The epidemiology, molecular characterization and clinical pathology of circovirus infections in pigeons – current knowledge

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ABSTRACT
The first cases of circovirus infections in pigeons were documented less than 25 years ago. Since then, circovirus infections have been reported on nearly all continents. The specificity of pigeon breeding defies biosecurity principles, which could be the reason for the high prevalence of PiCV infections. PiCV infections in pigeons lead to atrophy of immune system organs and lymphocyte apoptosis. Infected birds could be more susceptible to infections of the respiratory and digestive tract. PiCV has been associated with the young pigeon disease syndrome (YPDS). PiCVs are characterized by high levels of genetic diversity due to frequent point mutations, recombination processes in the PiCV genome and positive selection. Genetic recombinations and positive selection play the key role in the evolution of PiCV. A protocol for culturing PiCV under laboratory conditions has not yet been developed, and traditional vaccines against the infection are not available. Recombinant capsid proteins for detecting anti-PiCV antibodies have been obtained, and these antigens can be used in the production of diagnostic tests and subunit vaccines against PiCV infections. However, YPDS has complex etiology, and it remains unknown whether immunization against PiCV alone will contribute to effective control of YPDS.

1. Introduction
The pigeon circovirus (PiCV) or the columbid circovirus belongs to the genus Circovirus and the family Circoviridae (Mankertz et al. 2000). The circovirus infection in pigeons was first diagnosed in 1993 in the USA (Woods et al. 1993), but according to some reports, the virus had been documented earlier in Canada, the USA and Australia (Woods et al. 1994; Paré et al. 1999). The progress in molecular biology techniques facilitated rapid and accurate detection of infectious diseases. As a result, cases of PiCV infections in pigeons were subsequently reported in various countries and regions, including Northern Ireland (Smyth & Caroll 1995; Todd et al. 2001), Germany (Mankertz et al. 2000; Raue et al. 2005), Italy (Coletti et al. 2000; Franciosini et al. 2005), France (Abadie et al. 2001), Czech Republic (Taras et al. 2003), Belgium (Duchatel et al. 2005, 2006), Poland (Wieliczko et al. 2005; Stenzel et al. 2012, 2014a), Slovenia (Krapez et al. 2012), Hungary (Cságoła et al. 2012), United Arab Emirates (Ledwoń et al. 2011), Iran (Mahzouie et al. 2014), Taiwan (Liao et al. 2015), China (Zhang et al. 2011; Phan et al. 2013; Zhang et al. 2015a), Japan (Yamamoto et al. 2015) and the USA (Roy et al. 2003) (Figure 1). In most reports, the virus was detected in the pigeon species of Columba livia domestica, but in several cases, PiCV genetic material was also detected in Senegal doves (Streptopelia senegalensis) and collared doves (Streptopelia decaocto) (Kubiček & Taras 2005; Todd et al. 2008). Bird racing, pigeon exhibitions and other events that involve transport of foreign birds could contribute to the rapid spread of PiCV infections in pigeon populations. The PiCV is transmitted mainly horizontally through ingestion or inhalation of virus-contaminated fecal material and feather dust (Woods et al. 1993; Franciosini et al. 2005). The virus can also be transmitted vertically (Duchatel et al. 2005, 2006), but the role of this route has not yet been elucidated. Transport of large groups of domestic pigeons for racing, display of birds from various geographic regions during shows and exhibitions, and the inclusion of such birds in parent stock could contribute to the spread of pathogens between pigeons and could also lead to the production of new recombinant strains of PiCV as well as the psittacine circovirus (Julian et al. 2013).

2. Clinical pathology of PiCV infections
The pathology of PiCV infections in pigeons has not been fully elucidated to date, but PiCV is probably one of the factors that contribute to the young pigeon disease syndrome (YPDS) (Raue et al. 2005). The clinical presentation of PiCV infections in pigeons is highly varied. Recent research has demonstrated that PiCV alone is not capable of inducing symptoms of disease in pigeons. A combination of various immunosuppressive...
factors and conditionally pathogenic microorganisms is required for disease presentation. The type and intensity of clinical symptoms are largely determined by the type of confounding factor. In typical cases, the disease affects young pigeons in the summer months (between late May and early September in Europe, with peak prevalence during hot spells, trial flights and first racing competitions), although in Belgium, peak prevalence was noted in early spring which marks the beginning of the mating season for adult birds (Tavernier et al. 2000). Initial symptoms are relatively non-specific, and they include apparent foraging behavior where pigeons do not leave feeders and peck on feed, but when examined by palpation, their crops are empty or filled only with single seeds. This is followed by increased thirst and regurgitation from the crop. Crops are often filled with large quantities of greenish fluid (water combined with mucus and refluxed duodenal contents). Other symptoms include diarrhea and highly expressed, non-specific symptoms such as apathy, feather ruffling or reluctance to train. The health status of diseased birds rapidly deteriorates, and mortality rates in affected flocks can reach several dozen percent (Raue et al. 2005). Neurological disorders may develop 7–10 days after the appearance of the first symptoms of YPDS, including torticollis, involuntary circular movement and coordination problems, which can also point to concomitant infections with paramyxovirus (pigeon paramyxovirus serotype 1, PPMV-1). Sudden deaths are also reported. In young ornamental pigeons, the disease is far less frequently noted in the summer months, and its prevalence increases during the bird show season (November to February). The clinical presentation is similar, but infections are generally less severe. Circovirus infections often involve changes in plumage (Pass & Perry 1984; Soike et al. 1999; Soike et al. 2004; Sarker et al. 2015), but they are less commonly noted in pigeons than in parrots. Pigeon nestlings exhibit feathering complications, including lack of feather growth (in particular tail and wing feathers as well as larger covert feathers), deformed and depigmented feathers. Feathered birds may experience problems during successive feathering, including stunted growth of feathers to the length of 3–4 cm, necrosis and shedding of newly developed feathers which are often found on the floor of dovecotes (Dolka et al. 2016).

The syndrome was initially observed only in young domestic pigeons due to the fact that young birds have well-developed bursa of Fabricius (BF) containing numerous target cells for PiCV (Shivaprasad et al. 1994; Smyth & Caroll 1995; Gough & Drury 1996; Paré et al. 1999; Coletti et al. 2000; Abadie et al. 2001). At present, clinical cases of YPDS are noted not only in young birds, but also in pigeons older than 1 year (Stenzel et al. 2012). The PiCV also targets other organs of the immune system, including the thymus and spleen (Woods et al. 1993; Smyth & Caroll 1995; Coletti et al. 2000; Soike et al. 2001; Scullion & Scullion 2007). The virus or its genetic material have also been detected in the liver, kidneys, intestines, brain and skin (Smyth & Caroll 1995; Smyth et al. 2001; Soike et al. 2001; Ledwoń et al. 2011; Stenzel & Pestka 2014; Dolka et al. 2016). PiCV replicates in fast proliferating cells (epithelial cells) and immune system cells (lymphocytes and macrophages), and in this respect, it is similar to the psittacine circovirus (beak and feather disease virus, BFDV) (Raidal et al. 2015).
Intracytoplasmic inclusion bodies are detected in lymphoid tissue during histopathological analyses of pigeons infected with PiCV (Shivaprasad et al. 1994; Gough & Drury 1996; Coletti et al. 2000; Abadie et al. 2001). PiCV infections also led to atrophy of the immune system organs and the loss of lymphoid tissue in these organs, which is why PiCV is regarded as a putative immunosuppressive agent in pigeons (Coletti et al. 2000; Abadie et al. 2001; Todd 2003; Scullion & Scullion 2007). The mechanism of immunosuppression by PiCV has not been thoroughly investigated, but it appears that PiCV infections cause lymphocyte damage. Abadie et al. (2001) demonstrated a much higher percentage of apoptotic lymphocytes in the BF of birds infected with PiCV than in non-infected birds. A higher prevalence of accompanying infections of the respiratory tract caused by the pigeon herpesvirus, Chlamydia psittaci and Riemerella sp. (Soike et al. 2001; Stenzel et al. 2012; Stenzel et al. 2014b) and digestive tract infections caused by rotaviruses and picornaviruses (Soike et al. 2001) also suggest that PiCV could induce immunosuppression in pigeons. However, in some studies, PiCV was not correlated with the prevalence of selected viral and bacterial factors (Tavenier et al. 2000).

Several attempts have been made to verify the post-infectious immunosuppression hypothesis with the use of various experimental designs and molecular biology methods, including sequencing of PiCV genes and entire genomes. Schmidt et al. (2008) demonstrated that the disease cannot be induced in healthy pigeons that had been experimentally infected with PiCV contained in organ homogenates of infected birds that were vaccinated against PPMV-1 (pigeon paramyxovirus serotype 1) as an additional trigger. The cited authors did not observe differences in anti-PPMV-1 antibody titers in pigeons infected with PiCV and control group birds. In the above experiment the replication of the PiCV was detected based on the presence of characteristic inclusion bodies in lymphocytes and macrophages in immune system organs and the presence of PiCV genetic material. Similar results were reported by Duchatel et al. (2010), in whose study, a commercial vaccine against paratyphoid had no effect on the replication of the PiCV in naturally infected birds. Their findings could suggest that the infection caused by PiCV is not sufficient for the development of YPDS clinical symptoms. The authors concluded that additional factors are required for disease symptoms to develop and that PiCV strains could differ in pathogenicity.

3. Genome characterization and genetic diversity of PiCV

The organization of the PiCV genome is typical for the family Circoviridae and is very similar to the genome of the BFDV (Stenzel et al. 2014a). Similarly to other known vertebrate circoviruses, including the porcine circovirus (PCV) and the chicken anemia virus (CAV), the PiCV genome consists of single-stranded spherical DNA of approximately 2030 base pairs (bp). The PiCV genome is larger than PCV (approximately 1760 bp) and BFDV (approximately 1900 bp) genomes and is similar in size to the CAV genome (approximately 2000 bp) (Todd et al. 2001; Rosario et al. 2017). All circoviruses have an ambisense genome organization containing open reading frames (ORFs) on different strands of replicative form dsDNA. The only exception is the CAV genome which has a negative sense organization. The genome organization and structure of CAV differs from that of other circoviruses, which is why it was initially classified into the genus Gyrovirus. In a recent classification system, CAV was included in a new genus Cyclovirus (Rosario et al. 2017). All circoviruses have two major ORFs. The largest ORF, V1, which is located on the virion sense strand, encodes a protein responsible for viral replication (Rep protein), and the second largest ORF, C1, which is located on the complementary sense strand, encodes the viral capsid protein (Cap protein, CP) (Mankertz et al. 2000; Todd et al. 2001; Johne et al. 2006; Todd et al. 2008). The genome of all circoviruses also encodes other ORFs. For example, the CAV has three ORFs (all located on the virion sense strand) which encode proteins such as VP1 (capsid), VP2 (replication protein) and VP3 (apoptotic activity). The genome of other circoviruses encodes additional ORFs: BFDV encodes two additional ORFs on both viral sense and complementary sense strands, and PCV encodes an additional ORF on the complementary sense strand that encodes a VP3 protein with similar action to that of CAV VP3 (Todd et al. 2001; Hough et al. 2015). PiCV genomes encode three additional ORFs located on the complementary sense strand (Figure 2). The remaining ORFs encode PiCV...
proteins with unknown functions (Todd et al. 2008; Stenzel et al. 2014a). All circoviruses contain a conserved intergenic region between the start codons of genes encoding Rep and Cap proteins. This region contains a conserved nanonucleotide motif ‘(T/n)A(G/t) TATTAC’, where lower case nucleotides are observed at low frequency and ‘n’ represents nucleotides located at the apex of a potential stem-loop structure. This structure constitutes the origin of replication (Mankertz et al. 2000; Rosario et al. 2017). The pairwise identity between PiCV and BFDV or PCV is estimated at 57% and 35%, respectively, which is why these viruses were classified as different species (Mankertz et al. 2000; Todd et al. 2001).

Research has demonstrated that unlike the gene encoding Rep protein, the cap frequently undergoes mutations (Todd et al. 2008; Zhang et al. 2011; Cságola et al. 2012; Stenzel et al. 2014a; Liao et al. 2015; Zhang et al. 2015a). The similarity of nucleotide sequences of the cap gene in various PiCV strains is estimated at 87% on average (Figure 3), and the presence of separate clades, which were created based on a comparison of whole genome sequences, results from differences in the nucleotide sequence of capsid protein gene (Zhang et al. 2011; Cságola et al. 2012; Stenzel et al. 2014a; Yamamoto et al. 2015). Five main clades, termed A to E, have been identified based on differences in the sequence of the PiCV genome (Cságola et al. 2012; Stenzel et al. 2014a; Yamamoto et al. 2015). Clade A consists mainly of European isolates and a small number of American and Asian isolates. Clades B, C and D contain European and Asian isolates (Stenzel et al. 2014a; Yamamoto et al. 2015), whereas clade E is composed of the most genetically distant strains isolated from the domestic pigeon in the USA, the Senegal dove (Streptopelia senegalensis) and a recently described PiCV isolate from Japan (Yamamoto et al. 2015) (Figure 4). Interestingly, a phylogenetic analysis based on the sequences of PiCV rep revealed the presence of 2, rather than 5 main clades. The PiCV has a small genome and easily undergoes mutations, which is why the entire genome sequence should be examined in a phylogenetic analysis.

The evolution of circoviruses is based mainly on positive selection during contact with the host’s immune system, and it explains the relatively high level of genetic variation in PiCV isolates due to mutations in the cap. This type of adaptive host immunity is one of the primary drivers of positive selection in many viruses that infect vertebrates (Rigby et al. 1993; Benett et al. 2006; Esteban & Hutchinson 2011). A capsid is the protein shell of a virus, and it is exposed to the host’s immune cells, which stimulates a cascade of immune responses. Mutations that change the structure of CP are required to enable the virus to bind to target cell receptors, infect cells and avoid being neutralized by antibodies (Blanchard et al. 2003; Benett et al. 2006). An analysis of the nucleotide sequences of the cap in PiCV isolates revealed many mutations resulting from adaptive evolution (positive selection) of European isolates (Stenzel et al. 2014a), as well as a new genetic line of PiCV strains from Taiwan that had developed by positive selection (Liao et al. 2015).

A phylogenetic analysis of PiCV strains whose DNA was isolated from birds with YPDS and non-infected birds did not reveal mutations characteristic of viruses whose DNA was isolated from infected birds. No correlations were found between the genotype of PiCV
isolates and the severity of YPDS (Stenzel et al. 2014a). These observations confirm the hypothesis that additional factors, such as stress associated with weaning, racing competitions, vaccination and training, are required for YPDS symptoms to develop (Schmidt et al. 2008; Stenzel et al. 2014a).

4. Epidemiology of PiCV infections

The epidemiological data on PiCV infections give reasons for serious concern. The first report describing the prevalence of PiCV infections was published by Tavernier et al. (2000). The above authors performed microscopic evaluations of BF sections stained with hematoxylin and eosin. A PiCV infection was diagnosed based on the presence of inclusion bodies in BF cells. In the above study, only 19% of the analyzed young pigeons were infected with PiCV. Their results correspond with the findings of Soike et al. (2001) who with the use of electron microscopy method identified particles similar in shape and size to PiCV in 48% of the examined pigeon’s internal organs, whereas the percentage of positive results was approximately twice lower in a histological analysis. The results of molecular analyses characterized by higher sensitivity than histopathological techniques revealed much higher prevalence of PiCV infections. The first data relating to the prevalence of PiCV in Polish pigeons were published by Wieličko et al. (2005), and they revealed the presence of PiCV genetic material in 72% of racing pigeons in PCR assays conducted with the use of the method described by Roy et al. (2003). The above results are consistent with the findings of Stenzel et al. (2012) who relied on the method proposed by Freick et al. (2008) and detected the PiCV in 70% of the pigeons examined in 2010–2012 on average. The prevalence of PiCV was determined by age and health status, and it was the highest (close to 100%) in young birds showing non-specific symptoms of the disease (Stenzel et al. 2012). Asymptomatic infections were noted in 53% birds of all ages on average, and in 36% of adult domestic pigeons from breeding flocks (Stenzel & Pestka 2014; Stenzel et al. 2014a; Stenzel et al. 2016). Similar results were reported in studies of domestic pigeons conducted in Hungary (prevalence of less than 57%) and in China where PiCV DNA was isolated from 80.7% of diseased birds and 63.4% of asymptomatic birds (Zhang et al. 2011; Cságola et al. 2012). The PiCV genome copy number in the BF and blood serum was also significantly higher in infected

Figure 4. A neighbor-joining phylogenetic tree depicting the possible evolutionary relationships between 53 PiCV sequences from GenBank. The sequences of the cap gene (A), the whole genome (B) and the rep gene (C) are compared in the dendrograms. The isolates are marked as follows: accession number/isolate name/country where a given isolate was detected. The division of PiCV isolates into 5 clades reflects the high diversity in PiCV cap and explains the similarity of dendrograms A and B. The sequences were analyzed using MEGA 6 software. Each tree was produced using a consensus of 1000 bootstrap replicates.
birds with clinical symptoms of YPDS than in subclinically infected pigeons (Duchatel et al. 2011). The prevalence of PiCV infections in feral pigeons was similar to that noted in domestic pigeons in Slovenia at 74.3%, but the health status of the investigated birds was not described (Krapez et al. 2012). Interestingly, the prevalence of PiCV infections in healthy Polish population of feral pigeons, which were studied with the use of molecular methods developed by Roy et al. (2003) and Freick et al. (2008), was below 45% on average, which could indicate that natural selection is the main mechanism of controlling bird populations in urban areas (Wieliczko et al. 2005; Stenzel et al. 2014a).

The development of a recombinant antigen for serological analysis also produced rather interesting data. Daum et al. (2009) conducted a retrospective study of pigeon sera obtained in 1989, 1991 and 2008 and found anti-PiCV antibodies in 78% (61–90%) of the tested samples on average. A recent serological study of Polish domestic pigeons from selected breeding flocks revealed the presence of anti-PiCV antibodies in 72% birds on average, regardless of their infection status (average prevalence of 36%) (Stenzel et al. 2016). Somewhat different results were reported by Duchatel et al. (2011) who found anti-PiCV antibodies in 96% of the examined pigeons, but their study was performed on a very small population of 28 birds.

One loft races involving young pigeons have become highly popular in the last decade, and they provide valuable epidemiological data. In this pigeon sport, the winner is the fastest individual because all birds show symptoms of the disease, the PiCV genetic material is found in their immune organs; a histopathological examination reveals the presence of inclusion bodies in lymphocytes in the BF and spleen and when viral particles are identified under an electron scanning microscope (Duchatel & Szleszczuk 2011). In practice, PiCV infections are generally diagnosed based on clinical symptoms and the results of molecular analyses. Molecular analyses involve the amplification of both cap and rep genes or their fragments, but the more conservative rep region of the PiCV genome is more suitable for designing PCR primers (Table 1).

Treatment involves the supply of fluids to dehydrated birds, stabilization of parenchymal organs, administration of immunomodulatory drugs and substances that inhibit the proliferation of confounding factors. The main confounding factors in PiCV infections are *Escherichia coli*, *Klebsiella pneumoniae*, *Chlamydia psittaci*, *Riemerella* sp., *Chlamydia psittaci* and *Candida albicans* (Soike et al. 2001; Raue et al. 2005; Stenzel et al. 2014b). Domestic pigeons harbor highly antibiotic-resistant bacteria, and this fact should be taken into account during the treatment of accompanying infections (Scullion & Scullion 2010; Badouei et al. 2014; Stenzel et al. 2014c). Whenever possible, every therapeutic intervention should be based on an antibiogram. The effectiveness of treatment is determined by the antibiotic sensitivity of a given bacterial strain as well as the time elapsed from the onset of the first symptoms.

### Table 1. Primer sequences for molecular diagnosis of PiCV infections in pigeons. Most of the primers amplifying fragments of rep and cap and the intergenic region between these genes are marked with ‘*’.

| Target gene | Primer sequence (5′–3′) | Amplicon length (bp) | References |
|-------------|-------------------------|----------------------|------------|
| rep         | F: TTACCTCAATAYAAYCCT   | 548                  | Smyth et al. (2001) |
|             | R: CTTATACCTCCACCA      |                      |            |
| rep-cap*    | F: TGA AAG GTG CTG TGT CCA GG | 364                  | Todd et al. (2002) |
|             | R: GAT GCA TAT TIG TAA CCG GG |                    |            |
| rep         | F: GAT CTC AGC GAGGCT GTT GC | 388                  | Roy et al. (2003) |
|             | R: TCA CGG GAG AGC TGA ACT C |                     |            |
|             | R: CCGAAGTGCATGGTCACTAC | 150                  | Franciosini et al. (2005) |
| rep         | F: GCATAAGGGTGCCGCTGAAAAGG | 330                  | Todd et al. (2006) |
|             | R: ATTCGCGGCCGTCGCTGCT |                      |            |
| cap         | F: TGGAAGTTTTCATCGCACC | 325                  | Freick et al. (2008) |
|             | R: AGGGACAGGACAGCACCACACC |                      |            |
| rep         | F: GGT ACC CGC ATA AGG TGC CGG T | 139                  | Duchatel et al. (2009) |
|             | R: TIG ATC CGC CGG AAG AGC GCC T |                    |            |
A specific protocol for the prevention of PiCV infections in pigeons has not yet been developed due to the absence of vaccines targeting this pathogen. In recent years, attempts have been made to develop a recombinant PiCV capsid protein for diagnostic tests and a subunit vaccine against the PiCV (Daum et al. 2009; Duchatel et al. 2011; Lai et al. 2014; Stenzel et al. 2016). This vaccine could protect pigeons against PiCV infection and, possibly, lower the prevalence of YPDS. Unfortunately, the relevant research has not yet been conducted; therefore, the effectiveness of PiCV vaccines in combatting YPDS has not yet been confirmed. The hypothesis that a subunit vaccine against PiCV could be effective in preventing YPDS is based on studies where similar vaccines have been used for controlling porcine circovirus infections in pigs (Zhang et al. 2016; Li et al. 2016). Attempts have also been made to immunize parrots with the recombinant capsid protein of the psittacine circovirus, but the efficacy of the vaccine was not confirmed (Bonne et al. 2009). In view of the above, the only available solution for controlling the spread of PiCV infections involves the prevention of common viral and bacterial diseases in pigeons, the use of immunomodulators during vaccination and weaning, elimination of stress, and selection of parent stock based on the results of serological and molecular tests (Duchatel & Szeleszczuk 2011; Stenzel et al. 2011; Stenzel et al. 2016).

6. Conclusions
Circovirus infections pose a serious problem in pigeon breeding around the world. The prevalence of PiCV infections is relatively high, but stable. Breeding practices that violate biosecurity principles significantly contribute to the transmission of PiCV in pigeon populations. Vaccines against PiCV have not yet been developed. Due to the genetic diversity of PiCV and the presence of additional factors that cause YPDS, immunization with the PiCV antigen alone, aimed at reducing immunosuppression in pigeons, may be insufficient. In view of the high prevalence of subclinical infections and PiCV infections, the efficacy of such vaccines is also doubtful. Further research is needed to test the effectiveness of the proposed methods for the prevention of PiCV infections in pigeons.

Disclosure of interest
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