

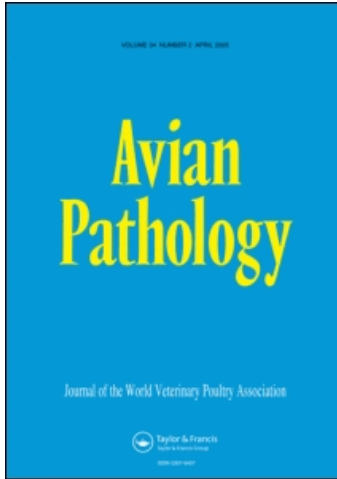
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# Diagnosis of goose circovirus infection in Hungarian geese samples using polymerase chain reaction and dot blot hybridization tests

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A polymerase chain reaction (PCR) and dot blot hybridization (DBH) test have been developed for the diagnosis of infection by a novel circovirus of geese (GoCV). These tests were applied to samples of bursae of Fabricius from sick and dead birds from commercial goose farms in Hungary. In this second report of the occurrence of circovirus infection in diseased geese, 103 of 214 (48.1%) and 37 of 150 (24.6%) birds, and 49 of 76 (64.5%) and 18 of 76 (23.7%) flocks were positive by PCR and DBH respectively. The sensitivity of the PCR test was such that 0.10 fg of virus DNA was detectable. The DBH test was less sensitive, only detecting larger amounts (40 pg) of DNA, but was used as a semi-quantitative method for detecting the presence of virus. The incidence of infection was affected by factors such as the age of the birds and rearing methods.

## Introduction

A circovirus-like infection of geese was first described by Soike *et al.* (1999) in a large commercial flock in Germany with a history of increased mortality and runting. Birds were affected from the first weeks of life by growth retardation and developmental and feathering disorders. Histopathological investigations of affected birds revealed varying degrees of lymphocytic depletion and histiocytosis in the bursa of Fabricius (BF), spleen and thymus. Globular or coarse granular cytoplasmic inclusion bodies were detected in cells of the bursal follicles, which, on ultrastructural examination, were found to consist of paracrystalline, multilayered or randomly packed arrays of icosahedral virus particles. The histological and ultrastructural appearance of the inclusions suggested that the geese were infected with a circovirus as they were similar to those described in psittacine

birds infected with beak and feather disease virus (BFDV) (Ritchie *et al.*, 1989) and in pigeons infected with pigeon circovirus (PiCV) (Woods *et al.*, 1993). Microbiological investigations detected the presence of co-infections with agents such as *Reimerella anatipestifer* and *Aspergillus* species and the authors concluded that circovirus-induced immunosuppression was the cause of the losses.

Circoviruses are non-enveloped, spherical viruses, 15–25 nm in diameter, which contain single-stranded, circular DNA genomes, 1.7–2.3 kb in size (Todd, 2000, Todd *et al.*, 2001a). Todd *et al.* (2001b) recently reported the cloning and nucleotide sequence determination of the genome of a novel circovirus recovered from the geese investigated by Soike *et al.* (1999) and provisionally named it goose circovirus (GoCV). On the basis of high nucleotide sequence identity and common genomic features shared with the previously-characterised porcine circovirus types 1 and 2 (PCV1,

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PCV2) and BFDV, GoCV is likely to be classified, together with PiCV, in the genus *Circovirus* of the family *Circoviridae* (Todd *et al.*, 2001b).

To date there are no reports describing the isolation and propagation of GoCV in cell culture, and the diagnosis of infection is currently dependent on histology or electron microscopy, both of which require specialist expertise or equipment. However, the availability of cloned virus-specific DNA and nucleotide sequence information has now facilitated the development of diagnostic tests based on the detection of virus DNA. This paper describes the development of dot blot hybridisation (DBH) and polymerase chain reaction (PCR) tests for the detection of GoCV DNA and their application for diagnosing GoCV infections of commercially-reared geese from Hungary.

## Materials and Methods

### *Samples*

A total of 214 BF samples were collected from sick or dead birds that were submitted for investigation to the Central Veterinary Institute, Budapest, Hungary. BF samples were selected for testing for the presence of circovirus because this tissue frequently showed characteristic histopathological changes, with intracytoplasmic inclusion bodies containing circovirus-like particles (Soike *et al.*, 1999). The first batch of 51 samples, obtained from 22 flocks, was collected from August to November 2000. The second batch of 163 samples, from 54 flocks, was collected from December 2000 to May 2001, when the birds were reared under different conditions. Information about the clinical signs and laboratory diagnoses was analysed where available, and information was also provided on the age of the birds, the mortality rate, the size of the flocks, and location of some farms. BF tissue from birds in most flocks in Batch 2 was examined histologically. A 2-sided Fisher's Exact test was applied when investigating possible associations between the results of the PCR tests and clinical signs or diagnoses.

### *Cloned circovirus DNAs*

Recombinant plasmids containing genomic DNAs of GoCV, and PiCV (Todd *et al.*, 2001b), PCV1 (Meehan *et al.*, 1997), chicken anaemia virus (CAV; recombinant plasmid pCAA5) (Meehan *et al.*, 1992), and canary circovirus (CaCV) (Todd *et al.*, 2001c) were used, together with the plasmid vector pBluescript (Stratagene, La Jolla, California), which contained no insert. A recombinant plasmid containing a 1664 bp fragment of the BFDV genome (Bassami *et al.*, 1998) was kindly provided by Dr Shane Raidal (Murdoch University, Perth, Australia).

### *DNA extraction*

DNA was extracted from approximately 25 mg of each BF tissue sample using the QIAmp DNA Mini Kit (QIAGEN Ltd., West Sussex, United Kingdom), according to the manufacturer's directions. The extracted DNA was eluted twice with 50  $\mu$ l sterile distilled water to give a final volume of approximately 100  $\mu$ l of extract.

### *Dot blot hybridization*

A 12.5  $\mu$ l volume of the BF extract, and three 10-fold serial dilutions were prepared for each BF DNA sample. The four samples were denatured and immobilised on nylon membrane as previously described (Todd *et al.*, 1991), except that Hybond N+ nylon membrane (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, United Kingdom) was used and DNA was irreversibly bound by exposing the membrane to 1200  $\mu$ J/cm<sup>2</sup> ultraviolet (UV) light in a cross linker (UVP, Cambridge, UK; Todd *et al.*, 2002). Hybridizations were performed using a 565 bp radioactively-labelled probe prepared as follows. The

recombinant plasmid containing the genomic GoCV DNA was treated with the restriction endonuclease *Eco*RI (Boehringer Mannheim, East Sussex, UK) to release the 1.8 kbp GoCV fragment. Following fractionation by electrophoresis on a 1% agarose gel, this fragment was purified using a QIAex II gel extraction kit (QIAGEN Ltd), and a 500-fold dilution was used as template to amplify a 565 bp GoCV-specific product. The oligonucleotide primers, PCR and cycling conditions used to amplify the 565 bp product were as described for primer set 1 (see below), except that 1  $\mu$ l of template DNA was used in the reaction mix. Approximately 25 ng of this purified plasmid-free product was labelled with [<sup>32</sup>P] dATP by the Megaprime DNA labelling system (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, UK) and the hybridization was carried out as previously described (Todd *et al.*, 1991, 2002). Briefly this involved hybridization in 6  $\times$  sodium citrate solution (SSC), 5  $\times$  Denhardt's solution and salmon sperm DNA (500  $\mu$ g/ml) for 17 h at 68°C, followed by two washes in 2  $\times$  SSC for 15 min at 68 °C, one wash in 2  $\times$  SSC, 0.1% sodium dodecyl sulphate for 30 min at 68 °C, and a wash in 0.1  $\times$  SSC for 10 min at 68 °C.

The approximate amount of GoCV DNA present in each sample extract was assessed and scored, according to whether a hybridization signal was detected at a given dilution of the extract. Hence, hybridization at a dilution of 1/1000, 1/100, 1/10 and undiluted, were given scores of 4+, 3+, 2+, and 1+ respectively. Where no signal was detected, the sample was scored as 0. The limit of detection by the DBH assay was determined by hybridization of aliquots of serial 10-fold dilutions (in water) of a pre-determined amount of cloned GoCV genomic DNA. As positive controls, at least four 10-fold dilutions of cloned GoCV genomic DNA were included in each assay. The specificity of the DBH assay was investigated by testing serial 10-fold dilutions of purified plasmids containing DNA of BFDV, CAV, CaCV, PiCV or PCV1, as well as the plasmid vector pBluescript. The first sample in each case contained approximately 100 ng of virus DNA.

### *Polymerase chain reaction*

Three different pairs of primers were evaluated for use in the PCR test, using 17 samples that were shown to be circovirus-positive by the DBH test. The primers were designed following examination of the genomic sequences of PiCV (Accession no. AJ298229), BFDV (Accession no. AF080560), and CaCV (Accession no. AJ301633), to select regions where the degree of identity that GoCV shared with the other circoviruses was minimal. Primer set 1 (5' TAA ATG CGA GTT TGA TGT GTC T 3' and 5' CAT TTA ACC CCT TCC AAA GAG T 3') was designed to amplify a 565 bp fragment (nucleotides 837 to 1401). Primer set 2 (5' CCA GTT CGT TGC TAA GAC AC 3' and 5' AAC AGG AAA CCA ATA CCA TTA C 3') was designed to amplify a 693 bp fragment (nucleotides 780 to 1472). Primer set 3 (5' TTA ATC AGT ACC TGA TAT TAA CC 3' and 5' AAG ACA CCG CAG CAC CTC T 3') was designed to amplify a 217 bp product (nucleotides 876 to 1092), which was encompassed by primer sets 1 and 2. The PCR amplification reactions were carried out using the Taq PCR Master Mix Kit (QIAGEN Ltd) according to the manufacturer's instructions. The 50  $\mu$ l reaction mixtures containing 50 pmoles of each primer and 5  $\mu$ l of extracted sample DNA were incubated at 94°C for 2 min, and then subjected to 35 cycles of 94°C for 30 sec, 60 °C for 30 sec and 72°C for 1 min, followed by a further extension period of 72°C for 7 min and refrigeration. With some BF samples, PCR amplification was applied to a 10-fold dilution of the sample extract in addition to the undiluted extract.

The sensitivity of the PCR test using primer set 1 was assessed by amplification of 5  $\mu$ l aliquots of serial (10- fold) dilutions of a known quantity of cloned GoCV genomic DNA. Three consecutive dilutions close to the limit of detection were included in each PCR test to ensure that the expected sensitivity was being achieved. The specificity of the PCR test was investigated by testing the ability of primer set 1 to amplify products of expected size from purified plasmids containing approximately 1 ng of inserts of DNA of CaCV, PCV1, BFDV and PiCV.

*Agarose gel electrophoresis and Southern blot hybridization*

The PCR amplification products in 25 µl of each 50 µl reaction mixture were separated by electrophoresis in 1% (wt/vol) agarose gels and the DNA bands, stained with ethidium bromide (0.5 µg/ml), were visualized using UV light. For hybridization, electrophoretically-separated DNAs were transferred onto Hybond TM N+ nylon membrane (Amersham Pharmacia Biotech Ltd., United Kingdom) by Southern blotting. Hybridization was performed as described for DBH, except that the probe was prepared using the 1.8 kbp GoCV genome, which had been amplified from the GoCV recombinant plasmid using the primers and PCR conditions previously described (Todd *et al.*, 2001b).

**Results***Sensitivity and specificity of the dot blot hybridization test*

When 10-fold dilutions of cloned GoCV genomic DNA in water were used to evaluate sensitivity, the DBH test routinely detected samples containing 40 pg or greater of GoCV DNA, but not 4 pg of target DNA. With similar dilution series of cloned BFDV, CAV, CaCV, PiCV and PCV1 DNAs, positive DBH signals were obtained only when relatively large amounts (10 ng or greater) of virus DNA was present, thus demonstrating a degree of cross hybridization. No signal was detected from samples of plasmid vector DNA.

*Application of dot blot hybridization to bursa of Fabricius samples.*

Of the 51 first batch BF samples tested by DBH, 17 (33.3%) were positive. Of these, 8 samples were recorded as 3+ and 4+ and considered to be strong positives (Figure 1a). Of the 163 second batch BF samples, 99 were tested by DBH, and of these 20 (20.2%) were positive (Table 1). Twelve were considered to be strong positives. Comparison of the signals obtained from test samples with those obtained using dilutions of the control recombinant GoCV plasmid indicated that some test samples, such as number 31 of batch 1, which were recorded as 4+, were likely to contain about 800 ng of circovirus DNA in each 100 µl sample of DNA from the original tissue extract. Using the DBH test, GoCV-positive birds were identified in 9 of 22 (40.9%) flocks sampled in the first batch, and in 9 of 54 (16.7%) flocks sampled in the second batch (Table 1). GoCV was detected in 37 of 150 (24.6%) samples tested by DBH.

*Selection of primers for polymerase chain reaction test*

Three pairs of primers, sets 1, 2 and 3, were tested for their abilities to amplify products of the expected size from first batch samples that were found to be circovirus-positive by DBH testing. Of the 17 first batch BF samples tested, 17 gave PCR products of the expected size with primer set 1, 15 gave bands of the correct size with primer set 2, and only 12 samples gave bands of the expected size

with primer set 3. On this basis, primer set 1 was selected for use in the PCR test for this investigation (Figure 1b). This primer set amplified a fragment that encompasses the 3' terminus of the rep gene, the 3' intergenic region, and the 3' region of the capsid protein coding gene (Todd *et al.*, 2001b). Southern blot hybridization confirmed that the 565 bp PCR products that were amplified from DNAs extracted from a selection of the BF samples by primer set 1 PCR were circovirus-specific.

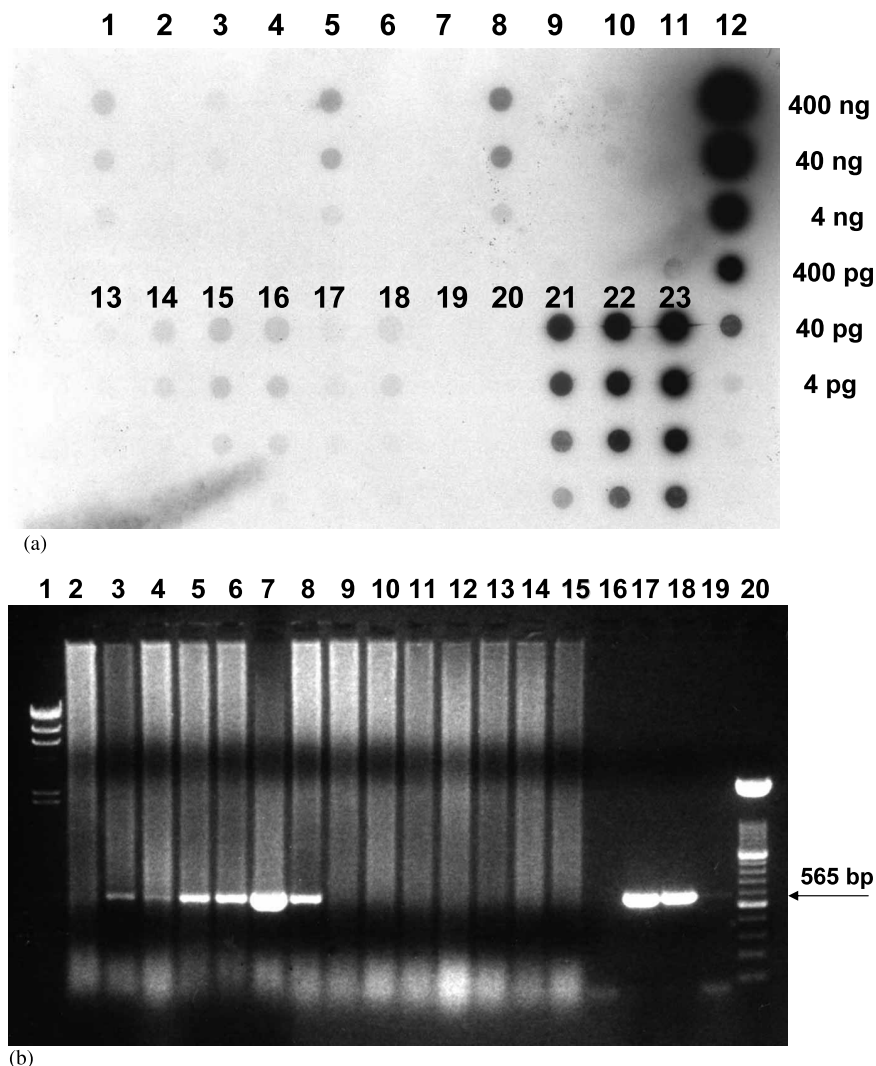
*Sensitivity and specificity of polymerase chain reaction test*

Using primer set 1, amplification of serially diluted (10-fold) cloned GoCV genomic DNA resulted in visible bands of the correct size being detected in agarose gels after ethidium bromide staining when as little as 0.10 fg of the DNA target sequence was used in the amplification mixture, but not when the next dilution, 0.01 fg, was used. Using dilution series prepared in water, PCR was approximately 10<sup>5</sup> times more sensitive than DBH. When the ability of primer set 1 to amplify products from plasmids containing approximately 1 ng of inserts of CaCV, PiCV, BFDV or PCV1 was tested no bands were visible with CaCV, PCV1, and PiCV DNA, but a very faint band of the correct size was observed with BFDV DNA, indicating some degree of similarity.

*Application of polymerase chain reaction test to samples*

When PCR amplification using primer set 1 was performed on the 51 first batch BF samples, clearly visible bands of 565 bp were detected in 28 (54.9%) samples. A further 8 samples (13.7%) were regarded as inconclusive as the PCR product appeared as a smear that extended from high molecular weight to the position of the expected 565 bp band. One sample was confirmed as positive when Southern blot hybridization was applied to DNAs amplified from the undiluted extract. Application of PCR to 10-fold dilutions of the remaining 7 sample extracts resulted in the detection of faint but visible bands of the expected 565 bp size. An additional twenty-seven first batch samples (which included those found to be negative) were further tested by PCR using diluted samples. With 14 samples, positive PCR products were obtained using both undiluted and diluted extracts. Twelve samples were negative using undiluted and diluted extracts. One sample was positive by PCR when undiluted extract was tested, but was negative by PCR when a diluted extract was used.

In total, 36 (70.6%) first batch samples were positive by PCR (Table 1). PCR testing of the 163 second batch samples using undiluted samples only resulted in 67 (41.1%) being recorded as positive.



**Figure 1.** (a). Detection of GoCV DNA in BF samples by DBH. For each BF extract, undiluted and three serial 10-fold dilutions were dotted onto membrane and hybridized with the  $^{32}\text{P}$ -labelled GoCV-specific probe. Lanes 1 to 11 and 13 to 23 contained BF DNA from goose sample numbers 2, 3, 27, 28, 30, 32, 38, 40, 48, 44, 47, and 43, 1, 4, 5, 6, 7, 8, 21, 31, 39, 42 respectively. Lane 12 contained a 10-fold dilution series of recombinant GoCV genomic DNA plasmid, the first sample containing approximately 400 ng of GoCV DNA. (b). Detection of GoCV DNA by primer set 1 PCR amplification in BF samples. Products were visualized by ethidium bromide staining following agarose gel electrophoresis. Lane 1 contained fragments of lambda phage DNA digested with Hind III included as size standards (Invitrogen). Lanes 2 to 15 contained PCR products amplified from BF DNAs from goose numbers 1 to 14 respectively. For control purposes lane 16 contained PCR mixture with distilled water, and lanes 17 to 19 contained PCR products amplified from serial 10-fold dilutions of recombinant GoCV genomic plasmid. Lane 20 contained 100 bp DNA ladder included as size standards (Boehringer Mannheim). The position of the 565 bp PCR product is shown.

Overall GoCV was detected by PCR in 103 of 214 (48.1%) samples tested. GoCV-positive PCR reactions were detected in samples from 20 of 22 (90.9%) flocks in the first batch, and 29 of 54 (53.7%) flocks in the second batch. All samples that were positive by DBH were positive by PCR.

#### *Clinical findings, laboratory diagnoses and histology and their relationship to PCR and DBH results.*

The clinical history was available for 33 flocks, all of which were within batch 2. Clinical signs included lameness, stunting, respiratory conditions, neurological signs and general malaise. Lameness

**Table 1.** PCR and DBH results for two different batches of geese.

Test	Batch 1		Batch 2		Total samples	Total flocks
	Samples	Flocks	Samples	Flocks		
PCR +ve	36/51 (70.5%)	20/22 (90.9%)	67/163 (41.1%)	29/54 (53.7%)	103/214 (48.1%)	49/76 (64.5%)
DBH +ve	17/51 (33.3%)	9/22 (40.9%)	20/99 (20.2%)	9/54 (16.7%)	37/150 (24.6%)	18/76 (23.7%)

**Table 2.** Laboratory diagnoses in GoCV-positive and GoCV-negative flocks.

	DBH +ve	DBH-ve/ PCR+ve	PCR +ve	PCR -ve
Reovirus	6	11	17	4
Polyomavirus	1	1	2	1
Derzsy's disease virus	2	2	4	3
Reticuloendotheliosis virus	0	2	2	0
Salmonellosis	1	1	2	5
Streptococcosis	0	4	4	2
Pasteurellosis	1	1	2	0
Pseudomoniasis	0	1	1	0
Mycoplasmosis	2	1	3	1
Candidiasis	2	0	2	0
Pneumomycosis	2	3	5	2
Cryptosporidium spp.	0	1	1	0
Erysipelas	1	0	1	0
Nephrosis	3	0	3	1
Enteritis	0	2	2	3
Pericarditis	0	1	1	0
Ricketts	0	4	4	5
Poisoning	0	1	1	0
Suffocation	0	0	0	1
No. of flocks with laboratory diagnosis	16	28	44	27

and stunting were the most common, and were observed in 21 and 7 flocks respectively. There were no associations between the occurrence of these or other clinical signs and GoCV infection.

Laboratory findings other than GoCV results were available for 71 flocks (Table 2). These included the diagnosis of reovirus, parvovirus and polyomavirus infections, and a range of bacterial diseases including salmonellosis, streptococcosis and pasteurellosis, as well as mycoses, mycoplasmosis and presence of *Cryptosporidium* spp. Ricketts was diagnosed in 9 flocks. Most of the infectious agents identified could be found in both GoCV-positive and GoCV-negative flocks, and at a similar prevalence in both types of flock, with the exception of reovirus infection and salmonellosis (Table 2). Of 21 flocks where reovirus infection was diagnosed, 17 flocks provided samples that were GoCV-positive. The prevalence of reovirus in GoCV-positive flocks (38.6%) was significantly higher ( $P < 0.05$ ) than that in GoCV-negative flocks (14.8%). However, the prevalences of salmonellosis in GoCV-positive flocks (4.5%) and GoCV-negative flocks (18.5%) were not significantly different.

Histological examination was carried out on the BF of birds from 52 flocks. Bursal lesions (mainly lymphocyte depletion) were found in birds from 75% of GoCV-positive flocks and from 50% of GoCV-negative flocks. However, inclusion bodies suggestive of circovirus were only found in birds from 3 flocks. These 3 flocks were positive for GoCV by both DBH and PCR. The clinical and diagnostic information relating to those geese that were GoCV-positive by DBH, and therefore likely to be more heavily infected with GoCV (see

Discussion), was closely examined for any association between GoCV infection and disease. The 37 DBH-positive samples were collected from 17 flocks. Of the 9 DBH-negative samples collected from these flocks, 7 were positive by PCR. In eleven flocks, high (2+, 3+, 4+) DBH scores were observed for samples from more than one of the birds sampled. The range of clinical conditions (except enteritis), co-infecting microbial pathogens, and histological findings described above were observed in DBH-positive birds. Since similar clinical signs and laboratory diagnoses were recorded for geese that were GoCV-negative (by DBH and PCR), no obvious association between DBH-positivity and other infectious conditions was apparent. Non-infectious diseases (rickets, poisoning and suffocation) were diagnosed in 11 flocks. Approximately half of these flocks were shown to be infected with GoCV, but none were positive by DBH. Ricketts occurred in 18.5% of circovirus negative flocks, compared to 9.1% of circovirus positive flocks.

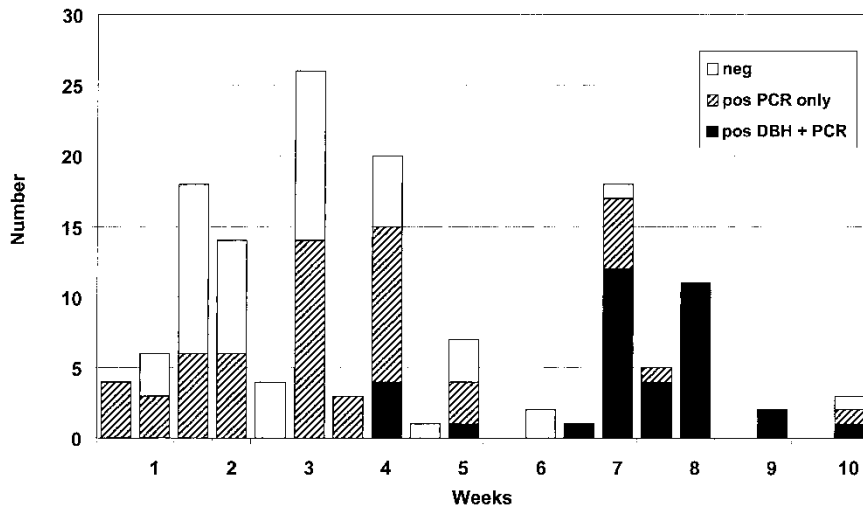
With the exception of the circovirus-like inclusion bodies found in birds from three infected flocks, similar histopathological findings were made in both DBH-positive and DBH-negative birds. Histological lesions were present in the BF from 88.9% of flocks from which DBH-positive samples were obtained, 68.4% of flocks from which DBH-negative, PCR-positive birds were obtained, and in 50% of flocks that were negative by PCR.

#### *Relationship of PCR and DBH results to age.*

The age distribution of the 145 birds that were tested for GoCV is shown in Figure 2. Forty four (84.6%) of the 52 birds that were negative by PCR were less than 5 weeks old. The 93 PCR-positive birds ranged from 3 days to 10 weeks of age, with 51 (54.8%) being less than 5 weeks, and 42 (45.1%) being 5 weeks or older. DBH-positive reactions were detected in birds as young as 4 weeks old, with the majority (30) of the 36 birds that tested positive by DBH being 7 weeks or older. All the birds that yielded samples with a 4+ score were at least 7 weeks old.

## **Discussion**

The application of DNA based detection methods has shown that GoCV infections are prevalent in diseased geese in Hungary. Infection was found in 48% of 214 birds and 65% of 76 flocks tested by PCR. Previously, there has been only one report of the occurrence of this infection, in Germany, but it may be that GoCV infection will be detected in goose-rearing countries worldwide. Infections with other circoviruses, for example BFDV (Gerlach, 1994), PCV2 (Allan & Ellis 2000), and PiCV (Woods *et al.*, 1994, Smyth & Carroll, 1995, Gough



**Figure 2.** Age distribution of birds tested for GoCV.

& Drury, 1996, Duchatel *et al.*, 1998, Coletti *et al.*, 2000, Soike *et al.*, 2001) are highly prevalent and widespread. The development of a serological test will be required to accurately determine the prevalence of GoCV infections, but this has been hindered mainly because GoCV has not yet been propagated in cell culture.

In the absence of serological tests, PCR is useful for detecting the presence of infection and has enabled the generation of data on the prevalence of infection in geese flocks in Hungary. Our results indicate that it could be applied usefully to goose flocks in other countries. However, although the high sensitivity of the PCR test is useful in detecting the presence of infection, this may lead to limitations in its use as an indicator of disease. The high sensitivity of PCR may also lead to false positives as a result of the PCR amplifying contaminating DNA, carried from one sample to another during necropsy or sample processing. False PCR-negative results may also be recorded for two reasons. In the first instance, the PCR primers may not amplify products where the sequences of the primers are not exactly identical to the genomic sequences of the GoCV isolates present in the samples. The difference in the abilities of the three primer sets tested in this study to amplify products of the expected size from known positive samples suggests there may be sequence diversity among GoCV isolates. Sequence analyses of additional isolates of GoCV from different geographical locations will be required to determine the extent of this variation and enable universal primers to be designed from conserved regions. Such sequence diversity is known to exist in PiCV isolates (Todd *et al.*, 2002). Secondly, the PCR test may not amplify target DNA in the presence of high levels of inhibitory contaminants. Nonetheless, the GoCV PCR test should prove valuable in diagnosing the presence of infection and in studies on the pathogenesis of the disease.

The DBH test does not have the technical drawbacks outlined above for PCR, but was found to be significantly less sensitive than the PCR test in detecting GoCV-positive samples. This reduced sensitivity may be advantageous in a test for diagnosing circovirus-associated disease as opposed to infection, since it has been shown in the case of post weaning multisystemic wasting syndrome caused by PCV2 that high levels of virus DNA correlate with the development of severe clinical disease (Rovira *et al.*, 2002). However, this has yet to be demonstrated with PiCV and GoCV by experimental reproduction of the disease. Any cross-contamination between samples is unlikely to present a problem with DBH, and the DBH test is not likely to be affected by minor sequence variation between isolates. The detection of virus DNA in BF samples by DBH indicates that comparatively high levels of virus were present in some birds. It is estimated that the presence of DBH-detectable DNA in extracts that were diluted 1000-fold signifies that virus DNA is present in some BF tissue samples at concentrations greater than 3 µg or 10<sup>12</sup> gene copies per g BF tissue. There are reports of detection of similar large amounts of PiCV DNA in BF samples from affected pigeons (Todd *et al.*, 2002). If high virus load is considered to be an indicator of disease, the DBH test may provide a convenient semi-quantitative method for demonstrating the presence of disease, rather than sub-clinical infection. Alternatively, virus DNA could be measured with greater precision using real-time PCR methods, although the costs involved may be prohibitive.

The results relating to the age distribution of the birds tested for GoCV are presented in Figure 2. Our finding that most (84.6%) birds that were negative by these DNA-detecting tests were less than 5 weeks of age suggests that protection against GoCV infections may be conferred by maternally-derived antibody (MDA), which might be expected to last until 3 to 4 weeks post-hatching. However, a

substantial proportion (55 of 103) of the birds of 5 weeks and younger were positive for GoCV by the PCR test, suggesting that MDA may not be present at protective levels in some birds. The detection of GoCV in birds of 1 week old or less raises the possibility that vertical transmission of the virus is possible. Of the samples analysed in this investigation, most (38 of 42) birds over 5 weeks of age were GoCV-positive by PCR. Of these 31 (73.8%) were positive by DBH. Our finding that most (31 of 36) of the birds positive for GoCV by the DBH test were older than 5 weeks suggests that the infections with this virus may be progressive, involving the production of increasing amounts of virus over a period of several weeks following the disappearance of MDA.

The difference in the proportions of positive and negative flocks between the two batches of samples may result from the birds being reared in different ways. The first batch samples, which showed a higher prevalence of GoCV infection by both PCR and DBH, were collected from August to November when the parent flocks and goslings were kept mainly in houses, and egg production was induced by artificial lighting. The second batch samples were collected from December to May, over the period in which the natural production cycle for geese occurs, when goslings were not confined to houses after 3 weeks of age. In this more natural outdoor rearing system the incidence of cross infection by the virus, and the stress levels of the birds due to overcrowding, may be lower compared to those in geese reared indoors.

While this paper provides useful information about the prevalence of GoCV infections, accurate assessments of the prevalence of circovirus-associated disease could not be made as other agents and syndromes were present in the flocks. No particular clinical history or other laboratory finding could be specifically associated with circovirus infection. In their report, Soike *et al.* (1999) concluded, on the basis of histopathological changes including lymphocyte depletion caused by circovirus, that a circovirus-induced immunosuppression was responsible for the growth retardation and increased mortality observed in the German goose-rearing farm investigated. However, in the present study, lymphocyte depletion was also observed in some GoCV-negative flocks. The detection of DBH-positive birds, particularly those which were scored 2+, 3+ and 4+, indicates that some birds were heavily infected with GoCV. On the basis of similar studies with PiCV and PCV2, it is very likely that these heavily-infected geese may be immunosuppressed leading to the disease scenario described by Soike *et al.* (1999).

In conclusion, a high incidence of GoCV infection has been demonstrated in diseased goose flocks in Hungary, but the role of GoCV as a causative agent of the problems encountered in

these farms has not been established. Experimental infection with GoCV under controlled conditions is needed in order to study the pathogenesis of infection, to determine the pathogenicity of the virus, and to determine any effect on various concurrent infections. Further work is also required to determine the levels of GoCV infection in goose-rearing countries worldwide. The outcome of such investigations would determine whether there is a requirement for vaccine development.

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## RÉSUMÉ

### Diagnostic de l'infection par le circovirus de l'oie, à partir d'échantillons d'oies hongroises, en utilisant la réaction d'amplification en chaîne par polymérase et un test de dot-blot hybridation

Une réaction d'amplification en chaîne par polymérase (PCR) et un test de dot-blot hybridation (DBH) ont été développés pour le diagnostic de l'infection à circovirus de l'oie (GoCV). Ces tests ont été appliqués à des échantillons de bourse de Fabricius d'oiseaux morts ou malades appartenant à des élevages d'oies en Hongrie. Dans cette deuxième publication, l'infection à circovirus chez les oies malades a été détectée à partir : — d'échantillons soit par PCR [103+/214, (48,1%)], soit par DBH [37+/150, (24,6%)] et — d'élevages soit par PCR [49+/176, (64,5%)], soit par DBH [18+/176, (23,7%)]. La sensibilité de la réaction PCR a été telle que 0,10 fg de virus a été détecté. Le test DBH a été moins sensible détectant des quantités supérieures à 40 pg de DNA mais a été utilisé comme une méthode semi-quantitative pour détecter la présence du virus. L'incidence de l'infection a été affectée par des facteurs tels que l'âge des animaux et les méthodes d'élevage.

## ZUSAMMENFASSUNG

### Diagnose einer Gänse-Circovirusinfektion in Proben von ungarischen Gänsen mit Hilfe von Polymerasekettenreaktions- und Dot-Blot-Hybridisierungstests

Ein Polymerasekettenreaktions (PCR)- und ein Dot-Blot-Hybridisierungstest wurden für die Diagnose der Infektion mit einem neuen Circovirus bei Gänsen (GoCV) entwickelt. Diese Tests wurden bei Proben der Bursa Fabricii aus erkrankten und gestorbenen Tieren von kommerziellen Gänsefarmen in Ungarn angewendet. In diesem zweiten Bericht über das Vorkommen von Circovirusinfektionen in erkrankten Gänsen waren 103 von 214 (48,1%) bzw. 37 von 150 (24,6%) der Tiere und 49 von 176 (64,5%) bzw. 18 von 176 (23,7%) der Herden positiv in der PCR oder der DBH. Die Sensitivität der PCR war so, dass 0,10 fg Virus-DNS nachgewiesen werden konnten. Der DBH-Test war weniger sensitiv, da er nur größere DNS-Mengen (40 pg) entdecken konnte, aber er wurde als semi-quantitative Methode für den Nachweis einer Viruspräsenz eingesetzt. Das Auftreten der Infektion wurde von Faktoren wie dem Alter der Tiere und der Aufzuchtmethode beeinflusst.

## RESUMEN

### Diagnóstico de la infección con circovirus del ganso en muestras de gansos húngaros mediante las técnicas de reacción en cadena de la polimerasa y de hibridación por dot blot

Se han desarrollado una técnica de reacción en cadena de la polimerasa (PCR) y de hibridación por dot blot (DBH) para diagnosticar la infección por un nuevo circovirus del ganso (GoCV). Estas técnicas se utilizaron en muestras de bolsas de Fabricio de aves enfermas y muertas en granjas de gansos en Hungría. En este segundo caso de infección por circovirus en gansos enfermos, 103 de 214 (48,1%) y 37 de 150 (24,6%) aves, y 49 de 176 (64,5%) y 18 de 176 (23,7%) de los lotes resultaron positivos por PCR y DBH respectivamente. La sensibilidad de la técnica de PCR fue tal que detectaba 0.10 fg de ADN. La técnica de DBH fue menos sensible, únicamente detectaba grandes cantidades (40 pg) de ADN, pero fue utilizada como una técnica semicuantitativa para detectar la presencia del virus. La incidencia de la infección se vio afectada por factores tales como la edad de las aves y el método de cría.