Rapid detection of H146-like goose calicivirus using a *Taq*Man-based real-time PCR assay

Min Zheng,[*,†] Su Lin,[*,†] Shizhong Zhang,[*,†] Xiuqin Chen,[*,†] Dandan Jiang,[*] Shaoying Chen,[*,†] Shao Wang,[*,†,1] and Shilong Chen[*,†,1]

*Institute of Animal Husbandry and Veterinary Medicine, Fujian Academy of Agriculture Sciences, Fuzhou, China; and †Fujian Animal Diseases Control Technology Development Center, Fujian Academy of Agriculture Sciences, Fuzhou, China

ABSTRACT H146-like goose-origin calicivirus (**H146-like GCV**) is a novel *Caliciviridae* family member in the *Sanovirus* genus that was recently discovered and proposed to cause runting-stunting syndrome and urate deposition in geese. At present, however, there is a lack of epidemiological information pertaining to the dynamics and distribution of H146-like GCV. The development of novel molecular diagnostic approaches capable of rapidly and accurately detecting this virus would support the strengthening, the prevention, and control of H146-like GCV infection. In the present study, we therefore used a *Taq*Man probe and primers specific for the viral nonstructural (**NS**) gene to develop a highly sensitive and specific PCR assay capable of detecting this H146-like GCV. The assay reproducibly detected 5.07×10^2 copies of a recombinant DNA plasmid containing the NS gene, with a dynamic range of 8 orders of magnitude (10^2–10^9 copies). Importantly, no cross-reactivity was observed with common viruses that affected waterfowl, and when we used this assay to evaluate clinical samples, we found it to be more sensitive and faster than traditional PCR. In summary, herein, we developed a novel *Taq*Man-based real-time PCR approach that could reliably detect and diagnose H146-like GCV. This tool will allow for the real-time diagnosis of H146-like GCV infections, enabling researchers to better understand the epidemiology and clinical presentation of this disease.

Key words: goose calicivirus, H146-like, real-time PCR, TaqMan probe, virus detection

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INTRODUCTION

Caliciviridae is a family of small nonenveloped viruses that have a small positive-sense polyadenylated 7- to 8-kb single-stranded RNA genome encoding 2 or 3 open reading frames (Desselberger, 2019). The amino acid sequences of the major capsid protein (**VP1**) and the nonstructural (**NS**) protein are used to subdivide avian caliciviruses into the *Bavovirus* and *Nacovirus* genera (Wolf et al., 2012).

Recent metagenomics work has enabled researchers to identify novel caliciviruses in the *Bavovirus* and *Nacovirus* genera in studies of waterfowl and shorebirds, respectively. In addition, researchers have identified goose-origin calicivirus (**GCV**) isolate H146 as a founding member of an unassigned genus (proposed name: *Sanovirus*), family *Caliciviridae* (Liao et al., 2014; Wang et al., 2017). At present, there are no reliable approaches to efficiently propagating avian caliciviruses in vitro, and few studies of the epidemiology of H146-like GCV among domestic geese have been conducted to date. Recent works suggested that H146-like GCV isolates were often detected in goslings exhibiting clinical signs of the runting-stunting syndrome and other symptoms similar to those observed in avian astrovirus infections (Lin et al., 2020). As such, there is clear value in the development of an accurate and efficient H146-like GCV-specific molecular diagnostic technique capable of enabling researchers to better understand the prevalence and transmission of H146-like GCV in areas of China where waterfowl breeding is common.

Considering the similarities between the clinical presentations associated with GCV and avian astrovirus, and the high economic impact of enteroviruses on the waterfowl production industry, it is necessary to develop a rapid and simple diagnostic method for H146-like GCV

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[1]Corresponding authors: 365415005@qq.com (SW); 75313894@qq.com (SC)

infection and surveillance. Herein, we describe the development of a novel TaqMan-based real-time PCR capable of detecting H146-like GCV in the proposed genus *Sanovirus*. This assay represents a valuable tool for both epidemiological studies and the detection of H146-like GCV in clinical specimens.

MATERIALS AND METHODS

Ethics and Statement

The animal welfare guidelines of the World Organization for Animal Health were observed in the present study, which received approval from the Animal Welfare Committee of the China Animal Health and Epidemiology Center. The Laboratory Animal Bioethics Committee of the Institute of Animal Husbandry and Veterinary Medicine approved all animal experiments conducted in this institution based on defined animal ethics guidelines and approved protocols (approval number: FAAS-IAHV-AEC-2018-0223). Geese were handled in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the Ministry of Agriculture of China, and the China Animal Health and Epidemiology Center.

Viruses

The H146-like goose calicivirus (**GCV**) isolate Makeng (**MA**) (GenBank accession no. MN068022) used in this study was prepared from the kidney homogenate supernatant obtained from a gosling with gout syndrome in Fujian Province, China. Goose astrovirus (**GoAstV**), goose paramyxovirus (**GPMV**), goose parvovirus (**GPV**), Muscovy reovirus (**MDRV**), duck hepatitis type I virus (**DHAV-1**), and duck tembusu virus (**DTMUV**) were isolated by our laboratory.

Design of Primers and a Probe

When an alignment of the genomic sequences of goose-derived and wild duck–derived caliciviruses was conducted using GenBank, a conserved region near the 5′ end of the NS protein–encoding gene in H146-like GCV was detected. The 5′ region (nucleotides 1,867–2,135) of the NS gene in the H146-like GCV MA isolate was then chosen and used to design conventional primers with the Oligo 6.0 program (Molecular Biology Insights, Inc., Cascade, CO). These primers were used for reverse transcription and cDNA amplification, with sequences being as follows: 5′-TGCATCTGGGACGAATTTGA-CAC-3′ (GCVF) and 5′-ACACCTGGGTTCTTCTT-CAT-3′ (GCVR). These primers were expected to amplify a 269-bp fragment. The Primer Express software (v 2.0; Applied Biosystems) was then used to prepare the NSQF/NSQR real-time PCR primers and the NSP TaqMan probe, which were predicted to generate a 134-bp amplicon. We used 5-carboxyfluorescein to label the 5′-end of this probe, while the 3′-end was labeled with the Black Hole Quencher 1 nonfluorescent quencher. Table 1 lists primers and probes used in the present study.

RNA Extraction and Reverse Transcription

H146-like GCV-positive samples of kidney tissue from geese with gout as identified using a previously reported conventional heminested RT-PCR approach (Canuti et al., 2019) were homogenized and combined with PBS to prepare a uniform suspension (HyClone, Beijing, China). Samples were then spun for 10 min at 12,000 at 4°C, with supernatants being collected. Caliciviral RNA in these supernatants was extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany), after which a One-Step PrimeScript RT-PCR Kit (TaKaRa Biotechnology, Dalian, China) was used to prepare cDNA based on provided guidelines. Extracted cDNA was stored at −20°C.

Standard Plasmid DNA Preparation

The NS gene was amplified via PCR in a reaction mixture containing 1 μL each of the forward and reverse primers (GCVF and GCVR; each 20 μmol/L), 5-μL cDNA template, 18-μL RNase-free dH_2O, and 25-μL Premix Taq (TaKaRa Biotechnology, Dalian, China). Thermocycler settings were as follows: 98°C for 10 s, 58°C for 30 s, 30 cycles of 58°C for 30 s, and finally 72°C for 5 min. Amplified PCR products were incorporated into the pMD18-T vector (TaKaRa Biotechnology, Dalian, China), which was then amplified in *Escherichia coli* JM109 (TaKaRa Biotechnology, Dalian, China) and purified using an E.Z.N.A. Plasmid Mini kit (Qiagen, Hilden, Germany). OD260 values were then used to measure the concentrations of this purified recombinant pMD18-NS plasmid, with numbers of copies being determined using an appropriate technique

Table 1. Primers and probe used in real-time PCR assay for H146-like GCV.

Gene	Primer-probe	Name	Sequence	Position[1]
NS	Forward	NSQF	5′-TGCATCTGGGACGAATTTGA-3′	1,867–1,886
	Reverse	NSQR	5′-ACGCTGGAGGTGAACATT-3′	1,983–2,000
	Probe	NSP	FAM-5′- ATTGAACTGCGACCTGGCTGAGAA-3′-BHQ1	1,950–1,973

Abbreviations: H146-like GCV, H146-like goose-origin calicivirus; NS, nonstructural.
[1]Nucleotide position was designated according to NS gene of H146-like isolate MA (GenBank accession no. MN068022).

(Lin et al., 2020). After preparation, this plasmid standard solution was stored at −20°C.

Real-Time PCR Standard Curve Preparation

An ABI Prism 7500 Fast device (Applied Biosystems, Foster City, CA) was used for this TaqMan assay together with a Premix Ex Taq Probe qPCR kit (TaKaRa Biotechnology, Dalian, China) based on provided directions. Each reaction was 20 μL in total volume and contained 2-μL recombinant plasmid, 10-μL Premix Ex Taq Probe qPCR (2×), 0.4-μL ROX Reference Dye II (50×), 0.5 μL of each primer (NSQF and NSQR at 10 μmol/L), 1 μL of probe (5 μmol/L of NSP), and 5.6 μL of dH_2O. Thermocycler settings were as follows: 95°C for 45 s; 40 cycles of 95°C for 15 s, and 60°C for 45 s. Recombinant pMD18-NS was included as a positive control in each run, whereas ddH_2O served as a negative control.

Specificity Analysis

The specificity of the TaqMan-based real-time RT-PCR assay was evaluated using the above conditions, with other goose viruses, including GoAstV, GPMV, GPV, MDRV, DHAV-1, and DTMUV, which were stored at our laboratory. Negative control (DNase-/RNase-free ddH_2O) was also contained in the run.

Sensitivity and Reproducibility Analyses

The limit of detection and efficiency of our reaction was evaluated by performing serial 10-fold dilutions of plasmid DNA in sterile water to yield a dilution series (5.07×10^0–5.07×10^9 copies/μL). Samples of plasmid DNA and blank control samples were then amplified in triplicate. The sensitivity of this real-time PCR assay was assessed via a heminested RT-PCR approach (Canuti et al., 2019). Intra-assay reliability was evaluated by simultaneously assessing 3 replicates of each of 3 dilution samples. Interassay reliability was assessed by repeating 3 independent experiments every other week. These interassay and intra-assay CV were determined by dividing the SD of each tested sample by its mean before multiplying the result by 100 (Lin et al., 2020).

Clinical Sample Analysis

In total, 94 clinical samples collected from diseased or deceased goslings with gout-like symptoms suspected of having been infected with H146-like GCV (including the spleen, liver, kidney, and intestinal tissue) were evaluated via both real-time and conventional PCR.

RESULTS

Standard DNA Template Establishment

Our initial H146-like GCV NS gene standard solution prepared using recombinant plasmid technology had a concentration of 162 ng/μL, allowing us to determine that there were 5.07×10^{10} copies/μL of the NS gene. A sequence analysis confirmed that the NS gene fragment encoded by this plasmid shared 100% identity with the expected NS sequence in the H146-like GCV MA isolate.

Standard Curve Preparation

After optimizing basic assay conditions, we prepared real-time PCR standard curves based on a linear regression analysis of threshold cycle (Ct) values vs. the number of NS gene copies in a given dilution sample across a series of 10-fold serial dilutions (5.07×10^9–5.07×10^1

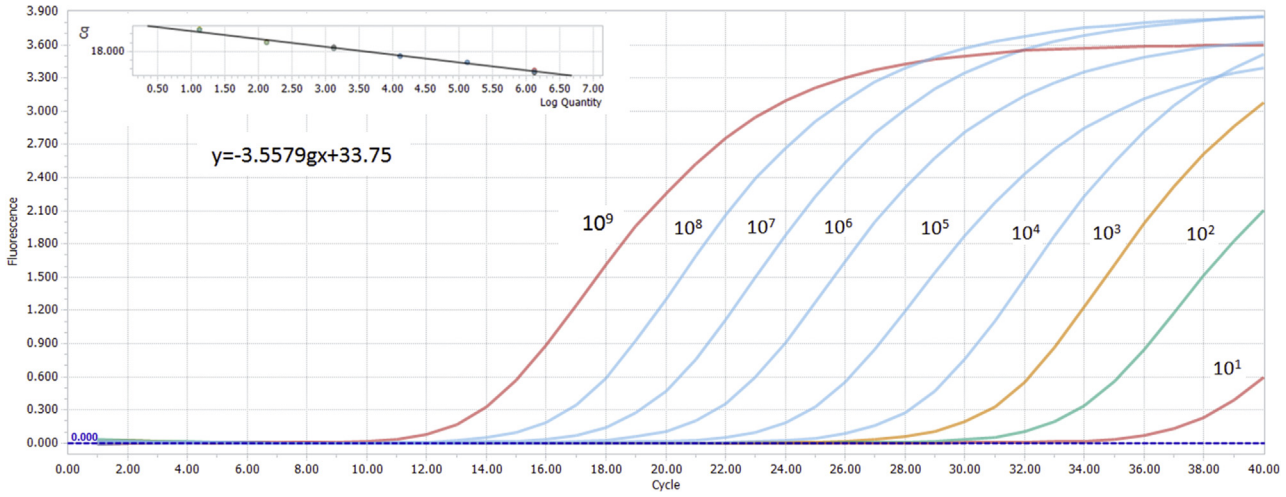

Figure 1. Standard curve for the real-time PCR. The X-axis represents copies of the plasmids, and the Y-axis represents the cycle threshold (Ct). The assay was performed using the TaqMan method on serial 10-fold dilutions of the plasmid DNA standard (5.07×10^9–5.07×10^1 copies), showing a linear relationship between plasmid concentration and threshold cycles. The standard curve produced using pMD18-NS was linear, with a correlation coefficient of 0.995 and a slope of −3.5579.

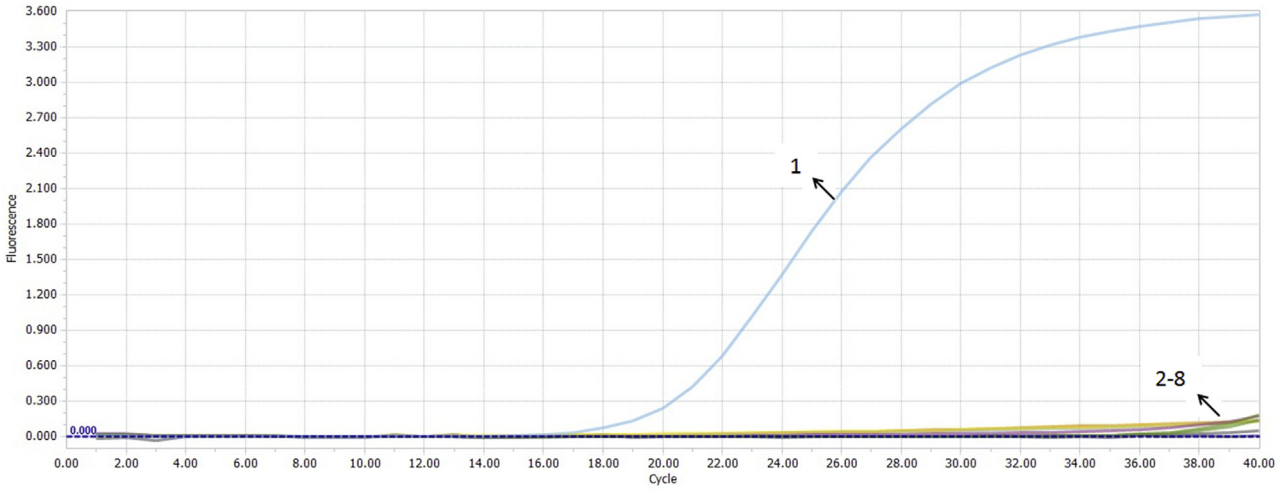

Figure 2. Real-time PCR specificity. The X-axis represents cycles, and the Y-axis represents the fluorescence data. 1: GCV MA (positive sample), 2 to 8: GoAstV, GPMV, GPV, MDRV, DHAV-1, DTMUV, and water control.

copies/μL). The resultant standard curve had a correlation coefficient (R^2) of 0.995 and a slope of −3.5579 (Figure 1), indicating that this H146-like GCV *Taq*Man PCR assay had an efficiency of 95%.

Assessment of the Specificity of This Real-Time PCR Assay

To evaluate the specificity of our TaqMan PCR assay, we evaluated its ability to detect the H146-like GCV NS gene in samples of H146-like GCV, GoAstV, GPMV, GPV, MDRV, DHAV-1, DTMUV, and ddH_2O (as a negative control). Although a positive test result was obtained for the H146-like GCV sample, as expected, all other samples yielded negative results, emphasizing that this assay specifically detects H146-like GCV (Figure 2).

Assessment of the Sensitivity of This Real-Time PCR Assay

Ten-fold serial dilutions of DNA standards (5.07×10^9–5.07×10^0 NS gene copies) were used to evaluate the sensitivity of our assay, revealing the limit of detection to be approximately 5.07×10^2 copies of viral DNA (Figure 3).

Assessment of the Reproducibility of This Real-Time PCR Assay

We next calculated interassay and intra-assay reproducibility CV for our TaqMan assay using 10-fold serial dilutions of pMD18-NS plasmid DNA (5.07×10^6–5.07×10^8 copies/μL). Three replicates of each sample were analyzed 3 times, with CV values being calculated via dividing the SD for each tested

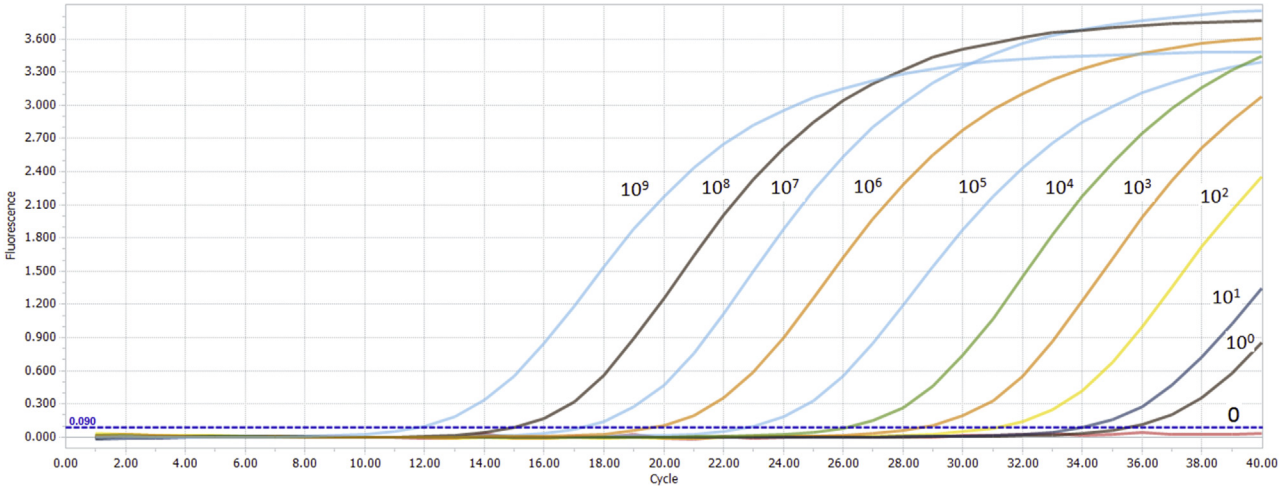

Figure 3. Sensitivity of the TaqMan real-time PCR assay for H146-like GCV detection. Ten-fold dilutions of the standard template pMD18-NS containing the target nucleotide sequence were amplified using the real-time PCR assay. Amplification plots of 5.07×10^9 to 5.07×10^0 copies/μL of pMD18-NS were detected by real-time PCR assay.

Table 2. Intra-assay and interassay reproducibility test of the TaqMan PCR.

Concentration of standard plasmid (copies/μL)	Intra-assay reproducibility (Ct)		Interassay reproducibility (Ct)[1]	
	$\overline{X}$ ± SD	CV (%)	$\overline{X}$ ± SD	CV (%)
5.07×10^8	14.222 ± 0.142	0.998	14.39 ± 0.218	1.514
5.07×10^7	17.714 ± 0.194	1.095	17.668 ± 0.129	0.730
5.07×10^6	20.626 ± 0.174	0.844	20.625 ± 0.229	1.110

[1]Three replicates of 10-fold serial dilution sample were tested. The interassay was tested for each 3 diluted samples every other week.

sample by its mean. Through this approach, we determined that this assay had an intra-assay CV of 0.844 to 1.095% and an interassay CV of 0.730 to 1.514% (Table 2).

Detection of H146-Like GCV in Samples From Infected Geese

The 94 collected clinical samples were tested to determine the feasibility of the *Taq*Man PCR assay. This analysis was conducted in parallel with the conventional PCR, which was also used to evaluate all of these samples. Conventional PCR yielded a 73.4% (69/94) rate of positive sample detection, whereas 86 of the 94 evaluated samples were H146-like GCV DNA positive, and our real-time PCR assay yielded a 91.5% (86/94) rate of positive sample detection (Table 3). For the GoAstV, 92 samples were positive, and the positive ratio was 97.9% (92/94). All the H146-like GCV–positive samples were also GoAstV positive, so the coinfection ratio of H146-like GCV and GoAstV was 91.5% (86/94) (Table 4). For the samples positive for both H146-like GCV and GoAstV, the H146-like GCV Ct values ranged from 22.70 to 32.56, whereas the GoAstV Ct values ranged from 24.47 to 34.08. The highest H146-like GCV concentration measured in any of these samples was 10^7 copies/g. Importantly, all samples that were H146-like GCV positive via conventional PCR were also positive in our *Taq*Man real-time PCR assay.

DISCUSSION

Several previously undiscovered astroviruses and caliciviruses have been identified in a range of wild bird species in recent years, *Anseriformes* and *Charadriiformes* being regarded as the wild reservoirs for such viruses (DeSouza et al., 2019; Liao et al., 2014; Wille et al., 2018). Several caliciviruses have been shown to circulate silently and infect a range of shorebirds, migratory birds, and domestic geese asymptomatically (Liao et al., 2014; Wille et al., 2018, 2019; Canuti et al., 2019). There is a need for the development of a reliable, efficient, and novel laboratory assay capable of detecting specific caliciviruses in clinical samples to determine whether geese suffering from astrovirus-associated gout in China exhibit simultaneous GCV infections. Such a tool would greatly advance current understanding of the epidemiology of GCV infections.

Owing to its use of fluorescent-labeled probes and the lack of any requirement for postamplification PCR product evaluation, real-time PCR functions as a higher sensitivity assay than conventional PCR. Although EvaGreen-based real-time PCR quantification approaches are inexpensive and easier to implement than *Taq*Man probe-based assays, the use of specific molecular probes markedly enhances assay specificity and allows for the simultaneous multiplexed PCR-based detection of a range of goose viral pathogens including GoAstV, goose hemorrhagic polyomavirus, GPV, novel duck-origin GPV, Muscovy duck parvovirus, duck enteritis virus, and DTMUV (Xuefeng et al., 2008; Yan et al., 2011; Woźniakowski et al., 2012; Niu et al., 2016; Xiao et al., 2017; Wan et al., 2018; Yin et al., 2020).

The NS gene has recently been shown to be relatively conserved in caliciviruses that infect waterfowl (Liao et al., 2014; Wang et al., 2017). Owing to this conservation, the *Taq*Man-based real-time PCR assay, primers, and probe described herein have the potential to be used for the universal detection of all such caliciviruses of the waterfowl origin. We designed these primers and probe sequences based on a comparison of conserved regions in 7 available nearly complete genomic sequences from duck and goose caliciviruses isolated in America, Australia, and China (Liao et al., 2014; Wang et al.,

Table 3. Detection of H146-like GCV in goose samples using *Taq*Man real-time PCR and conventional PCR.

Animal	Health status	Type of samples	No. of positive/no. of tested samples (%)	
			Real-time PCR	Conventional PCR
Geese	Diseased and deceased	Liver	14/16 (81.3%)	9/16 (56.3%)
		Spleen	15/18 (83.3%)	11/18 (61.1%)
		Kidney	42/42 (100%)	35/42 (83.3%)
		Intestinal tissue	15/18 (83.3%)	14/18 (77.8%)
		Total	86/94 (91.5%)	69/94 (73.4%)

Abbreviation: H146-like GCV, H146-like goose-origin calicivirus.

Table 4. Detection results of H146-like GCV in the archived clinical samples collected in 2018 and 2019 by the *Taq*Man real-time PCR.

		H146-like GCV		GoAstV		H146-like GCV + GoAstV		
Year	Sample number	Number	Ratio (%)	Number	Ratio (%)	Number	Ratio (%)	Clinical signs
2018	58	55	94.8%	57	98.3%	55	94.8%	RSS, gout
2019	36	31	86.1%	35	97.2%	31	86.1%	RSS, gout

Abbreviations: H146-like GCV, H146-like goose-origin calicivirus; RSS, runting-stunting syndrome.

2017; Wille et al., 2018, 2019; Canuti et al., 2019). Our final optimized assay exhibited an amplification correlation coefficient of 0.995 and a lower limit of detection of 5.07×10^2 copies/μL. Importantly, this assay was highly specific as no tested waterfowl viruses other than GCV yielded positive results when subjected to this real-time PCR assay. We further validated the practical utility of our *Taq*Man-based PCR assay by verifying its ability to detect H146-like GCV in 94 clinical samples collected in 2018 and 2019. All H146-like GCV-positive samples were originated from geese that were also GoAstV positive and had growth depression and gout clinical signs. The coinfection rate of H146-like GCV with GoAstV was 91.5% (86/94) in this study, which was first reported in the present study. The role of H146-like GCV and the coinfection of H146-like GCV and GoAstV in the runting-stunting syndrome and gout symptoms need to be further studied.

Our assay requires minimal sample preparation and yields replicable, accurate, and specific results within hours, making it ideal for the clinical detection of H146-like GCV in samples isolated from potentially infected geese. The specific pathogenicity of H146-like GCV remains to be fully understood, although its tissue-level distribution is thought to be closely linked to its potential pathogenic behavior. As such, the accurate measurement of H146-like GCV viral load in different tissues from H146-like GCV–infected geese may allow researchers to more fully understand the relationship between this virus and disease onset or progression.

In conclusion, our study is the first to outline a *Taq*Man real-time RT-PCR assay capable of specifically and rapidly quantifying H146-like GCV viral load in domestic geese. Overall, this assay has the potential to function as a valuable tool that will aid researchers in understanding the clinical and molecular epidemiology of H146-like GCV.

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DISCLOSURES

The authors declare no competing interests.

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