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Development of a PCR assay for detection and differentiation of Muscovy duck and goose parvoviruses based on NS gene characterization

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ABSTRACT. Muscovy duck parvovirus (MDPV) and goose parvovirus (GPV) have both been found to cause high mortality and morbidity in Muscovy ducklings. Specific detection is often rife with false positives due to high identity at the genomic nucleotide level and antigenic similarity between MDPVs and GPVs. In this study, significantly variable regions were found, via non-structural (NS) comparison, between MDPV and GPV NS genes; however, NS genes were conserved within the MDPV and GPV groups. A polymerase chain reaction (PCR) assay for detecting and differentiating MDPVs and GPVs was developed with more specificity based on the NS gene characterization. The assay detected as low as 10^3 DNA copies of both the MDPV and GPV strains, along with 549 separate base pairs (bp). No bands of the same size from other duck pathogens, including duck circovirus, duck enteritis virus, egg drop syndrome virus, duck-origin goose hemorrhagic polyomavirus, *Escherichia coli*, *Salmonella*, *Riemerella anatipestifer* and *Pasteurella multocida* were amplified. This indicates that this method for performing PCR provides a useful and reliable alternative tool for more precise differentiation of MDPV and GPV infection in clinical samples.

KEY WORDS: differentiation, goose parvovirus, Muscovy duck parvovirus, NS gene, PCR

There are two groups of waterfowl parvoviruses: Muscovy duck parvovirus (MDPV) and goose parvovirus (GPV). MDPV is mainly observed in three-week-old Muscovy ducklings. The Muscovy duck parvoviral disease, also known as "three-week" disease in China, causes locomotor dysfunction, stunting, and abnormal feather development and may result in death [5, 20]. MDPV was previously described by Lin in the 1980s in our lab in China [8]. The goose parvoviral disease, also known as Derzsy's disease, mainly affects one-month-old goslings and has a mortality rate as high as 90% [4, 28]. GPV infection was previously described by Fang in the 1960s in China [3].

Disease caused by GPV can be fatal, not only in goslings, but in Muscovy ducklings, swans and *Anser cygnoides*, whereas MDPV only causes disease in Muscovy ducklings [1, 9, 13, 18]. Recently, a novel GPV (designated N-GPV) was found in China, which caused Cheery Valley ducks to develop short beaks and dwarfism [2]. The newly emerging N-GPV exhibits pathogenicity primarily in domesticated ducks, including Cheery Valley ducks, Pekin ducks, mule ducks, Muscovy ducklings, Sheldrake ducklings and domestic goslings [6, 10, 11, 25]. In addition, N-GPV and novel MDPV variants, resulting from genetic recombination events between classic GPVs and classic MDPVs, have been discovered in China [17, 21, 22, 31].

Presently, the International Committee on Taxonomy of Viruses (ICTV) classifies MDPV, GPV and the variant virus as a single species (namely, *Anseriform dependoparvovirus* 1), in the genus *Dependoparvovirus*, the subfamily *Parvovirinae* and the family *Parvoviridae* (https://talk.ictvonline.org/taxonomy/). These viruses have single-stranded DNA genomes of approximately 5.1 kb in length. The genome in each of these viruses is flanked by identical inverted terminal repeats (ITRs) in both the 5′- and 3′-terminal ends, which can fold on themselves to form a palindromic hairpin structure. There are two open reading frames (ORFs) in both MDPV and GPV. The left ORF encodes non-structural (NS) protein, which is involved in viral replication and regulatory functions. The right ORF encodes three capsid proteins, VP1, VP2 and VP3, which are derived from the same gene via differential splicing. In addition, VP2 and VP3 contain the carboxyl-terminal portion of VP1 [5, 28, 29].

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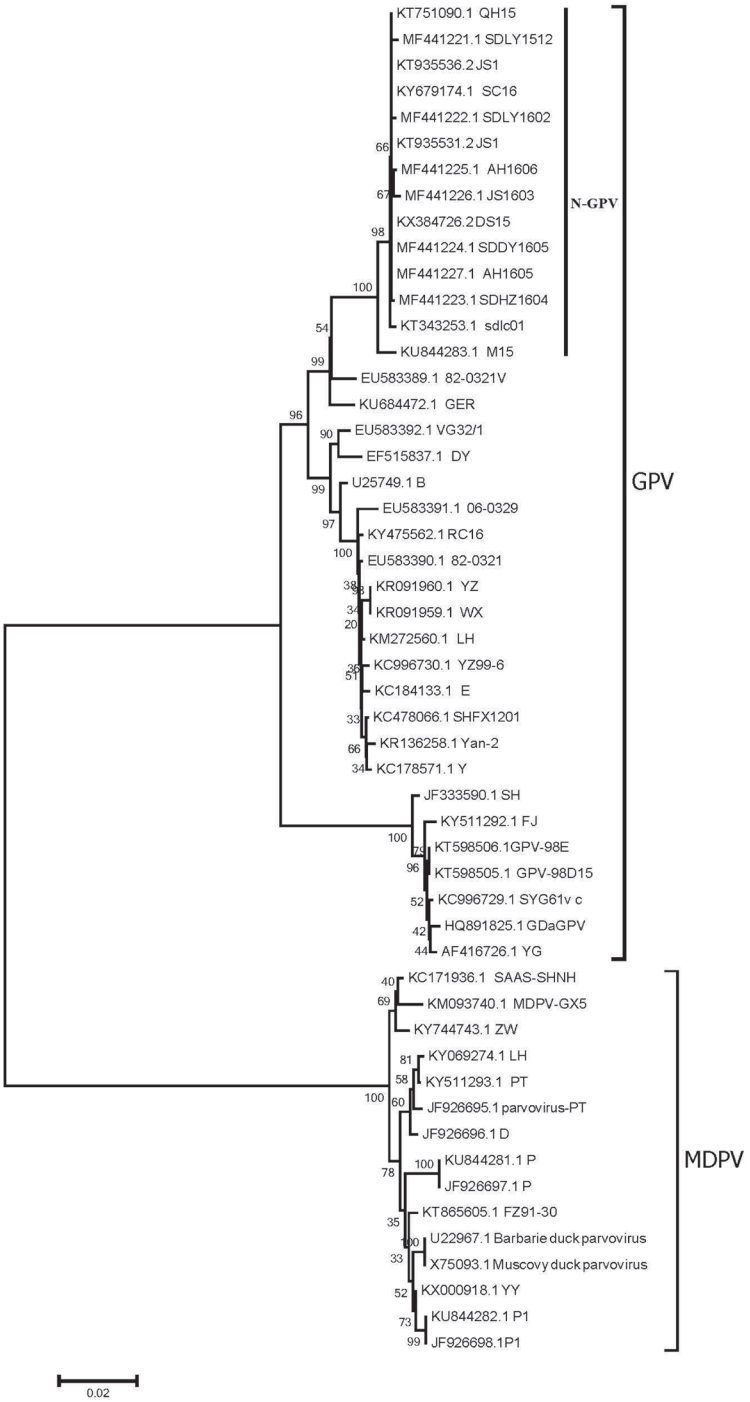

Fig. 1. Phylogenetic relationship between waterfowl parvoviruses based on alignments of the NS genes. The tree was generated with MEGA 6.0 software using the neighbor-joining method (bootstrap=1,000). The scale bar represents the number of nucleotide substitutions per site. Reference sequences obtained from GenBank are indicated by accession number and strain name.

Irrespective of relatively high homologies, antibody neutralization tests and host range differences have demonstrated that MDPV and GPV are obviously different. In a previous study, it was demonstrated that MDPV and GPV are pathogenic to Muscovy ducklings, even on the same Muscovy duck farms [1, 15, 22]. Therefore, differentiating MDPV and GPV in Muscovy ducklings is urgent. In the study, a PCR assay was developed, based on the NS gene, to provide an alternative tool to detect and differentiate MDPV and GPV with increased accuracy.

NS gene sequences from a total of 52 strains, including 15 MDPV and 37 GPV strains, were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/). A phylogenetic tree, based on the NS gene, was constructed by implementing neighbor-joining using MEGA 6. Bootstrap analysis was performed with 1,000 replications. The phylogenetic tree (Fig. 1) showed that the NS

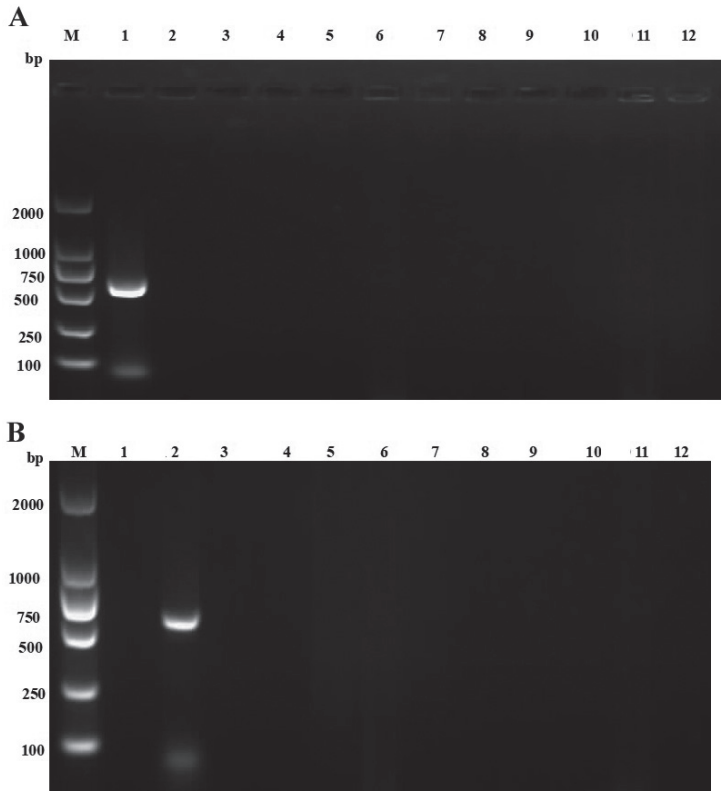

Fig. 2. Specificity test of the developed PCR assay. 2A: specificity test using MDPV-F and MGPV-R primers. 2B: specificity test using GPV-F and MGPV-R primers. Lane M, DL 2000 DNA Marker; Lane 1, MDPV; Lane 2, GPV; Lane 3, DuCV; Lane 4, DEV; Lane 5, EDSV; Lane 6, GHPV; Lane 7, *E. coli*; Lane 8, *S.S.*; Lane 9, R.A.; Lane 10, P.M.; Lane 11, negative control (nuclease-free water); Lane 12, healthy liver sample.

gene from the MDPVs shared a separate MDPV cluster, while the NS gene from the GPVs (including the N-GPV group) shared a different, separate GPV cluster. Sequence comparisons based on the NS gene were conducted using the Lasergene package with MegAlign by the ClustalW method. The nucleotide sequence identity was ≥98.1% in the MDPV cluster and ≥93.3% in the GPV cluster. However, between the GPV cluster and the MDPV cluster, the nucleotide sequence identity ranged from 81.0 to 83.4%.

Recently, we developed a method of PCR combined with restriction fragment length polymorphism (PCR-RFLP) to differentiate MDPV and GPV. This method shows a good specificity, sensitivity and repeatability [19]. However, this method requires an EcoR I digestion reaction after the PCR, making it more labor-intensive than conventional PCR.

Two specific forward primers (MDPV-F1 and GPV-F1) located on the NS gene of MDPVs and GPVs were selected. MDPV-F1 (5′-GATGAATGCTGTAGTGCAGGAGGA-3′) was designed using Oligo 7. All 15 MDPVs shared the same NS genes (position 603 to 626). GPV-F1 (5′- TTTGGC*H*GCCCCTTTACCTGATCC-3′) was designed using Oligo 7. All 37 GPVs shared the same NS genes (position 603 to 626), with the exception of position 609 (37 GPVs in this position shared 7C, 1A and 29T, and we used H to account for the respective variations). The differences between the MDPV and GPV primers are in bold type and underlined. The reverse primer MGPV-R (5′- ATTTTTCCCTCCTCCCACCA-3′), which was conserved between MDPVs and GPVs, was the same as that used in previous work [19]. The primers MDPV-F1 and MGPV-R can be used to amplify a specific 549-bp fragment of MDPV only, while GPV-F1 and MGPV-R can be used to amplify a specific 549-bp fragment of GPV in another PCR reaction tube.

Using these specific primers, a PCR assay for detection and differentiation of MDPV and GPV was performed in a total volume of 50 μl. The optimized components for each 50 μl reaction contained 25 μl DreamTaq Green PCR Master Mix (2×) (Thermo Scientific, Waltham, MA, U.S.A.), 1 μl primer-pair (MDPV-F1 and MGPV-R, or GPV-F1 and MGPV-R, separately, 10 μM each), 1 μl DNA template, and 22 μl nuclease-free water. The optimized PCR conditions for detection of MDPV (or GPV) were determined to be 95°C for 4 min (initial denaturation); 30 cycles of 95°C for 40 sec, 53°C for 30 sec, 72°C for 40 sec and a final extension step at 72°C for 7 min. The PCR products were subjected to electrophoresis on 1.0% agarose gels for analysis.

MDPV, GPV, duck circovirus (DuCV), duck enteritis virus (DEV), egg drop syndrome virus (EDSV), duck origin goose hemorrhagic polyomavirus (GHPV) [16], *Escherichia coli* (*E. coli*), *Salmonella* (*S.S.*), *Riemerella anatipestifer* (*R.A.*), *Pasteurella multocida* (*P.M.*) and negative controls (including nuclease-free water and a healthy liver sample) [19] were used to determine the specificity of the PCR assay. Viral DNA was extracted using the TIANamp Virus DNA/RNA kit (Tiangen, Beijing, China). Bacteria genomic DNA was extracted using the EasyPure Bacteria Genomic DNA Kit (Tiangen). In MDPV, MDPV-F1 and MGPV-R amplified 549-bp, however no amplification occurred using GPV-F1 and MGPV-R (Fig. 2A). GPV-F1 and MGPV-R amplified 549-bp in GPV, however no amplification occurred using MDPV-F1 and MGPV-R (Fig. 2B). No amplification occurred in any of

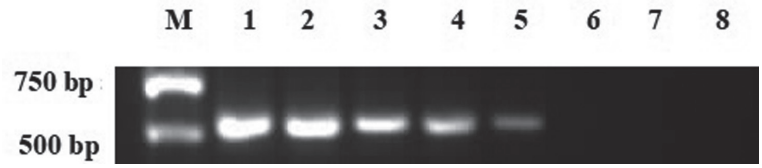

Fig. 3. Sensitivity test of the developed PCR assay. Lane M, DL 2000 DNA Marker; Lane 1, 10^7 DNA copies; Lane 2, 10^6 DNA copies; Lane 3, 10^5 DNA copies; Lane 4, 10^4 DNA copies; Lane 5, 10^3 DNA copies; Lane 6, 10^2 DNA copies; Lane 7, 10^1 DNA copies; Lane 8, negative control (nuclease-free water).

Table 1. Comparison results of clinical samples by PCR assay in this study with PCR-RFLP and virus isolation

Group	Samples[a)]	Age (day)	PCR assay in this study		PCR-RFLP[b)]	Virus isolation	Forward primer sequences[c)]
			MDPV-F1	GPV-F1			
MDPV positive	M1	9	MDPV (+)	GPV (−)	MDPV (+)	Unsuccessful	GATGAATGCTGTAGTGCAGGAGGA
	M2	3	MDPV (+)	GPV (−)	MDPV (+)	Successful	GATGAATGCTGTAGTGCAGGAGGA
	M3	18	MDPV (+)	GPV (−)	MDPV (+)	Unsuccessful	GATGAATGCTGTAGTGCAGGAGGA
	M4	7	MDPV (+)	GPV (−)	MDPV (+)	Successful	GATGAATGCTGTAGTGCAGGAGGA
GPV positive	G1	15	MDPV (−)	GPV (+)	GPV (+)	Successful	TTTGGCCGCCCCTTTACCTGATCC
	G2	9	MDPV (−)	GPV (+)	GPV (+)	Successful	TTTGGCTGCCCCTTTACCTGATCC
	G3	5	MDPV (−)	GPV (+)	GPV (+)	Unsuccessful	TTTGGCTGCCCCTTTACCTGATCC
MDPV and GPV co-positive	M5 and G4	12	MDPV (+)	GPV (+)	MDPV (+) and GPV (+)	Unsuccessful	GATGAATGCTGTAGTGCAGGAGGA TTTGGCTGCCCCTTTACCTGATCC
MDPV and GPV co-negative	F1	4	MDPV (−)	GPV (−)	MDPV (−) and GPV (−)	Unsuccessful	/
	F2	11	MDPV (−)	GPV (−)		Unsuccessful	/
	F3	9	MDPV (−)	GPV (−)		Unsuccessful	/
	F4	6	MDPV (−)	GPV (−)		Unsuccessful	/

a) MDPV positive samples are M1−M4, GPV positive samples are G1−G3, MDPV and GPV co-positive samples are M5 and G4, and MDPV and GPV co-negative samples are F1−F4. b) MDPV (+)=MDPV positive, GPV (+)=GPV positive; MDPV (−)=MDPV negative, GPV (−)=GPV negative. c) The positive samples were sequenced and analyzed. /=No sequence for negative samples.

the other eight pathogens (DuCV, DEV, EDSV, GHPV, *E. coli, S.S., R.A., P.M.*) or negative controls (Fig. 2A and 2B).

The limit of detection (LOD) of the PCR assay was evaluated using serial ten-fold dilutions of previously cloned pT-NS-MDPV and pT-NS-GPV [15]. The positive plasmids (pT-NS-MDPV and pT-NS-GPV) were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The copy numbers were then calculated using the formula by Yun *et al*. [27]. Ten-fold dilutions of the plasmids (pT-NS-MDPV and pT-NS-GPV), ranging from 10^7 to 10^1 DNA copies, were prepared to detect the PCR assay sensitivity. After agarose gel electrophoresis, the LOD of the PCR was 10^3 DNA copies for both MDPV and GPV (Fig. 3).

Twelve individual Muscovy ducklings (less than three-week) samples (livers) were collected from the Poultry Disease Treatment Center department of the Institute of Animal Husbandry and Veterinary Medicine of Fujian Academy of Agricultural Sciences (Fujian, Southeast China). The results showed that four (33.33%) samples (aged from 3 to 18 days) tested positive for MDPV; three (25%) samples (aged from 5 to 15 days) tested positive for GPV; and only one (8.33%) sample (aged 12-day) tested positive for both MDPV and GPV. Complete NS genes of positive samples that were previously amplified were used to verify the results [15]. The target PCR fragments were purified, T-A cloned, and then confirmed using the Sanger method (Sangon, Shanghai, China) (Table 1). In addition, differentiation between MDPV and GPV was tested in each of the 12 samples using the PCR-RFLP method [19] and the results were the same as those found using the PCR assay established in this study.

The total 12 samples were then used for virus isolation using 10-day Muscovy duck embryos collected from a Muscovy farm that had no previous history of MDPV or GPV outbreaks. Two MDPV isolates (designated as M2 and M4) and two GPV isolates (designated as G1 and G2) were successfully isolated (Table 1). These isolated viruses were tested via established PCR and then T-A cloned and sequenced to verify the results. These data indicated that the established PCR assay could be used for differentiating between MDPV and GPV, even in instances of co-infection.

Currently, differentiation between MDPV and GPV has been done via virus isolation, real-time PCR and PCR-RFLP [7, 14, 19, 24, 30]. Due to lack of complete genomic sequence data for GPV and MDPV, results using the above methods may be false if the primers used are referenced to unsuitable regions [21]. In the current study, a total of 52 NS gene sequences (15 MDPVs and 37 GPVs) from GenBank were compared. NS gene nucleotide identity was found to be high within both the MDPV cluster (≥98.1%) and within the GPV cluster (≥93.3%). However, the NS gene nucleotide identity between the GPV cluster and MDPV cluster ranged from 81.0 to 83.4%. These data indicate that false results could be obtained if the primers were not designed to target the specific variation regions. In order to solve this problem, MDPV specific primers (MDPV-F1) and GPV specific primers (GPV-F1)

were developed based on MDPV and GPV NS gene molecular characterization, respectively. Virus isolation and identification methods are labor-intensive, have low sensitivity and require a lot of time. Real-time PCR has an advantage over conventional PCR as it does not require agarose gel analysis to detect amplification fragments, however it does require specialized equipment and the high amplification efficiency may result in false positives [12]. PCR is a highly sensitive and specific test, which is widely used for virus detection [22, 23, 26]. Results confirming MDPV and/or GPV infection were obtained within 3h, including the time taken for DNA extraction, PCR assay and electrophoresis.

In conclusion, the present study describes a PCR assay for detecting and differentiating MDPV and GPV based on the genetic characterization of the NS gene. Amplification did not occur with DNA extracted from other pathogens that frequently affect ducklings in China, indicating that this novel method provides a useful and reliable and alternative tool for detecting and differentiating MDPV and GPV infection in clinical samples.

CONFLICTS OF INTEREST. The authors declare that they have no conflicts of interest.

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