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## Case Report—

## Goose Parvovirus and Circovirus Coinfections in Ornamental Ducks

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**SUMMARY.** Clinical observations and diagnostic procedures carried out to elucidate the cause of high mortality in 2–8-wk-old ornamental ducks (mandarin, wood, falcated, and silver teal ducks) are described. At necropsy, ducklings showed general pallor of skeletal and heart muscles, subcutaneous gelatinous transudates, pericarditis, ascites, and severe edema and hyperemia of lungs. Histopathologic examination revealed that the most important changes were located in the crop, bursa of Fabricius, and lungs with presence of amorphous basic intracytoplasmic inclusions. No bacteria or fungi could be detected from affected organs and ascitic fluid. Viral diagnosis included molecular detection for the presence of goose parvovirus (GPV), circovirus, avian influenza, herpesviruses, paramyxovirus, reovirus, and polyomavirus. Both GPV and circovirus could be detected by real-time PCR and nested broad-spectrum PCR, respectively. Phylogenetically, full-length nucleotide sequence of GPV showed a close similarity ranging from 95.6% to 97.9% with European and Asian pathogenic GPV. On the other hand, the detected circovirus showed nucleotide identity of 90% to 98% with goose circoviruses (GoCVs). This is the first report of GoCVs and GPV in ornamental ducks. The concurrence of GPV and GoCV infections is thought to contribute to the high mortality.

**RESUMEN.** *Reporte de caso-* Coinfección por el parvovirus de los gansos y circovirus en patos ornamentales: Reporte de un caso.

En este reporte se describen las observaciones clínicas y procedimientos diagnósticos llevados a cabo para dilucidar la causa de alta mortalidad en patos ornamentales (pato mandarín, pato joyuyo, cerceta de alfanjes y patos capuchino) de dos a ocho semanas de edad. En la necropsia, los patitos mostraron palidez general de los músculos esqueléticos y cardíacos, trasudados subcutáneos gelatinosos, pericarditis, ascitis, edema severo e hiperemia de los pulmones. El examen histopatológico reveló que los cambios más importantes se encontraron en el buche, la bolsa de Fabricio y en los pulmones con presencia de inclusiones intracitoplasmáticas básicas amorfas. No se pudo detectar bacterias u hongos a partir de órganos afectados y líquido ascítico. El diagnóstico viral incluyó la detección molecular para detectar la presencia de parvovirus de los gansos (GPV), circovirus, virus de influenza aviar, virus herpes, paramixovirus, reovirus, y poliomavirus. El parvovirus de los gansos y circovirus pudieron ser detectados por PCR en tiempo real y por PCR anidado de amplio espectro, respectivamente. Filogenéticamente, la secuencia completa de nucleótidos del parvovirus de los gansos mostró una gran similitud que va desde 95.6% a 97.9% con un parvovirus de los gansos patógeno de Europa y Asia. Por otro lado, los circovirus detectados mostraron una identidad en la secuencia de nucleótidos de 90% a 98% con circovirus de los gansos (Con las siglas en inglés GoCVs). Este es el primer reporte de circovirus de los gansos con parvovirus de los gansos en patos ornamentales. Se cree que la concurrencia de las infecciones por circovirus y parvovirus de los gansos contribuyeron a la elevada mortalidad.

**Key words:** goose parvovirus, goose circovirus, interspecies transmission, ornamental ducks, coinfection, sequencing, phylogenetic tree

**Abbreviations:** AAV2 = adeno-associated virus type 2; GoCV = goose circovirus; GPV = goose parvovirus; MDPV = Muscovy duck parvovirus; NJ = neighbor-joining; ORF = nonoverlapping open reading frame

Goose parvovirus (GPV) and Muscovy duck parvovirus (MDPV) belong to genus *Dependovirus*, family *Parvoviridae*, and cause Derzsy's disease in geese or 3-w disease in Muscovy ducklings (*Cairina moschata*). Infection with both parvoviruses has spread rapidly worldwide and causes high morbidity and mortality rates (7,34,35,45,47). Waterfowl parvoviruses have single-stranded DNA genomes of approximately 5100 bp that contain two large nonoverlapping open reading frames (ORFs). One ORF encodes the nonstructural protein known as nonregulatory protein (*Rep*), while the other ORF encodes VP1, VP2, and VP3 structural proteins (*capsid*). The VP2 and VP3 are contained within the

carboxyl terminal portion of VP1. The VP1 polypeptides of GPV and MDPV share an 88% amino acid sequence identity (3,37,46,48). In the affected geese and ducks the disease is characterized by anorexia, wheezing, locomotor dysfunction, myopathy of skeletal muscles, hepatitis, myocarditis, sciatic neuritis, and polio-encephalomyelitis (7). Other commonly observed lesions include atrophy of lymphoid organs (bursa of Fabricius, spleen, and thymus).

The emerging porcine parvoviruses and their coinfections with porcine circovirus type 2 were described in China (34) and Japan (26), indicating their cooperative roles in porcine circovirus-associated diseases. Circoviruses are characterized by small single-stranded circular DNA genomes approximately 2 kilobases in

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Table 1. History of outbreak.

Year	Duck species	Total no.	Mortality, no. (%)	Course of the disease (wk)	Surviving ducks	
					Dwarfism	White feathers
2013	Mandarin	200	190 (95)	3	-	-
	Wood	200	190 (95)	3	10	10
	Falcated	30	10 (33)	2	3	-
	Silver teal	50	10 (33)	2	-	-
	Muscovy	100	0 (0)	-	-	-
2014	Mandarin	150	150 (100)	3	-	-
	Wood	150	120 (80)	3	-	5
	Falcated	30	10 (33)	2	5	-
	Silver teal	50	10 (5)	2	5	5
	Muscovy	100	0 (0)	-	-	-
2015	Mandarin	160	100 (62.5)	5	5	-
	Wood	190	130 (68.4)	5	5	-
	Falcated	30	8 (26.6)	3	5	-
	Silver teal	60	20 (33.3)	5	10	-
	Muscovy	-	-	-	-	-

genome size. The family *Circoviridae* contains two genera: *Gyrovirus* and *Circovirus*. The genus *Gyrovirus* contains the chicken infectious anemia virus (24). The genus *Circovirus* contains several members such as porcine circovirus (6), psittacine beak and feather disease virus (25), pigeon circovirus (19,40), canary circovirus (23), raven circovirus (33), starling circovirus (16), finch and gull circovirus (38,39), ostrich circovirus (5), goose circovirus (GoCV) (32,40), mute swan circovirus (11), duck circovirus (12), and fox circovirus (1). In this study, the emerging of GPV and its coinfection with GoCV in ornamental ducks is described.

## MATERIALS AND METHODS

**Birds.** In 2013 a massive mortality of ducklings occurred in a large ornamental duck breeding facility located in Świętokrzyskie Voivodeship in southeastern Poland. All four ornamental duck species [mandarin (*Aix galericulata*), wood (*Aix sponsa*), falcated (*Anas falcata*), and silver teal (*Anas versicolor*) ducks] bred on the farm were affected, but the wood and mandarin ducks faced the highest losses of up to 95% mortality. Interestingly, none of the about 100 Muscovy ducklings, also present on the farm at that time, showed any signs of the disease. The first outbreak started in June 2013 and lasted for 2 to 3 wk. Ten wood ducks that survived the disease showed dwarfism and presence of abnormal white body feathers. A similar situation with very high mortality rates appeared in 2014 and 2015 (Table 1). In 2014, mandarin, wood, falcated, and silver teal ducks showed mortality rates of 100%, 80%, 33%, and 5%, respectively. In 2015, mandarin, wood, falcated, and silver teal ducks showed mortality rates of 62.5%, 68.4%, 26.6%, and 33.3%, respectively. The course of the disease ranged from 3 to 5 wk, and some of surviving ducklings showed dwarfism and feather depigmentation (white feathers). Dead ducklings from 2013, 2014, and 2015 were submitted for necropsy to the AviExpert Veterinary Clinic, Stanislaw Tokarzewski ul. Gajowa, Lublin, Poland. Autopsy examinations were done according to international guidelines and recommendations.

**Histopathologic examination.** During necropsy a selection of tissues of different organs were collected and fixed in 10% buffered formalin. The fixed tissues were sent to the Pathology Laboratory of the NOIVBD in Veldhoven (the Netherlands) for histologic examination. After arrival the samples were routinely processed, and paraffin sections were stained with hematoxylin and eosin and microscopically evaluated.

**Bacteriologic examination.** Swabs from cut surface of liver, lung, heart, and bone marrow were streaked on Columbia agar with 5% sheep

blood, MacConkey agar, xylose lysine desoxycholate agar, BBL™ Chromagar™ Candida, BBL Chromagar Orientation, Bile Esculin Azide Agar, mannitol salt agar, and Sabouraud dextrose agar (Becton Dickinson, Lublin, Poland) and incubated for 48 h at 37 C under aerobic conditions. Sabouraud dextrose agar plates were incubated at 37 C for 7 days.

**Detection of GPV in ornamental ducks.** RNA and DNA isolation from pooled samples ( $n = 5$ ) from lungs, livers, kidneys, brains, lymph nodes, or spleens was done using QiAamp cadior Pathogen Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer. For real-time PCR of GPV, GPV-VP2-s 5'-ACGGCACCTGCAAAAAAAAA-TAC-3' (location in VG/32/1 strain 2873-2895) and GPV-VP2-as 5'-CTGCCATTTTCAGATGCTGCC-3' (location in VG/32/1 strain nt 3019-3038) were used as forward and reverse primers, respectively, to amplify 165 bp (14). For each sample, a volume of 2.5 µl of the eluate was run in a 25 µl reaction on a Rotor-Gene Q real-time PCR cycler (Qiagen). The PCR reaction was done in a 25 µl final volume containing 12.5 µl Rotor-Gene SYBR Green PCR Mastermix, 2 µl GPV-VP2-s primer (10 pmol/µl), 2 µl GPV-VP2-as primer (10 pmol/µl), 0.3 µl SYBR Green (10-fold in DMSO was diluted to 1:1000 in DEPC-H<sub>2</sub>O), and 5.7 µl ddH<sub>2</sub>O. Samples were considered negative if no signal was observed during the 40 amplification cycles. A standard plasmid (pVL982) was constructed for standard curve preparation and DNA copy quantification as well as sensitivity and specificity determination (13). The cycler program was 95 C for 5 min, followed by 40 cycles of 95 C for 5 sec and 60 C for 10 sec. The melting temperature was 87 C.

**Coinfections of GPV in ornamental ducks.** All samples were tested for circoviruses using nested broad-spectrum PCR as described previously (11). For detection of polyomavirus, a nested broad-spectrum PCR was performed using VP1-1f and VP1-1r primers, followed by nested PCR with primers VP1-2f and VP1-2r targeting about 270 bp as described previously (16). For avian influenza, viral RNA was reverse transcribed under standard conditions with revertAid Reverse Transcriptase (Thermo Scientific, CITY, Germany) using the Uni-12 Primer (15). The cDNA was tested for influenza A virus by real-time PCR with IAV M1.2-Mix-FAM targeting the matrix (M) gene as described previously (14). Paramyxovirus was amplified using broadly reactive oligonucleotide primers according to Tong and others (41). Herpes viruses were tested according to van Deventer and co-workers using Consensus primer PCR (42). Briefly, the first PCR was performed using two upstream primers and one downstream primer in a multiplex format. The first PCR amplified 722 bp of polymerase gene, and the nested PCR amplified 250 bp. Samples were tested also for reovirus using consensus nested PCR as described by Wellehan (43).

Table 2. Description of GPV strains used for genetic analysis and their homology with currently detected GPV.

GPV isolates	Accession no.	Pathogenicity	Host	Country	Nucleotide identity % <sup>A</sup>	Amino acids identity %	
						Rep <sup>B</sup>	Cap <sup>C</sup>
VG32/1	EU583392	Vaccine	Goose	Germany	97.6	98.6	98.9
06-0329	EU583391	Pathogenic	Goose	Taiwan	95.6	98.2	97.8
82-0321V	EU583389	Vaccine	Goose	Taiwan	96	98.9	97.7
E	KC184133	Pathogenic	Goose	China	95.9	98.9	97.3
LH	KM272560	Pathogenic	Goose	China	95.9	99	98
sdlc01	KT343253	Pathogenic	Cherry Valley duck	China	96	96.8	99
Virulent B	U25749	Pathogenic	Goose	Hungary	97.9	98.9	98.1
WX	KR091959	Pathogenic	Goose	China	95.7	99	96.9
Y	KC178571	Pathogenic	Muscovy duck	China	96.2	98.9	98
Yan 2	KR136258	Pathogenic	Goose	China	96.2	98.9	97.3
FM	NC_006147	Pathogenic	Muscovy duck	France	81.8	90.6	87.7
MDPV-GX5	KM093740	Pathogenic	Muscovy duck	China	83.3	90.4	91.1
P	JF926697	Pathogenic	Muscovy duck	China	81.7	90.7	88.3
SAAS-SHNH	KC171936	Pathogenic	Muscovy duck	China	85.3	90.9	91.9

<sup>A</sup>Nucleotide sequences (nt 1–4792).

<sup>B</sup>Left ORF encodes the nonstructural protein (627 amino acids).

<sup>C</sup>Right ORF encodes VP1, VP2, and VP3 (732 amino acids).

**Full-length amplification of the GPV genome.** The full-length genomic sequence of the detected GPV was amplified in five partially overlapping DNA fragments using five pairs of primers (P1F, P1R; P2F, P2R; P3F, P3R; P4F, P4R; and P5F, P5R) as described by Chen and others (2). PCR conditions were as follows: 95 C for 5 min, 35 cycles of 95 C for 30 s, 55 C for 1 min, 72 C for 2 min, followed by a final extension at 72 C for 10 min. The GPV strain 32/1 was used as a positive control.

**Sequencing and sequence analysis.** The PCR products of GPV and circovirus were purified after agarose gel electrophoresis using the GeneJET Gel Extraction kit (Thermo Scientific) and cloned into CloneJET vector (Thermo Scientific). The purified plasmids were used for direct nucleotide sequencing using a Rhodamine Dye-Terminator Cycle Sequencing Ready Reaction Kit (Big Dye Terminator v1.1; Applied Biosystems, CITY, COUNTRY), followed by analysis in an ABIPRISM<sup>TM</sup> 310 Genetic Analyzer (Applied Biosystems). The sequencing data were initially checked by NCBI BLAST search, assembled, and edited using EditSeq (DNASTAR Inc., Madison, WI). Additional GPV and MDPV sequences available in GenBank were downloaded and subjected to ClustalW multiple sequence alignment to be compared with currently detected GPVs (Table 2). A nucleotide sequence identity matrix was calculated to determine the homology between the detected GPV and other selected reference isolates (Table 2). To assess the genetic relatedness among the GPV strains, phylogenetic trees were generated by the distance-based neighbor-joining (NJ) method using MEGA version 5.10. Bootstrap values were calculated based on 1000 NJ replicates of the alignment.

## RESULTS

**Gross lesions and bacteriologic examination.** At necropsy of the ducklings (2–8-wk-old), the most important features were general pallor of skeletal and heart muscles, subcutaneous gelatinous transudates, pericarditis, ascites (Fig. 1A), severe edema, and hyperemia of spleen (Fig. 1B) and lungs (Fig. 1C,D). Cultures of the affected organs and ascitic fluid showed no presence of bacteria or fungi.

**Histopathologic examination.** At the histopathologic examination the most important changes were located in the crop, bursa of Fabricius, and lungs. Irregular-shaped amorphous basic intracytoplasmic inclusions were seen in the basal layer of the mucosa of the crop

(Fig. 1E). Directly under the basal membrane a thick layer of inflammatory cells, mostly round-nuclear lymphoid cells, were present. In the bursa of Fabricius the outer cortex still contained lymphoid cells, but in the medulla most of the cells showed degeneration and karyorrhexis. In the layer of epithelial cells small irregular-shaped basophilic intracytoplasmic inclusions could be seen (Fig. 1F). The lungs were very compact due to congestion and contained almost no air. The parabronchi and many respiratory capillaries were filled with edema, hemorrhagic fluid, and erythrocytes. The trachea was lined with normal ciliated epithelium, which was covered with low protein containing fluid mixed with erythrocytes. In the liver a mild periportal round-nuclear infiltration was seen. The histology of thymus, myocardium, spleen, kidneys, proventriculus, duodenum, pancreas, small intestines, and ileocecal junction showed no major pathologic changes.

**Viral examination.** The five pooled samples were negative for polyomavirus, avian influenza, paramyxovirus, reovirus, and herpesviruses. However, 5/5 and 4/5 were positive for GPV and circovirus, respectively. Sequence analysis of circovirus showed nucleotide identity of 90% to 98% with GoCVs.

**Full-length genomic sequencing of GPV.** The detected GPV was amplified in five partially overlapping fragments by PCR. Amplification of GPV yielded PCR products of 176, 1389, 962, 1752, and 835 bp, respectively (Fig. 2A). The five fragments were assembled to build a full-length genomic sequence and designated GPV-GER. The sequence was submitted to GenBank with accession number KU684472. The phylogenetic tree based on nucleotide sequences (nt 1–4792) showed that GPV-GER was clustered, together with VG32/1 vaccine and pathogenic GPV strains in a distinct clade included in the GPV-related lineage (Fig. 2B). The GPV-GER contains two major ORFs: the left ORF (nt 537–2420), which encodes the nonstructural protein (*Rep*), contains 627 amino acids, and the right ORF (nt 2439–4637), which encodes the *capsid* protein (VP1, VP2, and VP3), contains 732 amino acids. Comparison using the complete genome sequence showed that the GPV-GER has a close similarity ranging from 95.6% to 97.9% and 81.7 to 85.3% with GPV and MDPV, respectively (Table 2). Amino acid sequences of *Rep* and *capsid* proteins were predicted from the nucleotide sequences. Analysis of the deduced amino acid

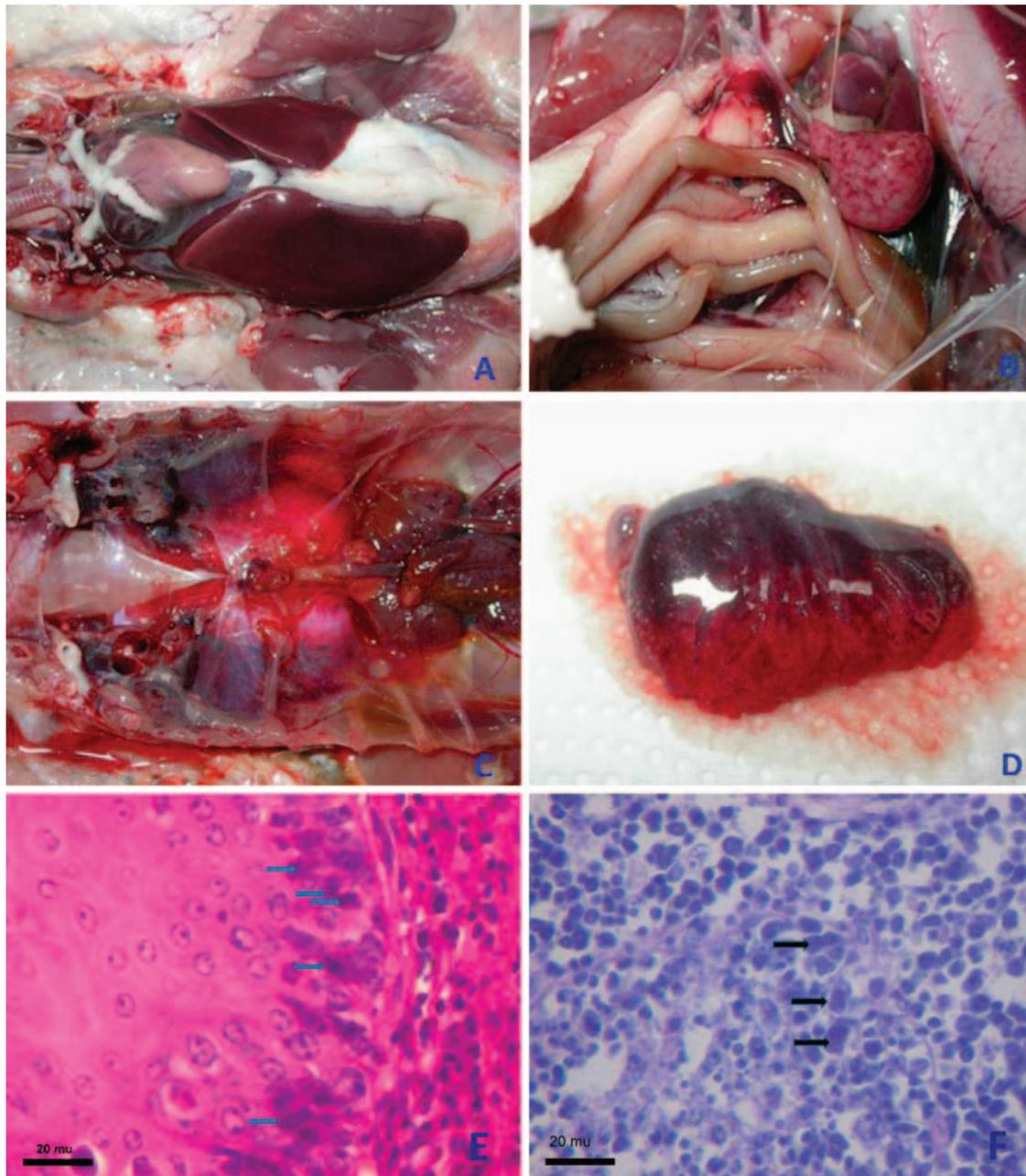


Fig. 1. Gross and histopathologic lesion of GPV and GoCV concurrent infection in ornamental ducks showing ascites (A), hyperemia of spleen (B), and lungs (C and D), a thick layer of inflammatory cells, lymphoid cells, and inclusion bodies in the crop (E) and bursa (F).

sequences of the *Rep* protein of GPV-GER revealed a homology of 96.8%–99% and 90.4%–90.9% with GPV and MDPV, respectively (Table 2). The highest amino acid homology (99%) was with pathogenic GPV (WX and LH strains). Compared with other GPV isolates, the *Rep* protein of GPV-GER has five amino acid substitutions: Q73R, S200T, A211V, T564S, and D/N609T. On the other hand, the determined amino acid sequences of the *capsid* protein showed a homology of 97.3%–99% and 87.7%–91.9% with GPV and MDPV isolates, respectively (Table 2). The highest amino acid homology was with pathogenic GPV strains [SDSDLC01 (99%), virulent B (98.1%)] and GPV vaccine [VG32/1 (98.9%)]. Compared with other GPV isolates, the *capsid* protein of GPV-GER

has one amino acid substitution K151T. The GPV-GER contains four potential N-glycosylation sites in *Rep* (<sup>150</sup>NKT<sup>153</sup>, <sup>225</sup>NYS<sup>228</sup>, <sup>360</sup>NWT<sup>363</sup>, and <sup>433</sup>NST<sup>436</sup>) and six in *capsid* (<sup>219</sup>NAS<sup>222</sup>, <sup>331</sup>NLT<sup>334</sup>, <sup>582</sup>NTT<sup>585</sup>, <sup>700</sup>NFS<sup>702</sup>, <sup>703</sup>NRT<sup>705</sup>, and <sup>712</sup>NET<sup>714</sup>).

## DISCUSSION

The GPV infection, also known as Derzsy's disease, goose hepatitis, or gosling plague, is an acute, contagious, and fatal disease among domestic goslings and Muscovy ducklings (4,8). Although the disease is confined to goslings and Muscovy ducklings, in addition, a distinct lineage of GPV was detected in

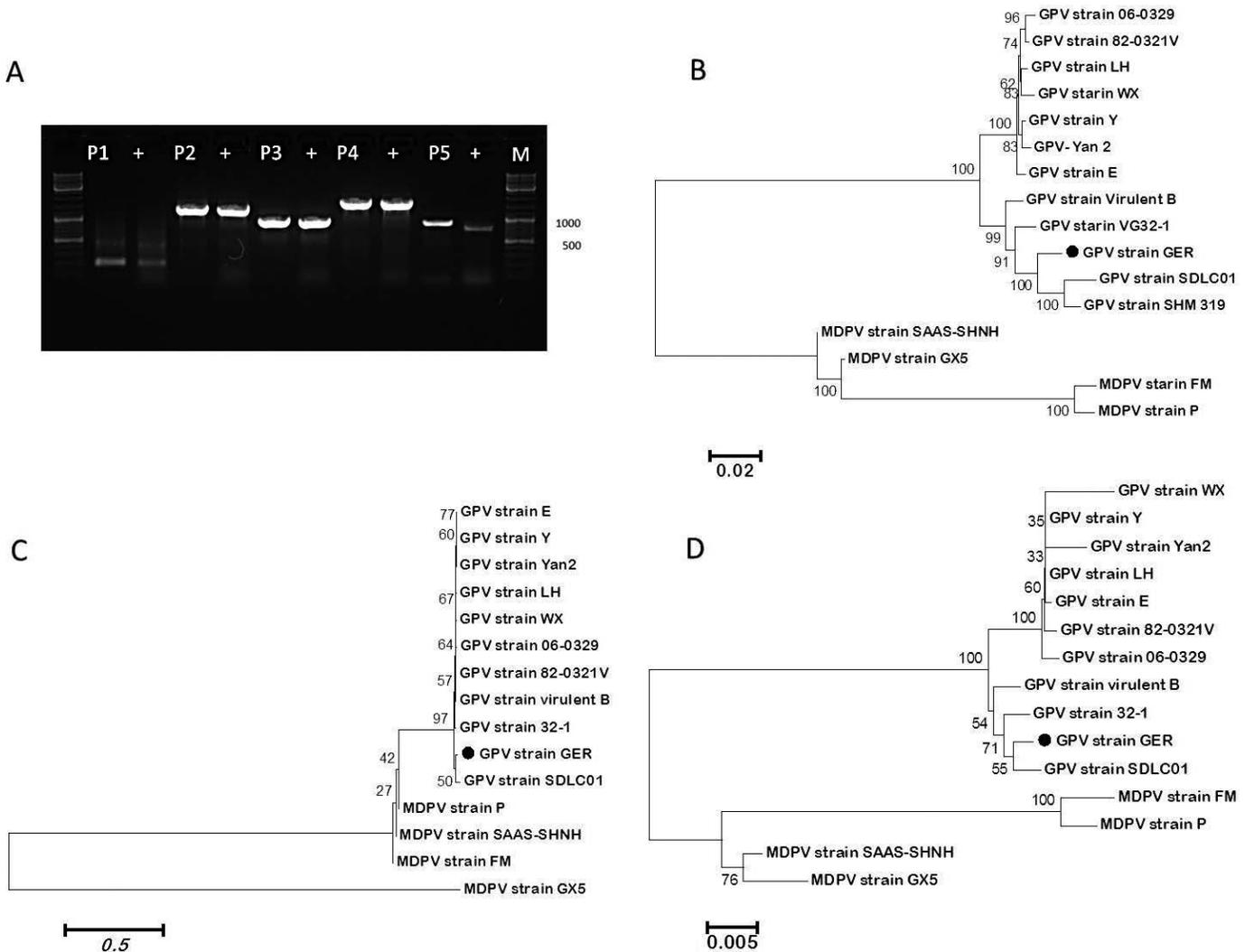


Fig. 2. (A) Full length amplification of GPV-GER. The genome was amplified in five partially overlapped fragments designated P1–P5. The GPV 32/1 vaccine was used as positive control (+). (B–D) Phylogenetic analysis of GPV-GER based on the nucleotide sequences of full length (B), *rep* (C), and *capsid* (D). The reliability of the tree was assessed by bootstrap analysis with 1000 replications. Bootstrap values  $\geq 70\%$  are shown. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood ( $-3031.4082$ ) is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches. Initial tree(s) for the heuristic search were obtained by applying the NJ method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted using MEGA6.

mule duck, a cross-breed of Pekin duck and Muscovy duck (22). Recently, GPV was detected in swan (28) and Cherry Valley ducklings (2). In this study, a case of GPV and GoCV in 2–8-wk-old ornamental ducks with high mortality rates was described. This suggests that species other than goose and Muscovy ducks could serve as a host for GPV. Muscovy ducks did not exhibit clinical disease, although they were reared with the four ornamental duck species (mandarin, wood, falcated, and silver teal ducks). According to the owner’s data, Muscovy ducklings were also not vaccinated against Derzsy’s disease. It was not possible to collect samples from Muscovy duck breeders; however, according to the owner’s data Muscovy duck breeders were not vaccinated against Derzsy’s disease. We assume that the Muscovy duck breeders were naturally infected with GPV and they were the original source of infection, and then the virus transmitted horizontally between different duck species.

It was reported that the concurrent infection of the flock with circovirus might be an independent risk factor through its presumed immunosuppressive effect that contributed to the outcome of infection in individual birds (7,32). Moreover, there is evidence of possible vertical transmission of circovirus from breeder ducks to ducklings (18), highlighting its potential for inducing immunosuppression in animals when very young. It is supposed that circovirus infections in ducks display similar symptoms to those observed in other domestic birds such as pigeon and geese. In wild birds, circovirus infections are associated with immunosuppression, leading to bird emaciation and an increased susceptibility to secondary pathogens (20,33,38,44). The possible role of these viruses in immunosuppression of the birds needs to be studied more comprehensively. To our knowledge, this is the first report of GoPV infection in ornamental ducks. In this context previous studies reported on the possible interspecies transmission of

circovirus (10,20,27). Transmission between free-living ducks or geese and their farmed counterparts is also considered possible (38). Kozdruń *et al.* (17) reported on the occurrence of coinfection with GPV and GoCV in geese flocks aging from 5 to 6 wk.

In our case the most important changes were located in the crop, bursa of Fabricius, and lungs with the presence of amorphous basic intracytoplasmic inclusions, as is known to be typical for circovirus infections. In previous studies the gross and histopathologic lesions of circovirus were not very consistent in naturally infected goslings; pathologic changes were found in the spleen and thymus (32). Inclusion bodies were also observed in lymphatic tissues, particularly in the bursa of Fabricius (7,31,32). However, no inclusion body detection was reported in experimentally infected goslings with GPV (9,28).

Sequence analysis of the currently detected GPV showed nucleotide identity of 95.6%–97.9% and 81.7%–85.3% with GPV and MDPV, respectively. The GPV-GER was clustered within the European and Asian GPV strains, which included pathogenic isolates (06-0329, LH, WX, Y, Yan-2, E, SDLC01, SHM319, and virulent B) and vaccine strains (VG32/1 and 82-0321V). The *Rep* and *capsid* proteins of GPV-GER have six unique amino acid substitutions and Q73R, S200T, A211V, T564S, and D/N609T in *rep* and *capsid* (K151T) proteins. However, the biological meaning of these mutations remains a question of value. Previous studies showed that the nucleotide sequences of GPV and MDPV had 70.2%–70.3% sequence similarity to that of adeno-associated virus type 2 (AAV2) and are thus closely related (47). Five residues (R484, R487, K532, R585, and K588) are involved in the receptor binding site of AAV2 (21). These basic amino acid residues are absent in GPV-GER and most analyzed GPVs, suggesting that other basic amino acids are included for receptor binding. Similar results were described by Shien *et al.* (30).

In the current study, GPV and its coinfection with GoCV were determined to be pathogenic based on the clinical pathologic findings in the field. However, the conducted diagnostics do not allow exclusion of other causes that could contribute also to the clinical picture and lesions on the farm. As an intervention strategy, all ducks were removed from the farm, the farm was disinfected, and new birds (mandarin, wood, falcated, and silver teal ducks) have been introduced. None of the “old” birds remained. In conclusion, the detection of GPV and GoCV in a disease outbreak in ornamental ducks suggests an increased susceptibility due to immunosuppression and demonstrates the possibility of interspecies transmission of GPV among different duck species.

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