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Short communication

Detection of a novel circovirus in mute swans (*Cygnus olor*) by using nested broad-spectrum PCR

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Abstract

Circoviruses are the causative agents of acute and chronic diseases in several animal species. Clinical symptoms of circovirus infections range from depression and diarrhoea to immunosuppression and feather disorders in birds. Eleven different members of the genus *Circovirus* are known so far, which infect pigs and birds in a species-specific manner. Here, a nested PCR was developed for the detection of a broad range of different circoviruses in clinical samples. Using this assay, a novel circovirus was detected in mute swans (*Cygnus olor*) found dead in Germany in 2006. Sequence analysis of the swan circovirus (SwCV) genome, amplified by multiply primed rolling-circle amplification and PCR, indicates that SwCV is a distinct virus most closely related to the goose circovirus (73.2% genome sequence similarity). Sequence variations between SwCV genomes derived from two different individuals were high (15.5% divergence) and mainly confined to the capsid protein-encoding region. By PCR testing of 32 samples derived from swans found dead in two different regions of Germany, detection rates of 20.0 and 77.3% were determined, thus indicating a high incidence of SwCV infection. The clinical significance of SwCV infection, however, needs to be investigated further.
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The members of the family *Circoviridae* are non-enveloped icosahedral particles with a diameter of 17–26.5 nm and a circular single-stranded DNA genome, approximately 2 kb in size (Todd et al., 2005). Currently, the family *Circoviridae* comprises the two genera *Gyrovirus* and *Circovirus*. The genus *Gyrovirus* contains the chicken anaemia virus as the only member (Noteborn et al., 1991). Within the genus *Circovirus*, 11 different viruses have been described so far. These include the two porcine circoviruses PCV1 and PCV2 (Meehan et al., 1997, 1998), as well as the circoviruses infecting several bird species: psittacine beak and feather disease virus (BFDV; Bassami et al., 1998; Niagro et al., 1998), canary circovirus (CaCV; Phenix et al., 2001), pigeon circovirus (PiCV; Mankertz et al., 2000; Todd et al., 2001b), goose circovirus (GoCV; Todd et al., 2001b), duck circovirus (DuCV; Hattermann et al., 2003), raven circovirus (RaCV; Stewart et al., 2006), starling circovirus (StCV; Johne et al., 2006), and the recently identified circoviruses infecting finch and gull (FiCV and GuCV; Todd et al., 2007).

The circovirus infections of birds have been linked to acute and chronic diseases with clinical symptoms ranging from depression and diarrhoea to immunosuppression and feather disorders (Todd, 2004; Raue et al., 2005). PCV2 is the causative agent of the postweaning multisystemic wasting syndrome and is associated with the porcine dermatitis and nephropathy syndrome (Chae, 2005), whereas PCV1 could not be associated with overt clinical disease (Allan and Ellis, 2000).

The viruses in the genus *Circovirus* possess an ambisense genome organization encoding the replication-associated (Rep) protein from the virus sense strand (open reading frame [ORF]-V1) and the capsid protein from the complementary sense strand (ORF-C1). Additional small ORFs have been recognized in some of the circoviruses (Todd et al., 2001b; Niagro et al., 1998; Johne et al., 2006). A stemloop structure containing a conserved nonamer sequence and involved in the initiation of the viral genome replication (Steinfeldt et al., 2001) is found in the genomes of all members of the genus *Circovirus*. Most

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of the circovirus species have been discovered by the application of different PCR assays targeting the ORF-V1 followed by genome amplification using inverse PCR (Todd et al., 2001a, 2007). In the case of starling circovirus, multiply primed rolling-circle amplification (RCA) has been applied for the detection and amplification of its genome (Johne et al., 2006).

In this communication, a nested broad-spectrum PCR was developed which should enable the sensitive detection of a broad range of different circoviruses including so far unknown circoviruses. Primer sequences have been selected by alignment of genome sequences of 11 circovirus species using the MegAlign module of the DNASTAR software package (Lasergene, USA). Primers Cv-s 5′-AGA GGT GGG TCT TCA CNH TBA AYA A-3′ and Cv-as 5′-AAG GCA GCC ACC CRT ARA ART CRT C-3′ were used for the first round of PCR, and primers Cn-s 5′-AGC AAG GAA CCC CTC AYY TBC ARG G-3′ and Cn-as 5′ACG ATG ACT TCN GTC TTS MAR TCA CG-3′ were used in the nested PCR, targeting the Rep-encoding region. The PCRs were performed using Taq DNA polymerase (PeqLab, Germany) and buffer Y (PeqLab) in a 25 μl reaction on a Thermal Cycler PTC-200 (MJ Research, USA). The optimized cycling protocol for the first round of PCR included 5 min of incubation at 95 °C, followed by 45 cycles each consisting of 94 °C for 30 s, 46 °C for 1 min and 72 °C for 1 min, and a final incubation at 72 °C for 5 min. The cycling protocol for the nested PCR was identical; however, an annealing temperature of 56 °C was used in this case.

DNA was isolated from organs of animals naturally infected with BFDV, PiCV, StCV, GoCV, and PCV2 using the DNeasy Tissue Kit (Qiagen, Germany) and subsequently tested by the nested broad-spectrum PCR. In all cases, a prominent band with the expected size of approximately 350 bp was observed after electrophoresis of the nested PCR products (Fig. 1A). Additional bands were also observed, however, their intensity was low as compared to the specific band. To demonstrate specificity, all of the bands at a position of 350 bp as well as selected bands at other positions were cloned into the vector pCR2.1 using the TOPO TA Cloning Kit (Invitrogen, Germany) and subsequently sequenced (not shown). In all cases, the 350 bp product sequences revealed similarities with ORF-V1-sequences of circoviruses by a GenBank database search using the BLASTN facility. In contrast, most of the sequences of bands with higher or lower molecular mass showed homologies to DNA sequences of mammalian, avian or bacterial species and were therefore considered as unspecific amplification products. In case of the BFDV- and PiCV-containing sample, a 0.6 kbp band was identified as the circovirus-specific product of the first round of PCR.

Samples derived from mute swans (*Cygnus olor*) found dead during an epidemic of sudden death in Germany in 2006 were also tested by the nested broad-spectrum PCR. DNA was extracted from approximately 25 mg of organ tissue (mixture of liver and spleen) using the DNeasy Tissue Kit (Qiagen, Germany). After electrophoresis of the secondary PCR products, a band with the expected size was detected in eight out of nine samples tested (Fig. 1B). The secondary PCR products of two samples were cloned and sequenced as above. Both sequences

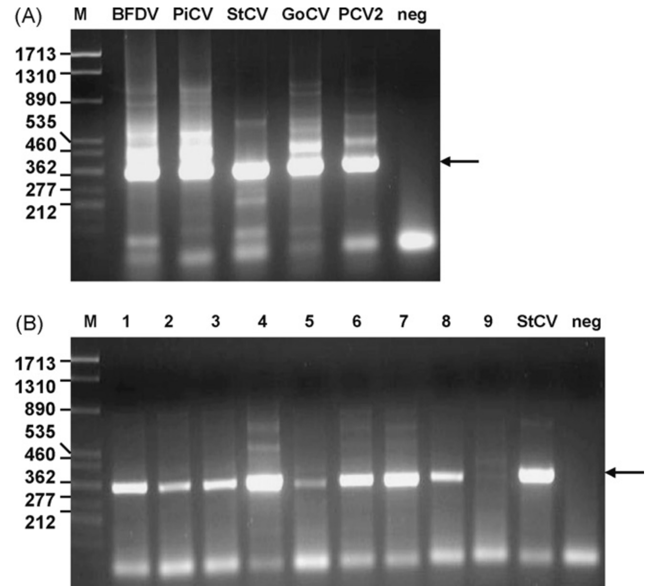

Fig. 1. Nested broad-spectrum PCR for the detection of circoviruses. Secondary PCR products obtained with DNA isolated from (A) animal organs infected with beak and feather disease virus (BFDV), pigeon circovirus (PiCV), starling circovirus (StCV), goose circovirus (GoCV) and porcine circovirus-2 (PCV2), or (B) organ samples of mute swans (1–9) found dead in Germany. M, molecular mass markers, indicated at the left, in bp; neg, negative control. The PCR products were separated on ethidium bromide-stained 2% agarose gels. The arrow indicates the position of the specific PCR product.

showed a similarity of 97.6% to each other. A search of the GenBank database using the BLASTN facility revealed similarities with ORF-V1-sequences of several circoviruses, but no identity with one of these. The novel circovirus thus identified was tentatively designated as swan circovirus (SwCV). Samples derived from 32 mute swans found dead in two regions of Germany and originally collected for a survey of avian influenza virus infections were screened using the nested broad-spectrum PCR. By this, SwCV was detected in 17 out of 22 samples (77.3%) from Saxony, and in 2 out of 10 samples (20.0%) from Mecklenburg-Western Pomerania (not shown). Two of the samples from Mecklenburg-Western Pomerania had been previously shown to contain highly pathogenic avian influenza virus using an RT-PCR specific for the influenza A virus M protein-encoding region. One of these samples was also positively tested for SwCV.

Based on the determined sequence, two primers (5′-GGG CAT GCA CGC CCT GAA GGC A-3′ and 5′-GTG CAT GCC CTT GTG ATA TTT G-3′) were constructed for the amplification of the remaining genomic sequences by inverse PCR. However, a specific PCR product could not be detected in any of the eight samples tested (not shown). Therefore, the multiply primed rolling-circle amplification (RCA) was applied to the DNA preparations as described by Johne et al. (2006) to amplify any circular DNA before use in PCR. Briefly, the TempliPhi 100 amplification kit (Amersham Biosciences, UK) was used with 1 μl of extracted DNA and 5 μl of TempliPhi sample buffer supplemented with 450 μM dNTPs for an initial denaturation at 95 °C for 3 min. After cooling on ice, 5 μl of TempliPhi reaction buffer and 0.2 μl of TempliPhi enzyme

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