

OCCURRENCE AND GENETIC DIVERSITY OF PIGEON CIRCOVIRUS STRAINS IN POLAND

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(Received 21 May 2013; accepted 31 October 2013)

Pigeon circovirus (PiCV) is an immunosuppressive agent widespread throughout the world, which causes a disease in pigeons called Young Pigeon Disease Syndrome. The aim of the study was to evaluate the prevalence of PiCV in Poland and investigate the genetic diversity relative to other known PiCV isolates. Samples from 152 pigeon flocks (88 flocks of racing pigeons and 64 flocks of fancy pigeons) from various regions of Poland were tested by polymerase chain reaction and an approximately 326-base fragment of the capsid protein gene (*Cap* gene) of the virus was amplified. The average viral prevalence was found to be 70.3% (76.13% in racing pigeons and 62.5% in fancy pigeons). Among the obtained positive samples, 21 were selected for sequencing and a phylogenetic analysis was performed. It was found that the majority of Polish PiCV isolates, to varying degrees, are related to isolates occurring in Europe. It was also observed that the *Cap* gene is variable and mutations often occur in it, which impacts the amino acid sequences in the capsid protein (nucleotide similarity averaged 86.57%, amino acid similarity averaged 89.02%).

Key words: Capsid protein gene, PCR, pigeon circovirus, phylogenetic analysis, Poland

Pigeon circovirus (PiCV, Columbid Circovirus – CoCV) belongs to the family *Circoviridae* (genus *Circovirus*). The genome of circoviruses occurring in pigeons constitutes a circular, single-stranded DNA containing approximately 2,000 bases forming two main and additive smaller open reading frames (ORFs). The larger ORF, ORF-V1 located on the virion sense strand, encodes a protein associated with viral replication (Rep), while the other, ORF-C1 located on the complementary sense strand, encodes the viral capsid protein (Cap protein, CP) (Mankertz et al., 2000; Johne et al., 2006). Previous relevant studies have demonstrated that in contrast to the gene forming ORF-C1, the gene forming ORF-V1 is highly conserved and mutations rarely occur in its sequence (Todd et al., 2008; Zhang et al., 2011).

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Pigeon circovirus infections are very common in the domestic pigeon population, and their clinical form occurring in young birds is called Young Pigeon Disease Syndrome (YPDS) (Smyth and Carroll, 1995; Paré et al., 1999; Raue et al., 2005; Duchatel et al., 2005, 2006; Zhang et al., 2011; Cságola et al., 2012; Stenzel et al., 2012). Circoviral infection in pigeons can be manifested in a wide range of signs including weight loss, decrease in racing performance, respiratory problems, diarrhoea, and occasional problems with plumage development (Raue et al., 2005). Pigeon circovirus infection leads to a loss in lymphoid tissue layer and lymphocyte apoptosis, which is the main cause of immunosuppression in the infected birds (Todd, 2000; Abadie et al., 2001; Raue et al., 2005). In addition, histopathological examination of the organs taken from sick birds has shown the presence of characteristic intracytoplasmic botryoid inclusion bodies in the cells of the lymphatic tissue (Caletti et al., 2000). A weakened immune response in infected pigeons predisposes the birds to opportunistic infections and superinfections by other viruses such as pigeon herpesvirus (PiHV) and pigeon adenovirus (PiAV) (Raue et al., 2005; Stenzel et al., 2012). Pigeon circovirus has been isolated from both healthy and symptomatic birds of all ages, which form the basis for the hypotheses that additional factors are needed for the development of YPDS, or that there are PiCV strains of different virulence (Schmidt et al., 2008; Zhang et al., 2011; Cságola et al., 2012; Stenzel et al., 2012).

The data presented in this paper are in part the continuation of a previous investigation into the occurrence of viral infections in pigeons in Poland (Stenzel et al., 2012). The aim of this part of the study was to evaluate the occurrence and genetic diversity of PiCV strains isolated from pigeons of different breeds in Poland.

Materials and methods

This study was conducted with the approval of the local ethics committee (number 71/2008, valid through 31 August 2012).

Samples

Experimental material was collected in 2011 from pigeons of 152 flocks (88 flocks of racing pigeons and 64 flocks of fancy pigeons) from different regions of Poland. The sample material consisted of cloacal swabs collected from pigeons. Moreover, if deaths were recorded in flocks, 0.2 g sections of internal organs (liver, spleen, bursa of Fabricius) were obtained during the postmortem examination of dead pigeons.

DNA extraction

Before attempting to isolate DNA, the cloacal swab samples were incubated in 300 µL phosphate buffered saline solution (PBS) at room temperature

for 30 min. The internal organ samples were homogenised in 500 μ L PBS using TissueLyser II (Qiagen, Germany). The remaining incubation fluid from the cloacal swabs and organ homogenate (200 μ L) for each sample was used in later stages of the DNA isolation process. DNA was extracted with the magnetic method using a Janus automated workstation (Perkin Elmer, USA) and a ready-made NucleoMag Tissue Kit (Macherey-Nagel, Germany) in accordance with the manufacturer's instructions. Eluted DNA concentration was measured using a NanoDrop (Thermo Scientific, USA) and then stored at -80°C until further use in the assay.

Polymerase chain reaction (PCR)

PCR was performed with the help of a HotStarTaq Plus Master Mix Kit (Qiagen, Germany). In order to detect PiCV genetic material, a 326-base fragment of *Cap* gene was amplified using primers developed by Freick et al. (2008): PiCV2-s 5'-TTGAAAGGTTTTTCAGCCTGGC-3' and PiCV2-as 5'-AGGAGACGAAGGACACGCCTC-3'. The contents of the reaction mixture were as follows: 10 μ L of HotStarTaq Plus Master Mix, 0.1 μ L each of the primers (concentration 100 μ M), 2 μ L of Coraload10x loading dye and 2 μ L of eluted DNA. The final reaction volume was made up to 20 μ L with RNase-free water. PCR was conducted in a Mastercycler II (Eppendorf, Germany) thermocycler under the following conditions: polymerase activation and initial denaturation at $95^{\circ}\text{C}/5$ min; followed by 30 cycles of: denaturation at $94^{\circ}\text{C}/60$ s, annealing at $60^{\circ}\text{C}/60$ s, chain elongation at $72^{\circ}\text{C}/90$ s and final elongation after the last cycle at $72^{\circ}\text{C}/10$ min. PCR results were analysed using capillary electrophoresis with the help of Bioanalyzer 2100 (Agilent Technologies, USA) and ready-to-use reagents Agilent DNA 1000 kit (Agilent Technologies, USA), developed for the separation and sizing of dsDNA fragments from 25 to 1,000 bp.

Sequencing and phylogenetic analysis

From among all the positive samples, 21 were selected for sequencing. Nucleotide sequencing of purified PCR products was carried out using the Big-Dye Terminator v3.1 kit (Applied Biosystems) and a 3730xl (Applied Biosystems) genetic analyser at Genomed Company (Poland). Nucleotide sequences were aligned using Lasergene version 8.1.5. (DNASTAR, USA). The sequences obtained in the current study were compared with 18 PiCV sequences from different parts of the world published in the GenBank database using the Clustal W method with the help of Mega 5 and Bioedit v. 7.0.0. software. The phylogenetic tree was generated using the distance-based neighbour-joining method with the help of Mega 5 software (Tamura et al., 2011). The bootstrap values were calculated for 1,000 replicates of the alignment. The nucleotide and amino acid identity matrices were created with BioEdit v. 7.0.0 software (Hall, 1999).

Accession numbers

Sequences of Polish PiCV isolates were submitted to the GenBank database to generate accession numbers. The obtained accession numbers were KC691675–KC691696. For easier identification in further analysis the isolate names were coded according to the scheme: PL_x_F or R, where the first two letters indicate that the country of isolation is Poland, 'x' denotes the sample number and the last letter indicates that the virus was isolated from fancy (F) or racing pigeons (R).

Results

PCR and the prevalence of pigeon circovirus in Poland

Among 152 examined samples, the presence of PiCV genetic material was determined in 107 of them, from which 67 (62.62%) came from racing pigeons and 40 (37.38%) from fancy pigeons. The total prevalence of PiCV infections in Poland in 2011 amounted to 70.39%. The obtained PCR products corresponded to the section spanning 60–385 of nucleotides in the *Cap* gene, which accounted for approximately 30% of this gene length.

Phylogenetic analysis

As is apparent from the dendrogram constructed on the basis of *Cap* gene fragment comparison, the analysed PiCV strains are clustered in 5 clades, which for publication purposes are defined by the contractual letters A–E (Fig. 1). As shown in Fig. 1, clade A includes the North American and European isolates which are closely related to most of the Polish isolates. Most of the Polish isolates consisted of small subclades of closely related viruses. Clade B comprises mainly European isolates (Belgian and Hungarian), and only 4 isolates are Polish. Clade C contains 2 European isolates and 4 closely related Polish isolates. None of the Polish isolates was related to the isolates belonging to clades D and E.

Analysis of nucleotide sequence in investigated gene fragments and amino acids in the coded proteins

Analysis of nucleotide sequences in the *Cap* gene fragment indicated the appearance of many mutations. All isolates classified into clades A, B and C have deletions of 3 nucleotides at position 157–159. At the same time, isolates classified as clades B, C and D have the deletion of 6 nucleotides at position 84–89, and Belgian and Hungarian isolates from clade B and isolate PL_168_F have the deletion of 3 nucleotides at positions 103–105. In addition, many nucleotide substitutions were found in the whole sequence of the analysed gene fragment. All of the identified nucleotide deletions reflected on the amino acid sequence as

a deletion of amino acids in positions 29–30 (clades B, C and D), 35 (Belgian and Hungarian isolates from clade B and isolate PL_168_F) and 58 (clades A, B and C) (Fig. 2). The percentage of nucleotide sequence similarity of the analysed *Cap* gene fragment within the tested Polish PiCV isolates averaged 86.57%, while the percentage of amino acid sequence similarity averaged 89.02% (Table 1).

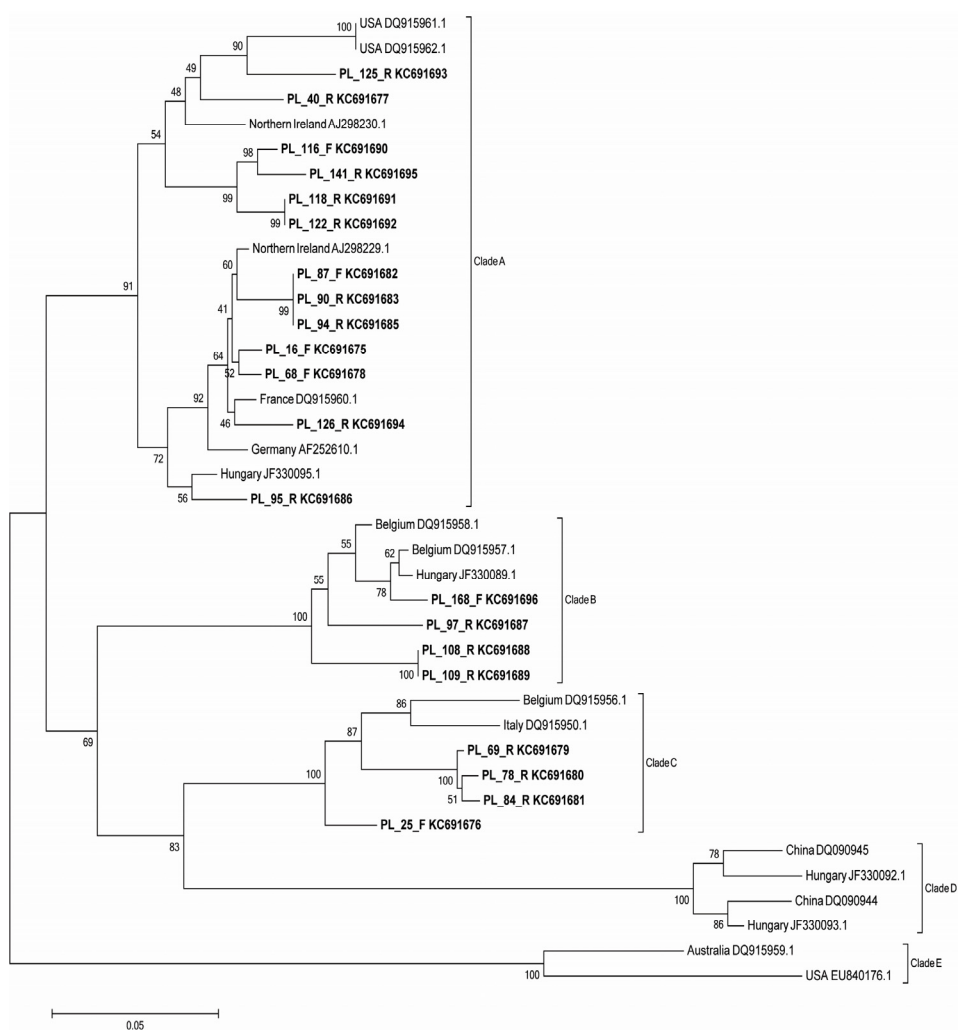


Fig. 1. Dendrogram based on the comparison of *Cap* gene fragments of PiCV included in the study. The isolates are represented by the name of the country where they were detected and the accession number. The Polish isolates are indicated in bold. Branch lengths represent the predicted number of substitutions and are proportional to the differences between the isolates. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches

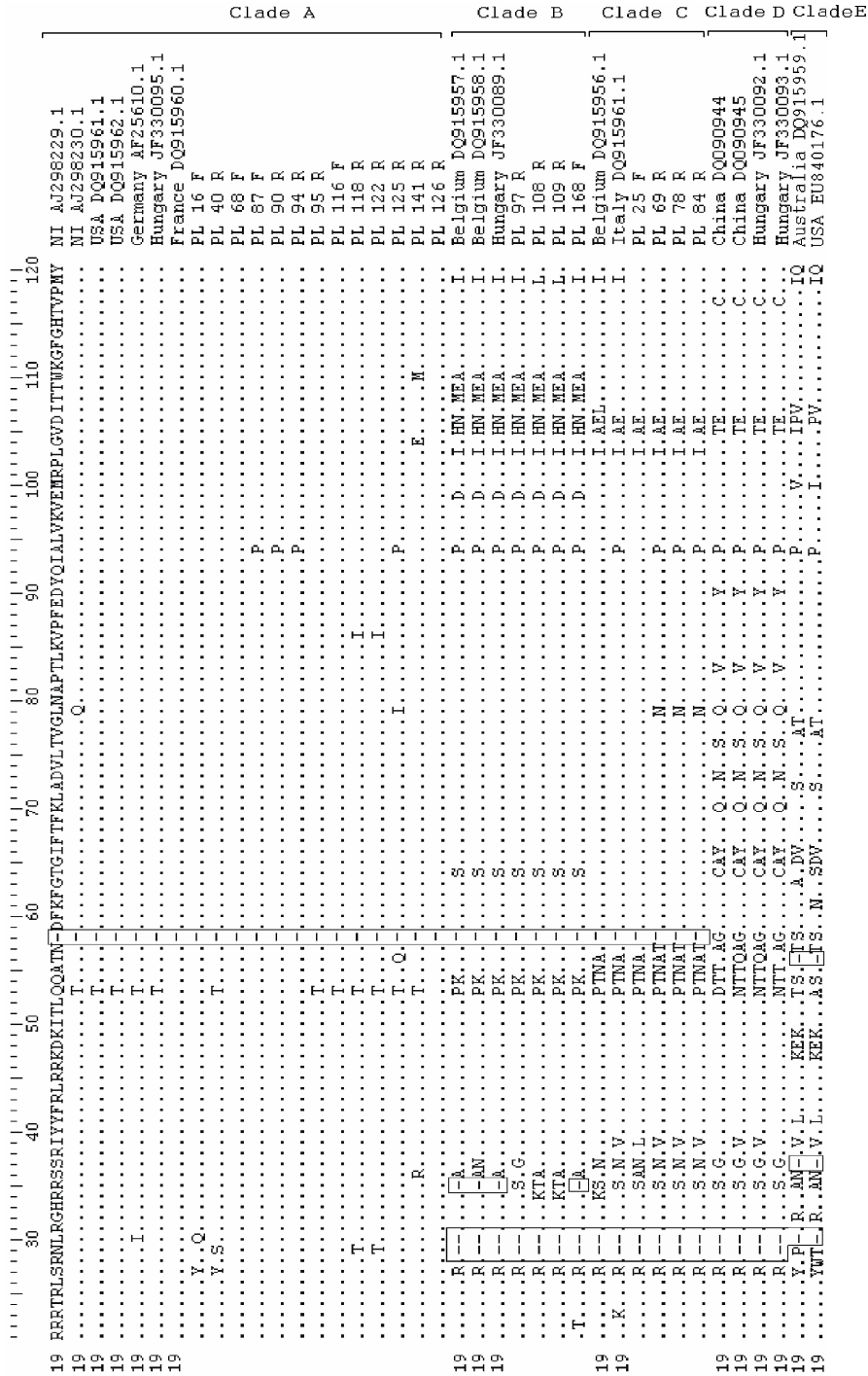


Fig. 2. Alignment of the analysed amino-acid fragments sequence of Cap protein among the examined PicV isolates. Amino acid deletions are boxed with solid lines. Sequences were grouped according to the phylogenetic tree

Discussion

Pigeon circovirus infections are very common all around the world (Smyth and Carroll, 1995; Paré et al., 1999; Caletti et al., 2000; Raue et al., 2005; Freick et al., 2008; Zhang et al., 2011; Cságola et al., 2012; Stenzel et al., 2012). The results obtained in this study indicate the high prevalence of PiCV infections in Poland. Phylogenetic analysis of Polish PiCV isolates presented in the study shows that although there is a high resemblance of nucleotide sequences in the investigated genes fragments (in average 86.57%), it is possible to extract different clades that have hypothetical common ancestors with PiCV strains available in the GenBank. By analysing the length of each branch of the phylogenetic trees, it can be stated that either the hypothetical common ancestors of the world's well-known PiCV strains and PiCV isolates from Poland studied in this investigation appeared in the distant past or that the virus mutates rapidly. The phylogenetic tree constructed on the basis of the *Cap* gene fragment is convergent with data presented by Cságola et al. (2012) wherein the authors divided the Hungarian isolates and isolates from all over the world into groups. To facilitate a comparative analysis, the same was performed in the present study by separating five clades of viruses. It should be noted that the character of such division is rather conventional and its aim is only to facilitate the interpretation of the results. By analysing the phylogenetic tree constructed on the basis of the *Cap* gene fragment, it can be stated that most of the Polish isolates are closely related mainly to European isolates. This may result from the fact that the import of pigeons from different European countries to Poland is very significant. Imported birds with a perfect pedigree are not used for racing but are kept as a reproductive flock. Reproductive flocks are often created with birds originating from different pigeon breeding facilities, which facilitates not only the transmission of the virus to uninfected birds in the flock but also the formation of recombinants (Cságola et al., 2012).

An analysis of amino acid sequences obtained by translation of the investigated gene fragments indicates that they are rather less volatile than the nucleotide sequences (approximately 89.02% of similarity).

So far, it is known that in circovirus infection of the pigeon organism, the arising antibodies are directed against the capsid protein (Daum et al., 2009; Duchatel et al., 2011). Based on previous studies the authors of this publication agree with certain hypotheses, which argue that changes in capsid protein structure may have an influence on the formation of resistance against this virus (Cságola et al., 2012). Pigeon circovirus infections do not cause large losses directly associated with the infection as observed in the case of paramyxoviruses (Aldous et al., 2004), although it is noteworthy that they are very strong immunosuppressants favouring the occurrence of associated infections which can in turn cause huge losses in flocks (Todd, 2000; Raue et al., 2005; Stenzel et al., 2012).

Taking into consideration the fact that PiCV infections are very common, development of a vaccine appears necessary. Because of the inability to prepare a laboratory culture of this virus, the best solution is to develop a recombinant vaccine (Bonne et al., 2009). The variability of the gene encoding the capsid protein (which may be reflected in its structure) may have an impact on the effectiveness of the potential vaccine. Because circoviruses easily undergo recombination, further investigation is needed to assess the genetic variation of a higher number of PiCV strains around the world.

Acknowledgement

We would like to thank Dr. Krzysztof Śmietanka of the National Veterinary Research Institute (Puławy, Poland) for his valuable advice.

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