo Fisher Scientific Co. (Shanghai, China), was used for *in vitro* transcription of RNA standards. Furthermore, the pLB-T Fast Cloning Kit, generously provided by Tiangen Biotech was used for cloning purposes. Primers and probes, critical for the detection of GoAstV, were synthesized and labeled by Sangon Biotech Company (Shanghai, China), ensuring specificity and efficiency in our detection assays. The Fluorescent qPCR Detector (CFX Connect™ Optics Module) was obtained from Bio-Rad Laboratories (Shanghai, China), which was instrumental in the real-time monitoring of the amplification process.

Primer and probe design and synthesis. To identify conserved regions within the genetic makeup of GoAstV, we employed bioinformatics tools DNAStar and DNAMAN to analyze and compare the gene sequences of 62 distinct GoAstV strains available in the National Center for Biotechnology Information database. Our focus was on the *ORF1b* gene, which encodes the RNA-dependent RNA polymerase, and is known for its conserved nature among the strains. Utilizing the *ORF1b* gene sequence of GoAstV as a reference, we designed a set of primers and probes tailored for the RT-RAA detection method. The design process involved a meticulous alignment of the genetic sequences to pinpoint regions that are conserved across the strains, ensuring the specificity and effectiveness of the primers and probes in detecting GoAstV. The sequences of the designed primers and probes are presented in Table 1, along with their corresponding gene locations and fragment lengths. These oligonucleotides were synthesized by Sangon Biotech Company, ensuring high-quality standards necessary for accurate and reliable detection of GoAstV in subsequent experiments.

Establish of standard. For the establishment of a plasmid standard, we utilized the extracted GoAstV RNA as a template to amplify the *ORF1b* gene fragment. This amplification was achieved using PCR primers, including F: 5'-TATTCGTATGATCTTGTGTGCT-3' and R: 5'-TCTTTAAC ATTATTGGGTGCAT-3', being designed to flank the target sequence. The PCR conditions were optimized as follows: An initial pre-denaturation step at 95.00 ˚C for 5 min,

Table 1. Sequences of primers and probe for real-time reverse transcription recombinase-aided amplification assays for goose astrovirus.

Primer and probe names	Sequence (5'-3')	Gene location	Fragment size (bp)
RAA-F1	TTATATTATTACCAACTGGGGAGGTTTGTA	938 - 967	30
RAA-F2	TTGTACAGTTAAGAAGGGAAATCCAAGTGG	963 - 992	30
RAA-R1	CGGCAATGACCCATGCTGTTTCCAATAAAG	1,060 - 1,089	30
RAA-R2	ACATTATCCCTGAGTAATCTGAGCGTCGGC	1,086 - 1,115	30
RAA-probe	TTCAACAACAGTGGACAACAATATGTGTAA[6FAM-dT] G [dSpacer] C [BHQ1-dT] GGCTCACC	999 - 1,048	50

followed by 35 cycles of denaturation at 94.00 ˚C for 30 sec, annealing at 59.00 ˚C for 30 sec, and extension at 72.00 ˚C for 60 sec, concluding with a final extension at 72.00 ˚C for 8 min and storage at 4.00 ˚C. The amplified *ORF1b* gene segment was subsequently cloned into the pLB-T vector, and the resulting positive plasmid was selected for further processing. The plasmid underwent single-enzyme digestion with the restriction enzyme XbaI, and the resulting DNA fragments were purified. This purified product served as a template for *in vitro* transcription using the MEGAscript™ T7 Transcription Kit, facilitating the synthesis of cRNA standards. The purity and concentration of the cRNA standards were assessed using a NanoDrop OneC Micro-volume Spectrophotometer (Thermo Fisher Scientific). The copy number of the cRNA was calculated using the following formula:

$$RNA\ concentration = \frac{(RNA\ concentration\ [ng\ L^{-1}] \times 10^{-9}) \times 6.02 \times 10^{23}}{Length\ of\ in\ vitro\ transcribed\ RNA \times 340}$$

These cRNA standards were then stored at – 80.00 ˚C to maintain their stability, ensuring their availability for future experimental use.

Screening of primers. The upstream and downstream primers designed for the RT-RAA assay (as detailed in Table 1) were systematically combined in pairs to formulate four distinct primer sets as follows: RAA-F1/RAA-R1, RAA-F1/RAA-R2, RAA-F2/RAA-R1, and RAA-F2/RAA-R2. To determine the most effective primer combination, the cRNA standard was diluted in a 10-fold serial dilution, and a working concentration of 1.19×10^5 copies *per* µL was utilized as a template in the fluorescence RT-RAA testing. This approach facilitated the assessment and selection of the primer set yielded the most reliable and efficient amplification results.

Optimization of reaction conditions. The RT-RAA assay was conducted using a CFX Connect™ real-time PCR thermal cycler, employing the fluorescence RT-RAA kit for the analysis. The reaction mixture, totaling 50.00 µL, consisted of the following components: A buffer (25.00 µL), primer F (10.00 µM; 2.00 µL), primer R (10.00 µM; 2.00 µL), probe (10.00 µM; 0.60 µL), nuclease-free water (12.90 µL), RNA sample (5.00 µL), and B buffer (2.50 µL). The procedure commenced with the preparation of a reaction mixture proportional to the number of assays planned, incorporating water, A buffer, primers, and probes. This mixture was thoroughly combined, and 25.00 µL was aliquoted into the detection unit tube pre-filled with reaction dry powder. To this, 5.00 µL of RNA sample and 2.50 µL of B buffer were added to the tube cap, being then sealed. The contents were mixed vigorously by inverting the tube five to six times, followed by a brief centrifugation at a low speed for 10 sec to ensure proper settling. The thermal cycling conditions were as follows: Initial pre-heat at 42°C for 40 sec, followed by amplification at 42.00 ˚C for 20 min. The results were interpreted based on the amplification curves; a peak time of ≤ 18 min (cycle threshold [Ct] value ≤ 36.00) indicated a positive result, while a peak time > 18 min (Ct value > 36.00) signified a negative result. To optimize the assay, a gradient of reaction temperatures (37 to 42.00 ˚C), reaction times (7 to 23 min), and concentrations of primers and probes (10.00 to 4.00 µM for primers and 10.00 to 3.30 µM for probes) was established. The optimal conditions were determined by comparing the slopes of the fluorescence amplification curves under these varying conditions, with the shortest reaction time and the most intense fluorescence signal being the criteria for optimization. Nuclease-free double distilled water was used as a negative control throughout the process.

Specificity test. Using the optimized reaction system, a nucleic acid solution of GoAstV at a concentration of 450 ng μL^{-1} was utilized as a positive control to validate the efficacy of the assay. Concurrently, nuclease-free double distilled water was employed as a negative control to ensure the specificity of the detection method. To further evaluate the specificity, the assay was challenged with nucleic acids from a panel of other avian viruses, including NDV, AIV-H9, GoCV, GPV, DTMUV, and FAdV-4. Each of these viral nucleic acids served as individual templates in the assay, allowing for a comprehensive assessment of the method's ability to specifically detect GoAstV without cross-reactivity with these closely related pathogens.

Sensitivity test. This experiment aimed to determine the limit of detection and assess the sensitivity of the optimized fluorescence RT-RAA method. A series of cRNA standards, spanning concentrations from 1.19 to 1.19×10^5 copies *per* µL, were used as templates. Nuclease-free double distilled water served as a negative control in these assays. By establishing the lowest concentration at which consistent detection was achieved, the sensitivity of the method was rigorously evaluated.

Repeatability test. To ascertain the reproducibility of the RT-RAA method, the optimized assay conditions were applied using the cRNA standard as a template. Nuclease-free double distilled water continued to serve as a negative control. The assay was performed in triplicate at three distinct cRNA concentrations to ensure reliability. The resulting Ct values were subjected to the analysis using the GraphPad Prism Software (version 8.0; San Diego, USA) to evaluate the consistency and precision of the method.

Clinical sample test. The clinical applicability of the GoAstV fluorescence RT-RAA method was assessed using a set of 40 clinical samples. Nucleic acids were extracted from these samples and used as templates in the RT-RAA assay. Concurrently, these samples were tested using conventional RT-PCR method for comparative analysis. The results from both methods were statistically evaluated using SPSS Software (version 26.0; IBM Corp, Armonk, USA) to confirm the consistency, and validate the clinical utility of RT-RAA method in diagnosing GoAstV infections.

Results

Primer screening. In accordance with the specifications of the RT-RAA nucleic acid amplification kit, two primer pairs were strategically designed for the detection of GoAstV. The evaluation was conducted using a GoAstV standard template at a concentration of 1.19×10^5 copies *per* µL with following four primer set combinations: RAA-F1/RAA-R1, RAA-F1/RAA-R2, RAA-F2/RAA-R1, and RAA-F2/RAA-R2. The selection of the optimal primer pair was based on the earliest peak time and the steepest slope of the amplification curve, as indicated in Figure 1. The RAA-F2/RAA-R1 primer set demonstrated the most robust fluorescence intensity in the shortest duration, signifying its selection as an optimal combination for subsequent RAA detection.

Optimal reaction conditions. This study meticulously optimized the concentrations of probes and primers, as well as the reaction time and temperature, to enhance the performance of the RT-RAA assay. A range of probe concentrations (10.00, 6.70, and 3.30 µM) and primer concentrations (10.00, 8.00, 6.00, and 4.00 µM) were tested, alongside various reaction times (7, 10, 13, 17, 20, and 23 min) and temperatures (37.00, 38.00, 39.00, 40.00, 41.00, and 42.00 ˚C). The comparative analysis of the fluorescence amplification curve slopes under these different conditions is presented in Figures 2, 3, and 4. The most pronounced amplification was observed with a probe concentration of 10.00 µM (Fig. 2A) and a primer concentration of 6.00 µM (Fig. 2B). The assay performed optimally at a reaction temperature of 39.00 ˚C (Fig. 3) and a duration of 20 min (Fig. 4). Balancing the guidelines provided in the kit manual with the practical considerations of experimental cost, the conditions deemed optimal were established at a primer concentration of 6.00 µM, probe concentration of 10.00 µM, reaction temperature of 39.00 ˚C, and a reaction time of 20 min.

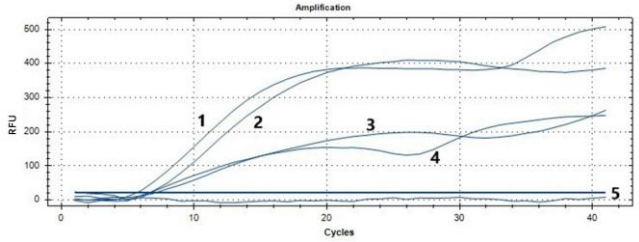

Fig. 1. Screening of primers for goose astrovirus detection by reverse transcription recombinase-aided amplification. RFU: relative fluorescence unit.

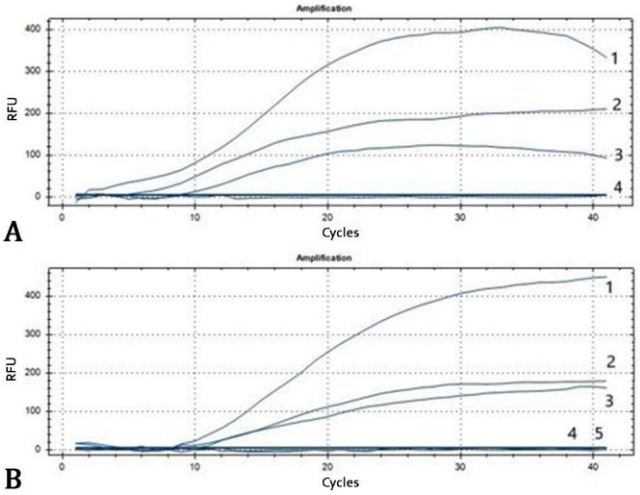

Fig. 2. Screening of the concentration of probe and primer for goose astrovirus detection by reverse transcription recombinase-aided amplification. RFU: relative fluorescence unit. **A)** Screening of the concentration of probe: 1: 10.00 µM; 2: 6.70 µM; 3: 3.30 µM; 4: Negative control; **B)** Screening of the volumes of primer: 1: 6.00 µM; 2: 10.00 µM; 3: 8.00 µM; 4: 4.00 µM; 5: Negative control.

Specificity test. The specificity of the GoAstV real-time fluorescence RT-RAA detection method was assessed using 5.00 µL of nucleic acids from GoAstV, NDV, AIV-H9, GoCV, GPV, DTMUV, and FAdV-4 as templates in the optimized RT-RAA reaction system. The results, being depicted in Figure 5, revealed that only GoAstV RNA produced a positive amplification signal, while all other templates and the negative control remained negative, as evidenced by the absence of amplification curves. These findings confirm the high specificity of the RT-RAA method for GoAstV detection in this study.

Sensitivity test. To evaluate the sensitivity of the RT-RAA assay, the standard GoAstV nucleic acid sample was subjected to a series of 10-fold gradient dilutions. The optimized RT-RAA reaction system was then utilized to determine the assay's minimum detection limit, being established at 1.19×10^2 copies *per* µL. This result indicates the assay's capability to detect low levels of GoAstV nucleic acid, as detailed in Figure 6.

Repeatability test. The repeatability of the real-time fluorescence RT-RAA method was determined by performing three replicate tests using GoAstV nucleic acid at three varying concentrations. The Ct values obtained from these replicates were analyzed using Prism Software, with the results being presented in Table 2. The coefficients of variation (CV%) for the replicates were 2.60, 3.67, and 3.42%, respectively, all falling within an acceptable range of less than 10.00%. These low CV% values indicate a high level of repeatability for the RT-RAA method.

Clinical sample test. This study involved the analysis of 40 clinical samples using both the RT-PCR and RT-RAA methods. The results, being summarized in Table 3, showed that the RT-RAA method identified 25 positive and 15 negative samples, being in full agreement with the outcomes of the RT-PCR method. A consistency analysis was conducted using SPSS Software, yielding a kappa value of 1 ($p < 0.001$), signifying perfect agreement between the two methods. The detection rate, sensitivity, and specificity of the GoAstV fluorescence RT-RAA method were all found to be 100%, demonstrating its effectiveness for clinical sample testing.

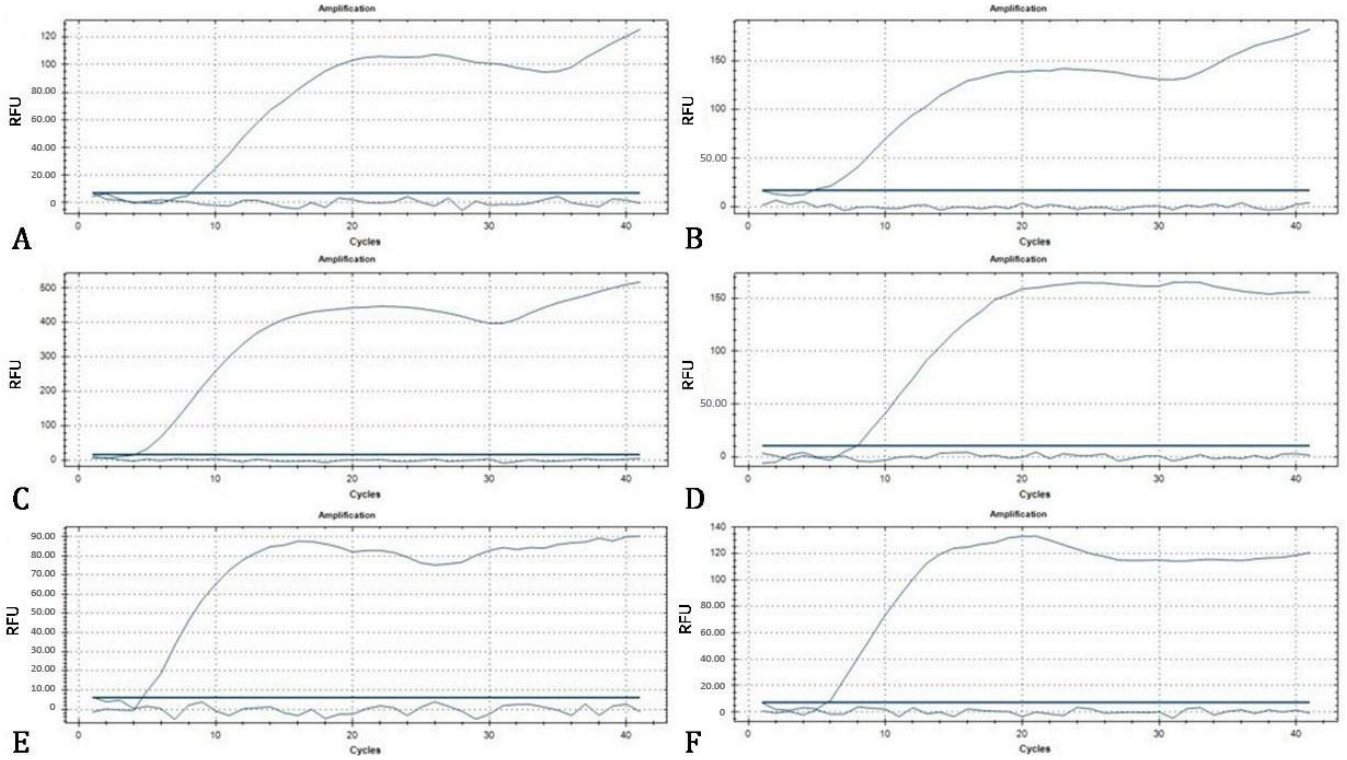

Fig. 3. Screening of reaction temperature for goose astrovirus detection by reverse transcription recombinase-aided amplification. RFU: relative fluorescence unit. The amplification time is set to 20 min. **A)** 37.00 ˚C; **B)** 38.00 ˚C; **C)** 39.00 ˚C; **D)** 40.00 ˚C; **E)** 41.00 ˚C; **F)** 42.00 ˚C. According to the speed of the peak time of the amplification curve and strength of the fluorescence signal, the reaction temperature is set to 39.00 ˚C.

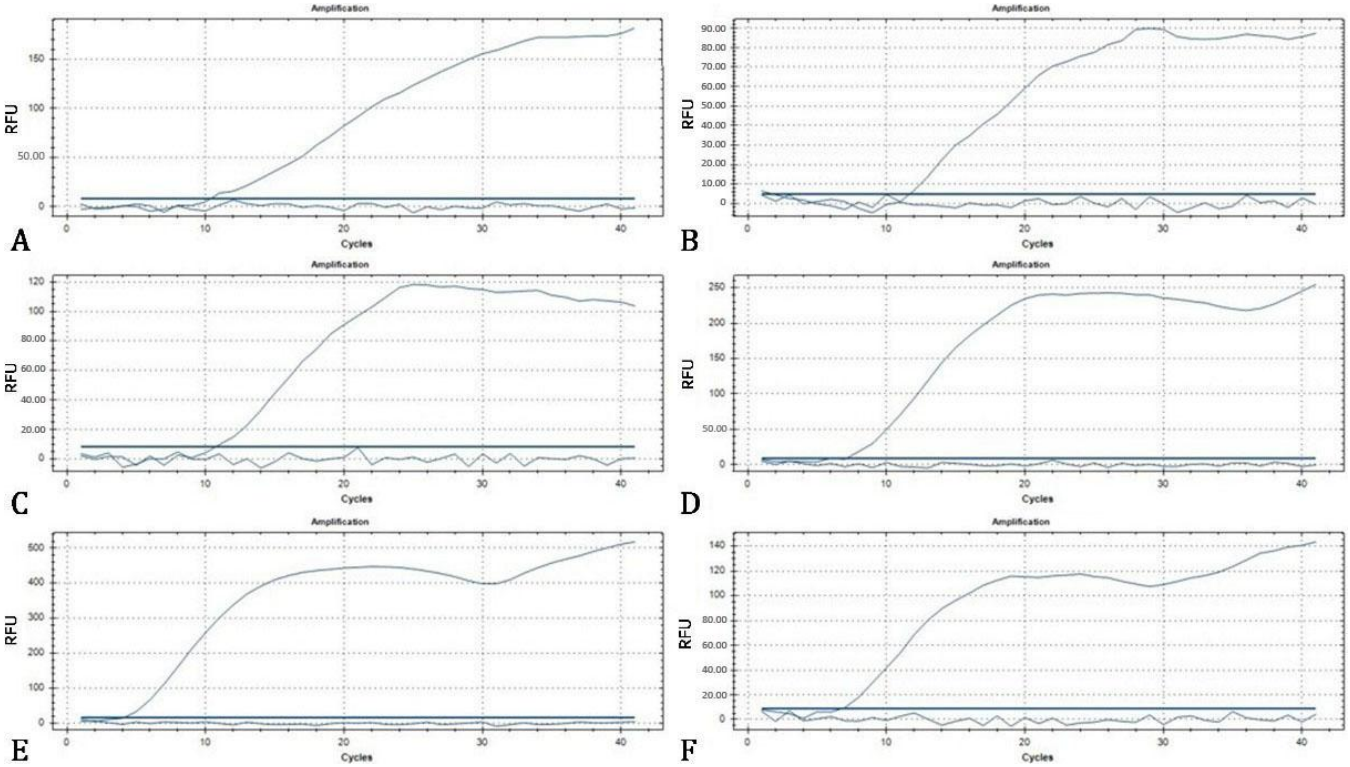

Fig. 4. Screening of reaction time for goose astrovirus detection by reverse transcription recombinase-aided amplification. RFU: relative fluorescence unit. Reaction temperature is set to 39.00 ˚C. **A)** 7 min; **B)** 10 min; **C)** 13 min; **D)** 17 min; **E)** 20 min; **F)** 23 min. According to the speed of the peak time of the amplification curve and strength of the fluorescence signal, the reaction time is set to 20 min.

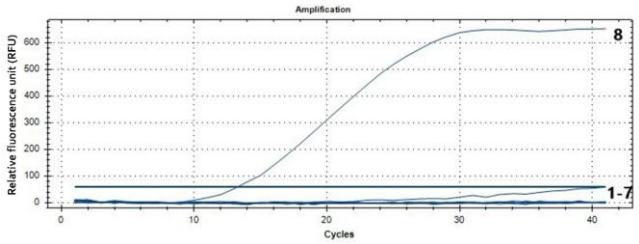

Fig. 5. Specificity analysis of real-time fluorescence reverse transcription recombinase-aided amplification detection method. RFU: relative fluorescence unit. 1-7: Negative controls for Newcastle disease virus, avian influenza virus H9 subtype, goose circovirus, goose parvovirus, duck Tembusu virus, and avian adenovirus type 4, respectively; 8: Goose astrovirus (GoAstV). Apart from GoAstV, no amplification curves were observed for other viral nucleic acids and the negative control.

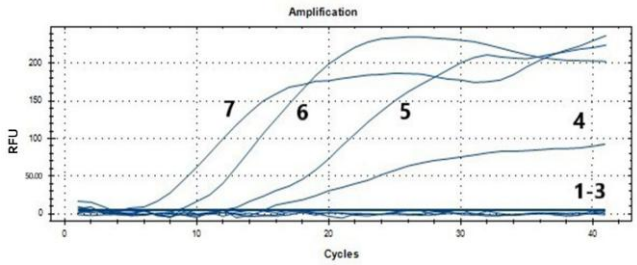

Fig. 6. Sensitivity test of real-time fluorescence reverse transcription recombinase-aided amplification detection method. RFU: relative fluorescence unit. The results showed that the lowest detection line was 1.19×10^2 copies *per* µL. 1: Negative control; 2: 1.19 copies *per* µL; 3: 1.19×10^1 copies *per* µL; 4: 1.19×10^2 copies *per* µL; 5: 1.19×10^3 copies *per* µL; 6: 1.19×10^4 copies *per* µL; 7: 1.19×10^5 copies *per* µL.

Table 2. Repeatability analysis of real-time reverse transcription recombinase-aided amplification assays for goose astrovirus.

Templates (copies *per* µL)	1	2	3	Mean ± SD	CV (%)
1.19×10^2	12.13	12.29	12.75	12.39 ± 0.32	2.60
1.19×10^3	8.66	8.10	8.17	8.31 ± 0.31	3.67
1.19×10^4	5.12	5.40	5.07	5.20 ± 0.18	3.42

SD: Standard deviation; CV%: Coefficient of variation.

Table 3. Clinical test results of two methods for goose astrovirus.

Clinical results		RT-PCR			Kappa	*p*-value of kappa	Performance characteristics	
		Positive	Negative	Total			Sensitivity (%)	Specificity (%)
RT-RAA	Positive	25	0	25	1	< 0.001	100	100
	Negative	0	15	15				
	Total	25	15	40				

RT-PCR: Reverse transcription polymerase chain reaction; RT-RAA: Reverse transcription recombinase-aided amplification.

Discussion

The recent upsurge in GoAstV infections among geese populations has led to extensive outbreaks. Affected goslings primarily display poor vitality, decreased feed intake, and excretion of white watery diarrhea. In severe cases, this can result in the death of goslings, causing considerable economic implications within the poultry industry. Currently, there are no effective drugs, vaccines, or other treatment options available to prevent and control infections and transmission caused by this virus, and prevention relies primarily on strict biosecurity measures and regular monitoring.[28] Despite the growing body of research on GoAstV, particularly regarding molecular epidemiology and detection methods, existing techniques, such as conventional PCR and qPCR, are primarily confined to laboratory use. This limitation necessitates the development of a detection method being not only highly specific and sensitive but also facile to operate, facilitating large-scale and field-based GoAstV screening. [26]

Current molecular biology techniques for GoAstV detection encompass conventional RT-PCR, real-time RT-qPCR, and multiplex RT-PCR. Previous studies have reported a sensitivity of 1.21×10^3 copies *per* µL for a conventional RT-PCR method developed by Li *et al.*, and 3.16×10^2 copies *per* µL for a real-time RT-qPCR method established by Zhai *et al.*[29,30] In this study, we achieved a detection sensitivity of 1.19×10^2 copies *per* µL with the RT-RAA method. This enhancement in sensitivity, coupled with the elimination of gel electrophoresis, significantly reduced the detection timeline. When juxtaposed with RT-qPCR, the RT-RAA method's reaction time was reduced to a mere 35.00%, allowing for rapid detection under isothermal conditions at 39.00 ˚C. The streamlined reaction process and unambiguous results render this method particularly suitable for on-site applications.

In this study, the RT-RAA method's efficacy was appraised using clinical samples with known backgrounds. Out of 40 samples analyzed, 25 tested positive, reflecting a 62.50% positivity rate, while 15 were negative, accounting for a 37.50% negativity rate, aligning with pre-existing data. The CV% for Ct values of repetitive GoAstV nucleic acid tests were consistently below 10.00%, underscoring the method's robust sensitivity and reproducibility. The absence of cross-reactivity with nucleic acids from other avian diseases further confirms the method's specificity.

In conclusion, the real-time fluorescence RT-RAA method introduced in this study integrates RT-RAA with fluorescent probes to facilitate rapid and real-time detection of GoAstV. The procedure is straightforward, requiring only the addition of the test sample and reaction

mixture to a dry tube, allowing the test to be completed within 5 to 30 min at a constant temperature of 39.00 ℃. This method has been proven to exhibit high sensitivity, excellent repeatability, and robust specificity. The validation through experimental and clinical sample analyses positions the RT-RAA method as a practical diagnostic tool with significant potential for both laboratory and clinical applications.

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Conflict of interest

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

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