Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2023.067

SHORT COMMUNICATION

Duplex SYBR Green I real-time RT-PCR for Simultaneous Detection of Goose Astrovirus Genotypes 1 and 2

Qi Zhai[1,2,3,4], Liang Zhao[1], Chun-Ling Jia[1,3,4], Xiu-Rong Zhou[1,3,4], Sheng-Jun Luo[1,3,4]*, Xiao-Hui Wen[1,3,4]* and Dian-Hong Lv[1,3,4]*

[1]Institute of Animal Health, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China
[2]Key Laboratory for prevention and control of Avian Influenza and Other Major Poultry Diseases, Ministry of Agriculture and Rural Affairs, P.R. China, Guangzhou 510640, China
[3]Key Laboratory of Livestock Disease Prevention of Guangdong Province, Guangzhou 510640, China
[4]Scientific Observation and Experiment Station of Veterinary Drugs and Diagnostic Techniques of Guangdong Province, Ministry of Agriculture and Rural Affairs, Guangzhou, 510640, China
*Corresponding author: (S-JL) 13808877470@163.com; (X-HW) wxh20060201@163.com; (D-HL) gdred@163.com

ARTICLE HISTORY (23-226)

Received: June 8, 2023
Revised: July 12, 2023
Accepted: July 14, 2023
Published online: August 06, 2023

Key words:
Goose astrovirus genotype 1 and 2 (GAstV 1 and GAstV 2)
Duplex SYBR Green I real-time RT-PCR (qRT-PCR)
Diagnostic tool
Waterfowl farming

A B S T R A C T

As the prevalence and complexity of goose astrovirus genotype 1 and 2 (GAstV 1 and GAstV 2) infections continue to rise in China's goose farming industry, a rapid, reliable and cost-effective diagnostic tool is urgently needed. Duplex SYBR Green I real-time RT-PCR (qRT-PCR) method was developed, which is capable of simultaneous detection of GAstV 1 and GAstV 2. We designed two primer pairs, targeting the viruses conserved genomic regions, facilitating the distinction of GAstV 1 and GAstV 2 through unique melting curve peaks. This method exhibited strong linear relationships in standard curves ($R^2 = 0.99$), and detection limits were 3.18×10^2 and 3.16×10^2 copies/μL for GAstV 1 and GAstV 2, respectively. With intra- and inter-assay variation below 2%, the assay exhibits excellent repeatability. This duplex qRT-PCR, displaying consistent detection rates comparable to a TaqMan-based method, offers a promising, rapid, and cost-effective diagnostic strategy for waterfowl farming.

To Cite This Article: Zhai Q, Zhao L, Jia C-L, Zhou X-R, Luo S-J, Wen X-H and Lv D-H, 2023. Duplex SYBR green I real-time RT-PCR for simultaneous detection of goose astrovirus genotypes 1 and 2. Pak Vet J, 43(4): 828-830. http://dx.doi.org/10.29261/pakvetj/2023.067

INTRODUCTION

Astroviruses, first isolated from humans, are found in various species, posing zoonotic risks due to their broad host range and cross-transmission. In 2016, severe gout outbreaks were observed in farmed goose herds in Eastern China, causing 2-50% mortality in young goslings. Identified in 2018, Goose astrovirus (GAstV) caused these outbreaks and has since spread across China, significantly impacting the goose farming industry (Niu *et al*., 2018).

GAstV is a small, non-enveloped virus from the Astroviridae family with a single-stranded positive-sense RNA genome and a diameter of 28-30 nm (Cortez *et al*., 2019). Two strains, GAstV 1 (FLX-like) and GAstV 2 (GD-like), cause similar symptoms (Liu *et al*., 2022). Existing studies primarily focus on diagnostic methods for GAstV 2. Although TaqMan-probe based real-time RT-PCR assays were developed for simultaneous GAstV 1 and GAstV 2 detection, the high cost is a drawback (Yi *et al*., 2022; Li *et al*., 2023). No reports exist of a low-cost SYBR Green I real-time RT-PCR for both strains. Hence, an affordable, practical and accurate molecular method for simultaneous detection is urgently needed. This novel method will shed light on GAstV's clinical status and provide tools for epidemiological research, disease prevention and control.

MATERIALS AND METHODS

Viruses and clinical samples: The GDYJ-21-01 strain of GAstV 1, the GDZJ-21-01 strain of GAstV 2, along with the Newcastle disease virus (NDV), avian influenza virus (AIV), goose circovirus (GoCV), goose parvovirus (GPV), and Tambusu virus (TMUV), which are all preserved by our laboratory, were utilized for specificity testing. Clinical samples were collected from goose farms located in Guangdong Province.

Table 1: Primers designed for duplex SYBR Green I real-time qRT-PCR.

Name	Sequences (5'→3')	Length (bp)
GAstV1-F	TGAAGAGATTGACCGCAAG	19
GAstV1-R	TCTGCACTCTCTGCCTG	17
GAstV2-F	TTGTGGATGACCTCTATATTAGTG	24
GAstV2-R	TACCGCATAAGAAACCTCAAC	21

Table 2: Detection of GAstV-1 and GAstV-2 in clinical samples by duplex SYBR Green I real-time qRT-PCR.

Positive rate	Samples (n=50)		Total
	cloacal swabs (n=39)	tissues (n=11)	
GAstV 1	2% (1/50)	4% (2/50)	6% (3/50)
GAstV 2	12% (6/50)	8% (4/50)	20% (10/50)
Coinfection	2% (1/50)	2% (1/50)	4% (2/50)

Primer design and standard plasmid DNA templates: The duplex SYBR Green I real-time RT-PCR assay primers were designed utilizing Beacon Designer 7 (PREMIER Biosoft International, Palo Alto, CA, USA). Detailed data about the primers is provided in Table 1. Tsingke Biotechnology synthesized two gene fragments containing the target sequences and cloned them into pUC57 vectors. The plasmids, verified through DNA sequencing, were utilized as standard plasmids, designated as pUC57-GoAstV1 and pUC57-GoAstV2, respectively.

Duplex qRT-PCR reaction condition optimization: The duplex qRT-PCR assays for GAstV 1 and GAstV 2 were consolidated into a single reaction system, incorporating primers, templates, and 2× HiScript® Ⅱ One Step qRT-PCR SYBR Green Mix (Vazyme Biotechnology Co., Ltd., Nanjing, China). This duplex reaction system was subsequently optimized, testing varying volumes of 10μM primers. During the optimization phase, the final primer concentrations in the system ranged from 100nM, 150nM, 200nM, to 250nM. Separately, 2μL of pUC57-GAstV1 and pUC57-GAstV2 templates were added to the same system. The optimal conditions for the duplex qRT-PCR system and primer concentrations were established using the Analytik Jena qTOWER3/G (Jena, Germany).

Duplex qRT-PCR specificity, sensitivity, and reproducibility: The specificity of this approach was assessed by conducting the duplex qRT-PCR under optimized conditions using nucleic acids from GAstV 1, GAstV 2, NDV, AIV, GoCV, GPV, and TMUV as templates; nuclease-free water was used as a negative control. The assay's sensitivity was ascertained via tenfold serial dilutions of GAstV 1 and GAstV 2 standard plasmids (pUC57-GoAstV1, 3.18×10^7 - 3.18 copies/μL, and pUC57-GoAstV2, 3.16×10^7 - 3.16 copies/μL), from which the limit of detection (LOD) was computed. The repeatability of the procedure was evaluated by conducting triplicate tests under optimized conditions with various concentrations of standard plasmids (3.18×10^3, 3.18×10^5, and 3.18×10^7 copies/μL for GAstV 1; 3.16×10^3, 3.16×10^5, and 3.16×10^7 copies/μL for GAstV 2). The coefficients of variation (CV) were calculated for both intra- and inter-assay variations in each experiment.

Clinical sample detection: The established duplex SYBR Green I qRT-PCR was used to analyze 11 cloacal swabs and 39 tissue samples from suspected GAstV-infected geese. Positive detection rates were compared to the TaqMan-based one-step real-time RT-PCR(Yi *et al*., 2022). The Sanger method confirmed the assay's reliability through randomly selected positive samples.

RESULTS AND DISCUSSION

Our study optimized a duplex qRT-PCR method for GAstV 1 and GAstV 2, demonstrating specificity, sensitivity, and reproducibility. The primers we designed produced distinct melting peaks for GAstV 1 and GAstV 2 (Tm values of 78.87 and 75.03℃, respectively) with no peaks for other pathogens, enabling the differentiation of these viruses (Fig. 1A and 1B). The method showed positive signals only for GAstV 1 and GAstV 2, with LODs of approximately 3.18×10^2 and 3.16×10^2 copies/μL, respectively (Fig. 1C). Coefficients of variation (CVs) were lower than 2%, indicating excellent reproducibility (Supplementary Table S1).

We evaluated this method using clinical samples from geese suspected of GAstV infection. Our method detected GAstV 1 and GAstV 2 in 6 and 20% of samples, respectively, with a 4% coinfection rate (Table 2). We found GAstV 2 infections were more frequent, consistent with prior epidemiological findings (Zhu and Sun, 2022). These results were consistent with those of a duplex TaqMan-based RT-PCR method, indicating our method's reliability.

Goose gout, often caused by GAstV, is a major disease in Chinese goose farms, leading to substantial economic losses (Li *et al*., 2022). The co-circulation of GAstV 1 and GAstV 2, with their similar clinical symptoms, complex evolution, and diverse transmission routes, contributes to the difficulty in disease control (Wei *et al*., 2020; Xu *et al*., 2023). Therefore, a rapid and reliable diagnostic method, like the one we've developed, is essential.

In conclusion, we developed a rapid, sensitive, and cost-effective duplex SYBR Green I qRT-PCR method capable of simultaneously detecting and differentiating GAstV 1 and GAstV 2. This method can aid in monitoring the spread of these viruses in China, providing early diagnostics for goose farming, thus contributing to healthier industry practices.

Conflict of interest: The authors state that there are no competing interests.

Acknowledgments: The following grants supported this study: National Key R&D Program of China (No. 2022YFD1801000), Special Fund for Scientific Innovation Strategy-construction of High-level Academy of Agriculture Science (No. 202110TD and R2020PY-JC001), Science and Technology Plan Projects of Guangdong Province (No. 2021B1212050021), and Guangdong Provincial Special Fund for Modern Agriculture Industry Technology Innovation Teams (No. 2023KJ119).

Authors contributions: QZ was responsible for designing the primers and probes, optimizing the reaction conditions, and drafting the manuscript. LZ, C-LJ, and X-RZ performed the diagnostic method testing. D-HL, X-HW, and S-JL designed the experiments and made revisions to the manuscript. All authors contributed to the article and have given their approval for the final submitted version.

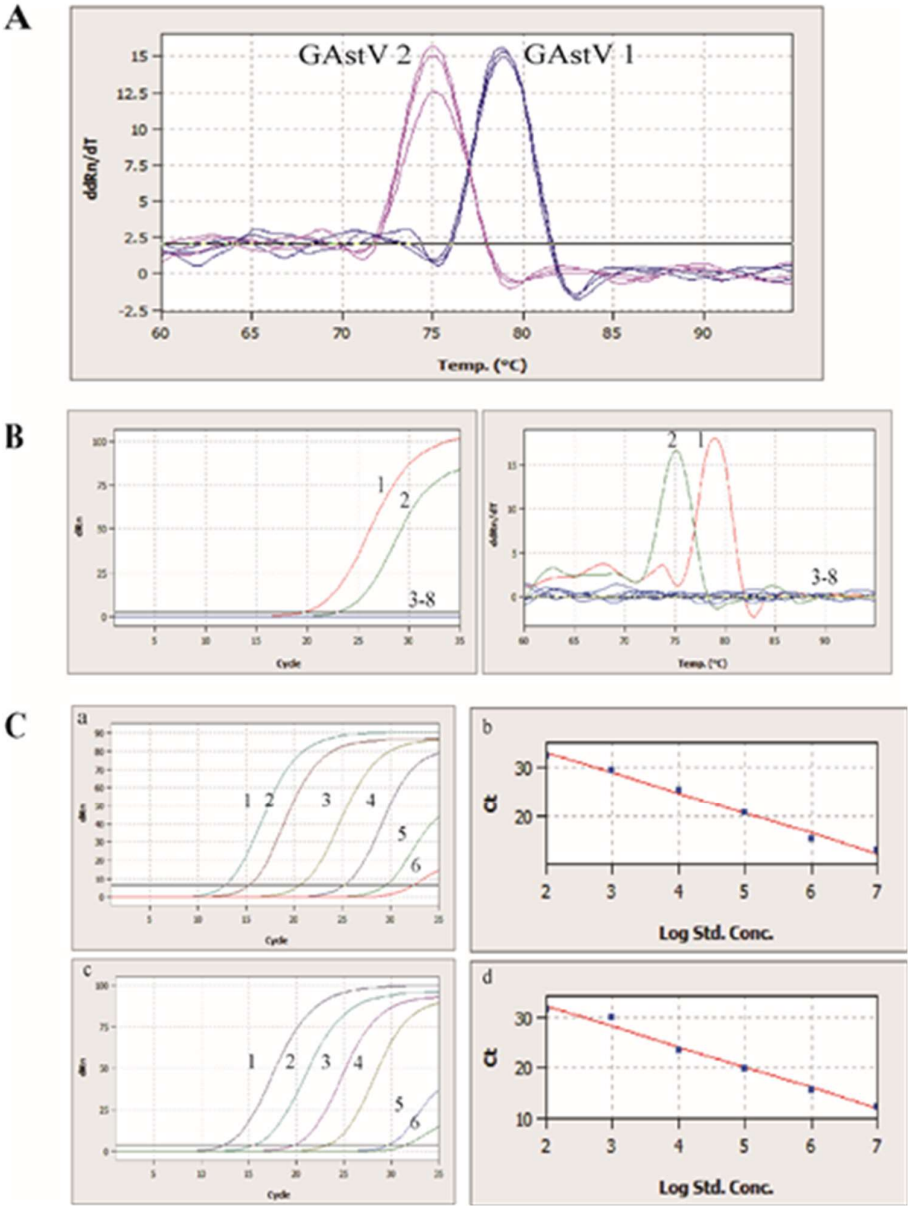

Fig. 1: Melting curves of duplex qPCR for GAstV 1 and GAstV 2 (**A**). Specificity analysis of duplex qPCR assay (**B**). 1 – 2: Positive templates of GAstV 1 and GAstV 2; 3 – 8: NDV, AIV, GoCV, GPV, TMUV, and nuclease-free water control. Sensitivity of the duplex qRT-PCR assay (**C**). (a): The amplification plot for the GAstV 1 plasmid; (b): Standard curve for GAstV 1, 1–6, 3.18×10^7–3.18×10^2 copies/μL standard plasmid; (c): The amplification plot for the GAstV 2 plasmid; (d): Standard curve for GAstV 2, 1–6, 3.16×10^7–3.16×10^2 copies/μL standard plasmid.

Supplementary Table S1: Intra- and inter-assay reproducibility of the duplex qRT-PCR assay.

Target pathogens	The concentration of standard plasmids (copies/μL)	Intra-assay		Inter-assay	
		$\bar{X}\pm SD$	CV (%)	$\bar{X}\pm SD$	CV (%)
GAstV 1	3.18×10^3	29.97±0.50	1.66	30.09±0.22	0.72
	3.18×10^5	20.68±0.12	0.56	21.05±0.24	1.15
	3.18×10^7	12.85±0.14	1.10	13.33±0.14	1.02
GAstV 2	3.16×10^3	29.67±0.11	0.36	29.18±0.43	1.47
	3.16×10^5	19.59±0.08	0.39	19.87±0.07	0.37
	3.16×10^7	12.34±0.15	1.18	13.01±0.07	0.52

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