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Short communication

Simultaneous detection of duck circovirus and novel goose parvovirus via SYBR green I-based duplex real-time polymerase chain reaction analysis

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ABSTRACT

Beak atrophy and dwarfism syndrome (BADS) is commonly caused by co-infection with duck circovirus (DuCV) and novel goose parvovirus (NGPV). Therefore, concurrent detection of both viruses is important for monitoring and limiting BADS, although such a diagnostic test has not been reported. In this study, we developed a duplex, SYBR Green I-based real-time polymerase chain reaction (PCR) assay to enable the simultaneous detection of DuCV and NGPV. The assay readily distinguished between the two viruses, based on their different melting temperatures (T_m), where the T_m for DuCV was 80 °C and that for NGPV was 84.5 °C. Other non-target duck viruses that were tested did not show melting peaks. The detection limit of the duplex assay was 10^1 copies/ μ L for both viruses. This method exhibited high repeatability and reproducibility, and both the inter-assay and intra-assay variation coefficients were <1.6%. Thirty-one fecal samples were collected for clinical testing using real-time PCR analysis, and the results were confirmed using sequencing. The rate of co-infection was 6.5%, which was consistent with the sequencing results. This duplex real-time PCR assay offers advantages over other tests, such as rapid, sensitive, specific, and reliable detection of both viruses in a single sample, which enables the quantitative detection of DuCV and NGPV in clinical samples. Using this test may be instrumental in reducing the incidence of BADS and the associated economic losses in the duck and goose industries.

1. Introduction

Duck circovirus (DuCV) is a member of the *Circovirus* genus within the *Circoviridae* family. It has been classified into two different genotypes (DuCV-1 and DuCV-2) based on the *Cap* gene and the complete genome, and has been associated with immunosuppressive effects since it was first reported in Germany in 2003 [1–5]. Subsequently, DuCV has been reported in many countries such as Hungary, the United States, and China [6–9]. DuCV has been detected in Cherry Valley ducks (Pekin ducks), Muscovy ducks, mule ducks, and wild ducks [3,7,9–11]. Ducks that are infected with DuCV present clinical symptoms that usually manifest as growth retardation, feathering disorders, and a poor body condition [1,2,12]. Notably, ducks with beak atrophy and dwarfism

syndrome (BADS) also show the abovementioned clinical symptoms [13].

Goose parvovirus (GPV) has a linear, single-stranded DNA genome that is approximately 5000–5100 nucleotides long [14]. According to a previous study, GPV can infect only geese and Muscovy ducks, but not other species of ducks [15]; however, the novel goose parvovirus (NGPV) was recently identified and was shown to be capable of infecting Cherry Valley ducks (Pekin ducks) [16–18]. The clinical symptoms caused by GPV include lethargy, stunting, anorexia, locomotor dysfunction, and watery diarrhea, which can lead to high mortality in geese and ducks that are less than one month old [19].

BADS was first reported in China in 2015 and has already caused enormous damage to the goose and duck industries [16,20]. The typical

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clinical symptoms of BADS are beak atrophy, tongue protrusion, strong growth retardation, paralysis, and diarrhea [13]. Subsequently, NGPV was isolated and identified as the pathogen responsible for BADS in ducks [16,21]. However, NGPV was detected in only 20% of samples from ducks with typical BADS symptoms in a subsequent study [22]. DuCV can cause immunosuppression in ducks, making infected ducks more prone to co-infection with other bacterial and viral pathogens, such as NGPV [2,11]. Many cases of co-infection with circovirus and parvovirus have been documented in swine, canines, and wild carnivores [23–26]. Based on these observations, it is urgent to study whether ducks are also commonly co-infected with both viruses, which highlights the need for an assay that can detect NGPV and DuCV simultaneously.

Rapid, reliable, and sensitive detection of infectious diseases at an early stage is important for infectious disease prevention and control. SYBR Green I-based real-time polymerase chain reaction (PCR) assays offer many advantages, including sensitivity, rapidity, and convenience, especially for the early detection of infectious diseases. These advantages make SYBR Green I-based assays an excellent choice for simultaneously detecting NGPV and DuCV in field samples. Many duplex real-time PCR assays have been developed to detect different viruses; however, to our knowledge, no effective assay has been reported that can clarify the co-infection status of NGPV and DuCV at the same time. Therefore, in this study, we established a duplex SYBR Green I-based quantitative real-time PCR assay to detect both of these viruses simultaneously.

2. Materials and methods

2.1. Viruses and clinical samples

Thirty-one fecal samples were collected between January 2019 and May 2020 from different duck farms located in eastern China. The samples were obtained from ducks of different ages that showed symptoms typical of BADS. An NGPV virus strain (accession number MT646163) and a DuCV virus strain (accession number MY646346) were isolated from one of the duck farms, verified by sequencing, and maintained in our laboratory.

2.2. Nucleic acid-template preparation

The fecal samples were vortexed in phosphate-buffered saline at a density of approximately 0.1–0.2 g/mL and centrifuged at 12,000×g for 10 min. The resulting suspensions were collected in new RNase-free centrifuge tubes. DNA was extracted from each sample using the TIA-Namp Virus DNA/RNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. The DNA samples were stored at –80 °C until use.

2.3. Preparing plasmid standards for real-time quantitative PCR

To amplify the DuCV *rep* gene and the NGPV *VP1* gene, two pairs of primers were designed using Primer Premier 5.0. The partial *rep* gene of DuCV was amplified with the forward primer: 5'-GCTGAAGCGA-CATCCGCTGTGA-3' and the reverse primer: 5'-TTATTGGGAACGG-GAGGGTACATGC-3', and cloned into the pMD-19T vector (TaKaRa Bio). The recombinant plasmid was named pMD-19T-*rep*-DuCV and verified by sequencing at Sangon Biotech (Shanghai, China). The *VP1* gene of NGPV was also amplified with the forward primer: 5'-ACT-CAAAACAGATATGTCTACTTTT-3' and the reverse primer: 5'-CGGTGGCTCCTCCGCTTGTACTGC-3', and cloned into the pMD-19T vector to produce the pMD-19T-*VP1*-NGPV plasmid. The recombinant plasmids were quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Dreieich, Germany). The DNA concentration in each sample was calculated using the following formula: DNA concentration (copy number/μL) = (6.02 × 10²³ copies/mol × plasmid concentration [g/μL] × 10⁻⁹)/([DNA length in nucleotides × 660 g/mol]).

Each plasmid was serially diluted 10-fold and stored at –20 °C until use.

2.4. Designing primers for SYBR green I-based real-time PCR

Primers for amplifying DuCV were synthesized based on a conserved region in the *rep* gene [7], where the sequence of the forward primer was 5'-GTATCACTGACCGTTACC-3' and that of the reverse primer was 5'-GCTCTTTGTTGCTAGTTATG-3'. The primer pair was used to amplify a partial region of the DuCV *rep* gene, with a length of 93 base pairs (bp). The primers used to amplify the NGPV were synthesized based on the *VP1* gene [27], where the sequence of the forward primer was 5'-AATTTC AATGAGGTAGACA-3' and that of the reverse primer was 5'-ATTACTCCAGATGTTCCA-3'. The primer pair was used to amplify a partial region of the NGPV *VP1* gene, with a length of 171 bp. The primers were synthesized by Sangon Biotech.

2.5. Optimization of the duplex DuCV/NGPV SYBR green I-based quantitative real-time PCR assay

The duplex DuCV and NGPV SYBR Green I-based quantitative real-time PCR assay was optimized by modifying the PCR thermocycling conditions, as well as the primer and SYBR Green I concentrations, in order to obtain the best detection results.

2.6. Singular and duplex quantitative real-time PCR

Singular real-time PCR was performed in 20-μL reaction mixtures containing 10 μL of SuperReal PreMix Plus (Tiangen, China), 0.25 μM each of the forward and reverse primer, and 1 μL of the plasmid DNA template. The final volume was brought up to 20 μL using double-distilled H₂O (ddH₂O). Amplification was performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the following thermocycling program: 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Fluorescence signals were automatically collected at the end of each cycle.

The singular real-time PCR assays for DuCV and NGPV were combined into one reaction system containing SuperReal PreMix Plus, primers for both the viruses, and templates. The duplex reaction system was then optimized using different volumes of primers, and the optimal volumes of the templates were determined. Melting-curve analysis was performed to determine the melting-temperature (T_m) values of the DuCV- and NGPV-specific amplicons, in order to distinguish between each product. Standard, unknown, and negative-control samples were analyzed in duplicate in neighboring wells, and the results are presented as average T_m values from duplicate wells.

2.7. Sensitivity, specificity, and repeatability of the duplex real-time PCR assay

To determine the limit of detection of the duplex real-time assay, we performed real-time PCR for both viruses separately, using 10-fold serial dilutions of standard plasmid templates with concentrations ranging from 1 × 10⁸ to 1 × 10¹ copies/μL. For the duplex real-time PCR assays, we detected each virus separately or both viruses together. The lowest concentration of each plasmid DNA template for which the detection rate was ≥95% was defined as the limit of detection in each case.

To rule out potential false positives caused by other pathogens that might be present in the samples, we tested the duplex real-time PCR assay on DuCV (accession number: MY646346), NGPV (accession number: MT646163), duck astrovirus (DuAstV) (accession number: JX624774), Muscovy duck parvovirus (MDPV) (accession number: MH807698), duck plague virus (DPV) (AV1222 Vaccine strain), and Newcastle disease virus (NDV) (La Sota Vaccine strain) templates.

Several concentrations of the standard DuCV and NGPV plasmids (10⁷, 10⁵, and 10³ copies/μL) were tested to evaluate the reproducibility of the duplex real-time PCR assay. The coefficient of variation (CV) was

calculated from the real-time PCR data for each plasmid concentration tested.

2.8. Analysis of clinical samples

We tested 31 fecal samples from different duck farms in the Anhui Province, specifically from ducks that showed clinical symptoms characteristic of BADS. The clinical test results obtained with our duplex real-time PCR assay were evaluated by comparison with the results of a singular real-time PCR assay, which was developed previously [7,28]. All positive samples detected by either method were confirmed by sequencing, which was performed at Sangon Biotech.

3. Results

3.1. Construction of standard curves for the DuCV and NGPV plasmids

Partial regions of the DuCV *rep* gene and the NGPV *VPI* gene were amplified (Fig. 1a) and cloned separately into the pMD-19T vector. Both recombinant plasmids were sequenced by the Sangon Company and confirmed by performing BLAST and MegAlign analysis. The results showed high similarities with those for reference strains. Then, the recombinant plasmids were used as standard DNA templates for DuCV and NGPV. Linear standard curves were constructed using the standard DuCV and NGPV plasmids at dilutions ranging from 10^{10} to 10^3 copies/ μL . The standard curve equation for the DuCV plasmid was $y = -3.343x + 44.113$, with an R^2 value of 0.998 (Fig. 1b). The standard curve equation for the NGPV plasmid was $y = -3.538x + 46.736$, with an R^2 value of 0.999 (Fig. 1c).

3.2. Optimization of the duplex real-time PCR assay

Melting-curve analysis was performed with the duplex real-time PCR assay to distinguish between the amplification of DuCV and NGPV. Distinct melting-curve peaks were observed for DuCV ($T_m: 80.5 \pm 0.5$ °C) and NGPV ($T_m: 85 \pm 0.5$ °C), as shown in Fig. 2a and Fig. 2b, respectively.

Subsequently, the duplex real-time PCR assay was performed in a total volume of 20 μL ; the reaction mixtures contained 10 μL of Super-Real PreMix Plus, 0.6 μL each of the forward and reverse DuCV primers, 0.4 μL each of the forward and reverse NGPV primers, 1.0 μL of the DuCV or NGPV cDNA template, and 6.0 μL of ddH₂O. Thermocycling was performed with an initial hold step at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The target fragments formed after duplex real-time PCR showed distinct melting curves. Fig. 3 shows that DuCV and NGPV amplification were easily distinguished based on the specific T_m values for DuCV (80 °C) and NGPV (84.5 °C).

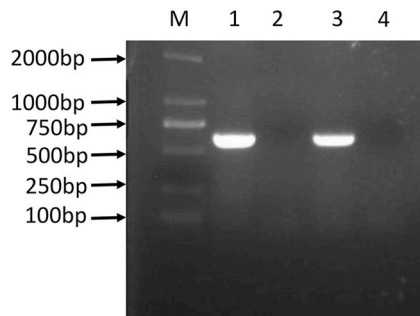


Fig. 1a. Agarose gel image with amplification of *rep* and *VPI* genes. M: DL2000 DNA marker, Lane 1: the partially amplified DuCV *rep* gene, Lane 2: negative control; Lane 3: the partially amplified NGPV *VPI* gene, Lane 4: negative control.

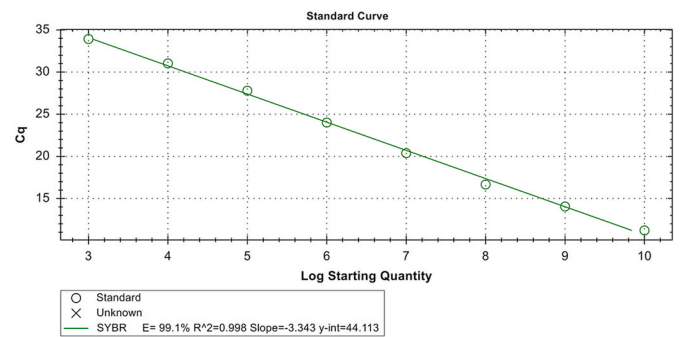


Fig. 1b. Standard SYBR Green I-based quantitative PCR curves generated by plotting the mean Cq values from triplicate samples versus the concentrations of DuCV plasmid DNA standards, which were serially diluted 10-fold over concentrations ranging from 1×10^{10} to 1×10^3 copies/ μL . The coefficient of determination (R^2) and the line equation of the regression curve (y) were calculated using a CFX96™ Real-Time PCR Detection System.

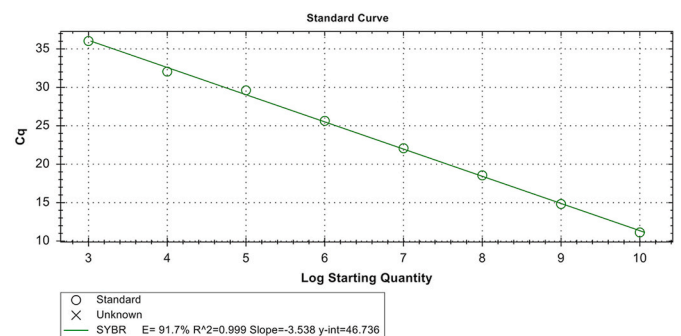


Fig. 1c. Standard SYBR Green I-based quantitative PCR curves generated by plotting the mean Cq values from triplicate samples versus the concentrations of NGPV plasmid DNA standards, which were serially diluted 10-fold over concentrations ranging from 1×10^{10} to 1×10^3 copies/ μL . The coefficient of determination (R^2) and the line equation of the regression curve (y) were calculated using a CFX96™ Real-Time PCR Detection System.

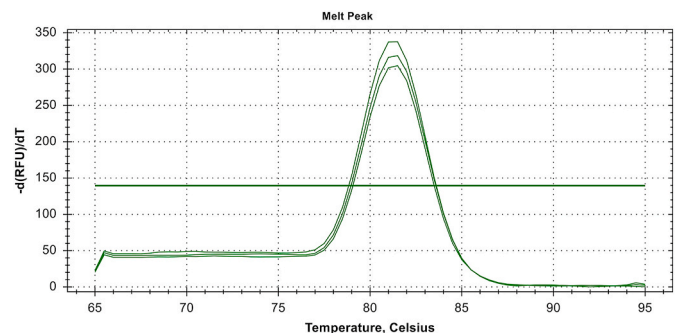


Fig. 2a. Melting-curve analysis for the singular SYBR Green I-based real-time PCR assay for detecting DuCV.

3.3. Sensitivity, specificity, and reproducibility of the duplex real-time PCR assay

Using the optimized system and the plasmid standards for each virus at concentrations ranging from 1×10^8 to 1×10^1 copies/ μL , we found that the real-time assay could detect positive samples at the lowest concentration (1×10^1 copies/ μL) (Fig. 4a and 4b).

The duplex real-time PCR assay was tested with samples positive for DuCV and NGPV, or other duck pathogens (NDV, DPV, MDPV, and DuAstV). As shown in Fig. 5, the target viruses were detected and other

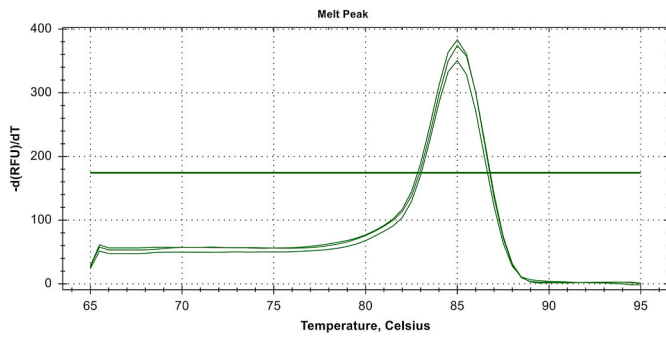


Fig. 2b. Melting-curve analysis for the singular SYBR Green I-based real-time PCR assay for detecting NGPV.

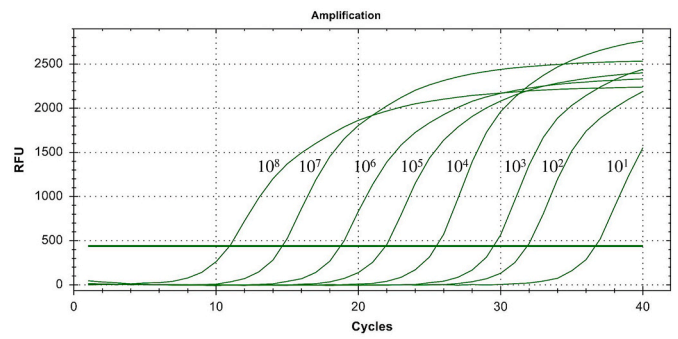


Fig. 4b. Sensitivity analysis for NGPV. Amplification curve of SYBR Green I real-time PCR using the standard plasmids of NGPV. The recombinant plasmid standards of NGPV was used as a template after ten-fold diluted, ranging from 1×10^8 copies/ μ L to 1×10^1 copies/ μ L.

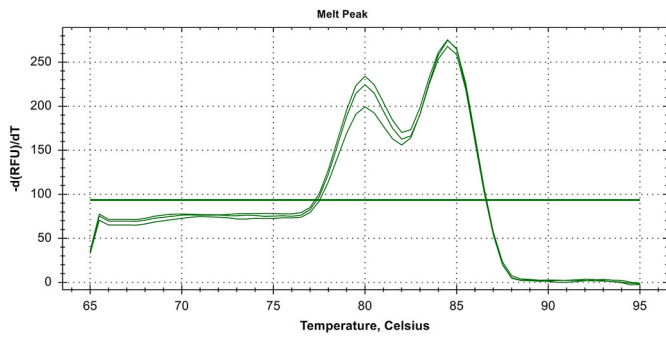


Fig. 3. Melting-curve analysis for the duplex SYBR Green I-based real-time PCR assay for detecting DuCV and NGPV.

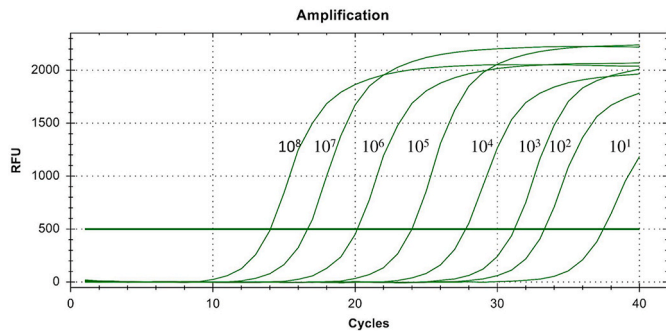


Fig. 4a. Sensitivity analysis for DuCV. Amplification curve of SYBR Green I real-time PCR using the standard plasmids of DuCV. The recombinant plasmid standards of DuCV was used as a template after ten-fold diluted, ranging from 1×10^8 copies/ μ L to 1×10^1 copies/ μ L.

pathogens were negative, indicating that the duplex real-time PCR assay showed good specificity.

To assess the assay reproducibility, three parallel experiments were performed with the DuCV and NGPV standard plasmids at each dilution. The intra- and inter-assay CV values for DuCV ranged from 0.42% to 0.98% and 0.73%–1.52%, respectively, and the intra- and inter-assay CV values for NGPV ranged from 0.34% to 0.86% and 0.69%–1.57%, respectively (Table 1). Thus, the duplex real-time PCR assay was reliable and showed high reproducibility for detecting DuCV and NGPV in the same tube.

3.4. Virus detection in clinical samples

Thirty-one fecal samples were collected from ducks exhibiting clinical symptoms of BADS at different duck farms. Using the duplex real-time PCR assay, 5 of the 31 samples tested were positive for DuCV, 2

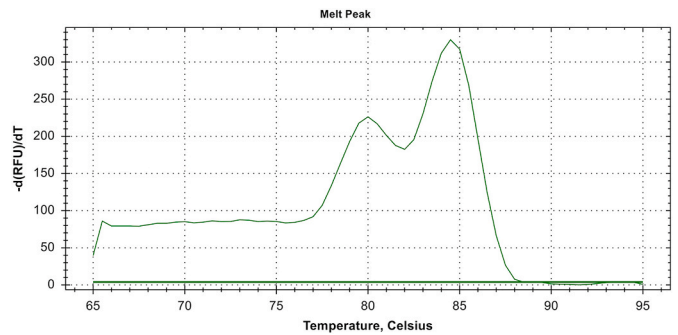


Fig. 5. Melting-curve analysis for evaluating the specificity of the duplex SYBR Green I-based real-time PCR assay. No cross-reactions were detected with duck astrovirus, Muscovy duck parvovirus, duck plague virus, Newcastle disease virus, or ddH₂O.

Table 1

Intra- and inter-reproducibility assay of DuCV.

category	DNA standard (copies/ μ L)	Mean (Ct)	SD	CV (%)
Intra-assay	1×10^7	14.82	0.062	0.42
	1×10^5	21.63	0.071	0.45
	1×10^3	27.81	0.178	0.64
	1×10^1	34.77	0.340	0.98
Inter-assay	1×10^7	14.64	0.107	0.73
	1×10^5	21.66	0.174	0.80
	1×10^3	28.55	0.415	1.40
	1×10^1	34.56	0.528	1.52

Intra- and inter-reproducibility assay of NGPV

Category	DNA standard (copies/ μ L)	Mean (Ct)	SD	CV (%)
Intra-assay	1×10^7	14.81	0.050	0.34
	1×10^5	21.68	0.088	0.41
	1×10^3	27.73	0.222	0.80
	1×10^1	34.81	0.300	0.86
Inter-assay	1×10^7	14.64	0.103	0.69
	1×10^5	21.65	0.181	0.83
	1×10^3	28.72	0.370	1.21
	1×10^1	34.51	0.543	1.57

were positive for NGPV, and 2 were positive for both DuCV and NGPV. These results were in accordance with the sequencing results. The results of our duplex real-time PCR assay were in 100% agreement with singular real-time PCR. Thus, the duplex real-time PCR assay described in this study can be applied for the differential diagnosis of DuCV and NGPV in duck farms with high specificity, sensitivity, and reproducibility.

4. Discussion

Cross-species virus transmission from wildlife reservoirs poses remarkable health risks to humans and animals, as exemplified by the current coronavirus pandemic. After several cross-species spreads, the coronavirus eventually infected humans, resulting in serious effects in terms of morbidity and mortality [29–32]. Many other viruses can also cross species barriers, including parvoviruses and circoviruses. Parvoviruses have been found in geese, ducks, and canines, and circoviruses have been found in ducks, swine and canines [19,33–37].

According to previous studies, BADS is usually caused by co-infection with NGPV and DuCV [13]. However, NGPV showed a low detection rate in samples from ducks with typical BADS symptoms in a subsequent study [22]. As an immunosuppressive virus, DuCV can reduce the body's resistance to invading viruses; thus, when ducks are simultaneously infected with both viruses, the toxic effects of NGPV can be greatly enhanced, which increases the risk of ducks developing BADS [2,11]. To further study whether co-infection with NGPV and DuCV increases the risk of ducks developing BADS, there is an urgent need for an assay that enables the simultaneous detection of both viruses [2,11,16,21]. SYBR Green I-based assays are cheaper than TaqMan assays and offer similar rapidity, specificity, sensitivity, and reproducibility when combined with melting-curve analysis. These factors made SYBR Green I-based detection a good choice for our duplex real-time PCR assay. Moreover, SYBR Green I-based real-time PCR had been widely used as a diagnostic method because of its apparent advantages over conventional PCR in terms of its simplicity, efficient detection, and sensitivity, which enable high-throughput screening even in the presence of fewer copies of a viral genome [38].

Duplex SYBR Green I-based real-time PCR has been used in numerous applications. In general, duplex real-time PCR offers many benefits as a detection method. Accurate detection at a low concentration enabled the diagnosis and prevention of porcine diarrhea at an early stage. Some virulent strains may cause severe symptoms at a low titer and, thus, a sensitive detection method is indispensable in such situations. SYBR Green I-based assays are more rapid, can be implemented more simply, generally enable detection in a single step, and produce results that are consistent with those of other methods, which reflects the greater stability of SYBR Green I-based methods [39,40]. The model exemplified in this study can be extended to the differential diagnosis of other mixed viral infections, which highlights its general significance and applicability to multiple areas of veterinary medicine [41].

In this study, a duplex SYBR Green I-based real-time PCR assay was successfully developed to amplify the *rep* gene of DuCV and the *VP1* gene of NGPV. This assay easily distinguished both two viruses based on their different T_m values, which were 85 ± 0.5 °C for NGPV and 80.5 ± 0.5 °C for DuCV. Furthermore, the detection limit of the assay was 10^1 copies/ μ L in both cases, which renders it more sensitive than the conventional PCR technique. Thirty-one fecal samples were collected to study the prevalence of both viruses, and the co-infection rate was found to be 6.5%. This is the first report describing co-infection with NGPV and DuCV. The method developed in this study could be helpful for controlling co-infection with both viruses across duck farms in China and limiting the prevalence of BADS.

In conclusion, we described the development of a SYBR Green I-based real-time PCR assay that can detect DuCV and NGPV simultaneously in a single sample. In summary, this assay can accurately reflect epidemiological data such as scale of the disease, rate of transmission, and severity of the epidemic, so as to be beneficial in monitoring and preventing the spread of the disease. The assay enables fast and stable detection of both viruses; therefore, it may serve as a reliable tool for field investigations of DuCV and NGPV co-infection. The application of this assay for clinical detection will not only improve the detection capacity, but will also reduce the workload and cost, thereby benefiting clinicians and epidemiologists.

Compliance with ethical standards

All experiments were compliant with the ethical standards of Anhui Agricultural University.

Author statement

Yong Wang and Yongqiu Cuiconceived of the study, carried out the experiment and drafted the manuscript, contributed equally to this work. Yeqiu Liparticipated in the data collection and analysis. Shudong Jiang involved in drafting of the manuscript. HuaLiu and Jing Wang participated in statistical analysis. Yongdong Li conceived of the study, revising the manuscript critically. All authors have read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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