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Detection of virus particles resembling circovirus and porcine circovirus 2a (PCV2a) sequences in feces of dogs

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ABSTRACT

During routine electron microscopy of fecal samples from diarrheic dogs dated from 2000 virus particles resembling circovirus in shape and size were detected in two samples (V2177/00; V3374/00). Polymerase chain reaction (PCR) using primers specific for porcine circovirus type 2 (PCV2) amplified DNA recovered from both samples. Sequencing of PCR amplificates (V2177/00) obtained with PCV2-specific primer pairs revealed a genome size of 1768 bp. The nucleotide sequence was highly similar (98% nucleotide identity) to the PCV2a reference sequence.

Circoviruses (CV) are 15–20 nm large icosahedral virus particles harboring a circular single stranded DNA of about 1.7–2.3 kb. Taxonomically, they belong to the family *Circoviridae* which also comprises the proposed genus *Cyclovirus* and the genus *Gyrovirus* (Li et al., 2010). First evidence of their existence was their detection in a porcine kidney cell line (PK15) leading to the name porcine circovirus type 1 (Tischer et al., 1974). More than two decades later a second porcine circovirus, PCV2, was detected and made responsible for a couple of syndromes including post weaning multisystemic wasting syndrome, porcine dermatitis and nephropathy syndrome and PCV2-associated reproductive failure which were summarized later under the so-called porcine circovirus associated disease (PCVAD) (Limsaranrom et al., 2015; Meng, 2012; Pearodwong et al., 2015; Shin et al., 2015). Currently four genotypes namely PCV2a, PCV2b, PCV2c and PCV2d-mPCV2b are recognized (Franzo et al., 2015). Numerous circoviruses also exist in birds, the most important of which are psittacine beak and feather disease virus and pigeon circovirus (Todd, 2004). Due to the increasing availability and use of deep sequencing techniques other circovirus sequences have been identified in environmental specimens and were recovered also from fish, arthropods and amphibians (Blinkova et al., 2009; Lőrincz et al., 2012; Rosario et al., 2009, 2011; Tarjan et al., 2014). In canines a circovirus (DogCV) has been described first in 2012 (Kapoor et al., 2012). It was found in a serum sample from an apparently healthy dog. A related DogCV was demonstrated a short time later and suspected to be associated with necrotizing vasculitis and granulomatous lymphadenitis in dogs (Li et al., 2013). In Germany a DogCV has been mentioned first in 2015 (Gentil, 2015). We herein report the detection of circovirus and PCV2a sequences in feces of dogs from 2000.

Fecal samples from diarrheic dogs sent to our laboratory by veterinary practitioners were routinely checked by electron microscopy for viruses (Herbst and Krauss, 1988). Samples were suspended 1:10 (w/v) in Eagle's Minimal Essential Medium (Biochrom, Berlin). After clarification at 1,000 ×*g* for 5 min the supernatant was concentrated by ultracentrifugation for 1 h at 200,000 ×*g* (Beckmann L5V). A droplet of the suspended pellet was then adsorbed onto formvar coated copper grids (Plano GmbH, Wetzlar, Germany) for 15 min and subsequently stained with phosphor tungstate acid for 1 min. The grids were allowed to air dry and were then examined by electron microscopy using a Zeiss EM 10/CR.

DNA was extracted with the Qiagen Viral RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Resulting DNA was eluted in 60 μl of elution buffer and stored at −20 °C until use. PCV2-specific DNA was amplified with primers PCV-2IS and PCV-2IAS (Larochelle et al., 2000) in a Tgradient thermal cycler (Biometra, Goettingen, Germany). The total PCR volume was 20 μl consisting of 10 μl of QIAamp Multiplex Master Mix (Qiagen, Hilden, Germany), 2 μl of each primer (final concentration 0.2 μM) and 2 μl of extracted DNA. PCR was performed with the following cycling conditions: DNA polymerase activation for 15 min at 95 °C followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 90 s and an extension at 72 °C for 30 s. PCR products were analysed on 1.5% agarose gels stained with ethidium bromide (0.4 μg/ml), photographed and stored as digital image.

For sequencing, the PCV2 genome was amplified in two PCRs generating amplicons of 824 and 1174 bp, respectively, with primers PCV2-1f

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(5′-GCAGCACCTCGGCAGCACCTC-3′), PCV2-1r (5′-GAGGAGTACCATTC CAACGGGGTCTG-3′), PCV2-2f (5′-TGGT(G/T)GTTATTGATGACTTTT ATGG-3′) and PCV2-2r (5′-GTTGGGGTCCGCTTCTTC-3′). Sequencing of amplicons was done by LGC Genomics (Berlin, Germany). PCR conditions were the same as above, only the annealing temperature was 61 °C and extension time was 1 min. Sequences were visualized and edited with ApE (A plasmid Editor, Wayne Davis, University of Utah, Version 1.17; http://biologylabs.utah.edu/jorgensen/wayned/ape/). Alignment of sequences was performed with ClustalX (Version 2.0, Conway Institute UCD, Dublin, www.clustal.org). All other sequence manipulations were done with BioEdit (Version 7.0.5.3, http://www.mbio.ncsu.edu/BioEdit/page2.html). The nucleotide sequence has been deposited in GenBank with the accession number KX352445.

Round non-enveloped virus particles of about 15 nm in diameter were detected by electron microscopy in two fecal samples (V2177/00 and V3374/00) out of a number of altogether 417 samples of diarrheic dogs examined for possible enteric viruses in 2000. Both samples were from patients aged three and 4 months suspected for canine parvovirus infection. Further details were not provided. As shown in Fig. 1A and B full and empty virus particles were visible. They were composed of a moderate number of capsomers giving the surface of the nucleo-capsids an irregular appearance. Based on their shape and size they were identified as circoviruses. There have been no further cases where virus particles resembling those in Fig. 1 could be clearly identified. Apart from this unexpected finding coronavirus particles were additionally detected in sample V2177/00. No other viruses were observed in V3374/00. Studies focusing on bacterial and parasitic pathogens were not performed.

DNA extracted from both samples was amplifiable with PCV2-specific primers. To confirm that it may be PCV2 the whole circovirus genome of sample V2177/00 was amplified by two PCRs generating overlapping fragments that were then sequenced. Concatenated sequences had a size of 1768 bp that is concordant with the size of PCV2. Comparison with sequences deposited in GenBank confirmed it to be PCV2 with an identity of 98% to the PCV2a reference sequence (acc. no. AF055392). All subsequent comparisons refer to that sequence. Most of the single nucleotide polymorphisms (SNPs; n = 47 in the complete genome) were located in the capsid gene (n = 31) resulting in a sequence identity of 95.6% for the capsid gene. Eleven of the SNPs were non-synonymous causing amino acid (aa) substitutions at following positions (p.): p.12F > Y, p.47A > T, p.59A > R, p.63S > T, p.72L > M, p.75N > K, p.76L > I, p.169R > S, p.180K > R, p.191A > G, and p.200T > A. Ten of the aa substitutions altered the aa composition of known epitopes in the regions 47–63, 69–83, and 165–200 (Mahé et al., 2000; Lekcharoensuk et al., 2004).

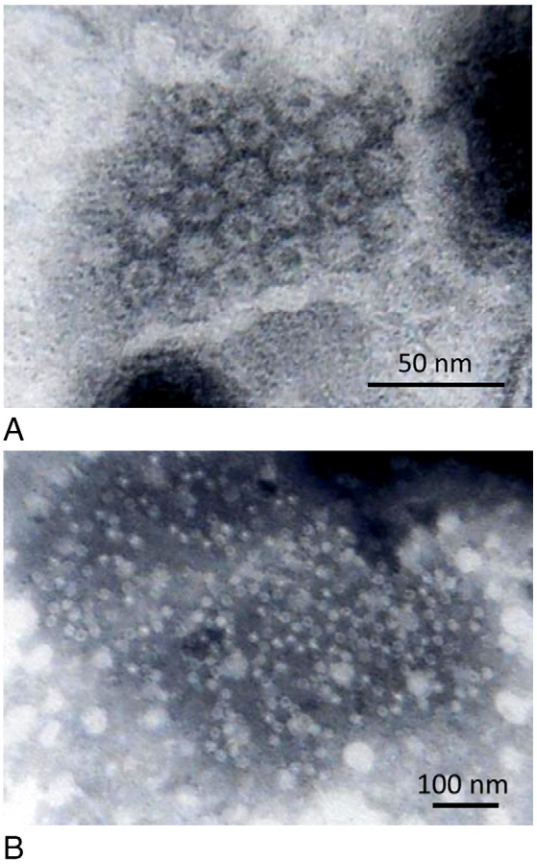

Fig. 1. A. (V2177/00) and B. (V3374/00) Electron micrographs of negatively stained circovirus particles in fecal samples of diarrheic dogs.

To our knowledge this is the first time that PCV2 has been reported in dogs. Although PCV2 specific amplicons have been obtained in two cases, there is only clear evidence of this virus in sample V2177/00. Regarding the 417 samples of diarrheic dogs examined for virus in 2000 this report seemingly represents an accidental finding. There are no indications for the source of the virus. However, the detection of PCV2 in rodents, cattle and calves (Halami et al., 2014; Lőrincz et al., 2010; Nayar et al., 1999) makes the transmission of the pathogen to non-porcine hosts possible, but, the way remains unclear. It remains also unclear whether the PCVs demonstrated in the dog feces only passed through the gut or were actually able to infect the host. The search for antibodies against PCV2 might be valuable in clarifying this question, but were not performed in this study. However, results of antibody testing to PCV in non-porcine species did not yield clear results (Tischer et al., 1995). The clinical relevance of the present finding is very difficult to assess since studies for possible bacterial or parasitic pathogens were not available. Only in case of sample V2177/00, however, it seems very likely that the additionally detected coronavirus rather than the circovirus was responsible for diarrhea.

Considering genome size and sequence the recently reported DogCV differs from the herein reported PCV2a. Hence at least two different circoviruses obviously occur in dogs. Not only the genome sizes of DogCV (2063 nt; GenBank accession no. KC241982) and PCV2a (1768 nt) are different, but both also belong to two distinct clades within the group of mammalian circoviruses (Li et al., 2013). However, PCV2 genomes are known to be highly variable resulting in currently four genotypes, PCV2a-d (Franzo et al., 2015). Within PCV2 there was a shift from PCV2a to PCV2b which occurred around 2003 in the pig population. Whether DogCV, first described in 2012, may have undergone a similar shift during the last two decades originating from PCV2a or is a distinctly different circovirus could be answered in a retrospective study, examining fecal samples from dogs not only for the presence of PCV2 but also for other circoviruses.

Conflict of interest

None.

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