PIGEON CIRCOVIRUS: BACULOVIRUS EXPRESSION OF THE CAPSID PROTEIN GENE, SPECIFIC ANTIBODY AND VIRAL LOAD MEASURED BY REAL TIME POLYMERASE CHAIN REACTION

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ABSTRACT

Pigeon circovirus infections occur in both young and adult racing and meat pigeons and have been reported worldwide. In the present study, the ORF C1 capsid gene of pigeon circovirus was expressed in insect cells and the expressed protein was used in an indirect immunofluorescence assay to determine antibody titres in naturally infected pigeons. The viral load of sera and the Bursa of Fabricius were also measured by real-time polymerase chain reaction. Twenty seven of the 28 serum samples tested were found positive for antibodies to pigeon circovirus capsid protein (titres from $\log_2 4$ to $\log_2 8$). The circovirus was detected in serum and Bursa of all young pigeons by real time polymerase chain reaction, but serum of all adult pigeons (1 year old or older) was negative. Viral loads in the serum ($6.56 \times 10^8 \pm 8.18 \times 10^8$ copies genome/µl) and in the Bursa ($4 \times 10^{10} \pm 3.87 \times 10^{10}$ copies genome/milligram tissue) of sick young pigeons were significantly higher when compared to those in the serum ($4.52 \times 10^7 \pm 1.35 \times 10^8$ copies genome/µl) and in the Bursa ($6.64 \times 10^9 \pm 1.12 \times 10^{10}$ copies genome/milligram tissue) of clinically healthy pigeons. This suggests that the detection of high levels of virus may be associated with the clinical status of the birds.

INTRODUCTION

Pigeon circovirus (PiCV) infection occurs in both racing and meat pigeons, and has been reported worldwide (1, 2, 3, 4, 5, 6, 7, 8, 9, 10). This infection is common in pigeons aged between 1 day and 6 months of age (11, 12). A broad range of clinical signs, including lethargy, weight loss, respiratory distress, diarrhea, poor racing performance and mortality are seen in infected pigeons. In racing pigeons this combination of clinical signs is commonly described as "young pigeon disease syndrome" (YPDS), with the type and severity of the clinical signs depending on the secondary pathogens present (13, 14). YPDS has clinico-pathological features in common with post-weaning multisystemic wasting syndrome (PMWS) with which porcine circovirus type 2 (PCV2) is associated. The apparent predisposition to secondary infections is suggestive of immunosuppression. While the primary effect of chicken anaemia virus (CAV), a *Gyrovirus* of the *Circoviridae* family, is depletion of the T cells in the thymus with minimal effect on the B cells in the Bursa of Fabricius, the PCV2 and PiCV appear to deplete both the B and T cells (15, 16).

Initially, the diagnosis of the PiCV infection was mainly based on histology, sometimes confirmed by electron microscopy. However the availability of cloned PiCV DNA and nucleotide sequence data has allowed the development of *in situ* hybridization, dot blot hybridization and polymerase chain reaction (PCR) assays (17, 18, 19, 20, 21, 22), which can detect very small amounts of viral DNA. PiCV DNA can be found both in healthy and sick birds (20), in the absence of the microscopic observation of the characteristic botryoid inclusions which are pathognomonic for PiCV infection. Botryoid inclusions are found in lymphoreticular tissues. In 32 Bursa of Fabricius (BF) samples from normal and sick pigeons, these inclusions were observed in 41 % of samples whereas 84 % were found positive by PCR (20). A real-time PCR assay based on SYBR Green chemistry, has been developed for the quantification of PiCV DNA in various samples (23). Testing of diseased pigeons showed

that the viral loads were high in BF (up to 2.07×10^9 copies/mg), liver (up to 2.88×10^8 copies/mg) and spleen (up to 5.57×10^8 copies/mg). In liver samples, the viral load was significantly higher in sick pigeons than in apparently healthy ones. This is also observed for PCV2 for which there is a strong correlation between high PCV2 genomic load in tissues and PMWS (24).

So far, there are no reports describing the isolation and propagation of PiCV in cell culture and there are no virus-specific antisera for detecting virus antigen. Recently, an indirect enzyme-linked immunosorbent assay for the detection PiCV-specific serum antibody was developed and may be a useful tool to PiCV diagnosis (25). As there is a lack of information relating to the antibody status versus PiCV genomic load in sera of healthy or affected PiCV naturally infected birds. The aim of the present work is to produce a PiCV capsid which can be used to develop a specific, sensitive method for detecting antibodies to PiCV, and to develop the knowledge of the PiCV biology in applying this test to serum samples collected from symptomatic or asymptomatic pigeons whose viral load was measured by real time PCR.

MATERIALS AND METHODS

Test Samples and DNA extraction

Samples were obtained from 28 racing pigeons derived from one loft containing 140 birds that had a five-year history of PiCV infections and YPDS. Samples were collected from 6 adult pigeons (Table 1, # 1 to 6), 6 eight-month-old pigeons (Table 1, # 7 to 12) and 16 young pigeons aged 2 weeks to 4 months (Table 1, # 13 to 28), four of which presented with signs of YPDS (Table 1, # 20, 24, 25 and 27). Blood samples were collected by venipuncture of the medial metatarsal vein and sera were collected. The BF was collected from 15 pigeons (Table 1, # 13, 15 - 28). Viral DNA was extracted from BF and serum samples using the QIAmp DNA mini kit (Qiagen Benelux b. v., Venlo, The Netherlands) according to the manufacturer's instructions. For serum, 50 µl were used as starting material respectively, while about 50 mg tissues were homogenized for extraction. Extracted DNA's were eluted in 100 µl buffer and stored at – 20° C until use. Remaining tissue samples were fixed in 10% neutral buffered formalin. Formalin-fixed samples were dehydrated, embedded in paraffin wax and sectioned at 4 µm for examination by light microscopy. All sections were stained with hematoxylin and eosin (HE).

Table 1: Results of conventional and SYBR Green real-time for sera and BF and antibody titres

Pigeon N°	Age	BI	PCR	QPCR[a]	PCR	QPCR[b]	log$_2$ IIF titres
				Sera		BF	
1	5 y.	NT	neg	neg	NP	NP	7
2	5 y.	NT	neg	neg	NP	NP	6
3	5 y.	NT	neg	neg	NP	NP	5
4	4 y.	NT	neg	neg	NP	NP	6
5	3 y.	NT	neg	neg	NP	NP	5
6	1 y.	NT	neg	neg	NP	NP	5
7	8 m.	NT	P	1.5×10^2	NP	NP	6
8	8 m.	NT	P	3.3×10^3	NP	NP	8
9	8 m.	NT	P	1.5×10^2	NP	NP	8
10	8 m.	NT	neg	neg	NP	NP	7
11	8 m.	NT	neg	neg	NP	NP	5
12	8 m.	NT	neg	neg	NP	NP	8
13	4 m.	X	P	1.14×10^5	P	1.78×10^7	7
14	4 m.	NT	P	6.72×10^5	NT	NT	5
15	3 m.	neg	P	2.66×10^4	P	4×10^8	6
16	3 m.	neg	P	4.26×10^5	P	5.83×10^9	4
17	3 m.	neg	P	3.59×10^4	P	8.5×10^9	7
18	2 m.	X	P	2.28×10^5	P	3.5×10^8	6
19	2 m.	neg	P	4.41×10^7	P	8.7×10^9	6
20*	2 m.	XXX	P	5.47×10^8	P	3.2×10^{10}	8
21	6 w.	neg	P	2.66×10^5	P	2.78×10^6	7
22	5 w.	neg	P	3.26×10^5	P	6.34×10^5	6
23	5 w.	XX	P	4.51×10^8	P	3.79×10^{10}	6
24*	4 w.	XXX	P	1.84×10^9	P	7.83×10^9	5
25*	4 w.	XX	P	3.51×10^7	P	9.6×10^{10}	4
26	4 w.	(X)	P	4.32×10^5	P	1.13×10^{10}	5
27*	4 w.	XXX	P	2.02×10^8	P	2.41×10^{10}	neg
28	2 w.	neg	P	5.11×10^5	P	4×10^2	4

NP: not present
NT: not tested
*: affected pigeons
BI: botryoid inclusions
(X): minimal
X: mild
XX: moderate
XXX: severe
[a]: quantitative PCR, number of genome copies per µl sera
[b]: quantitative PCR, number of genome copies per mg tissue
NT: not tested
P: positive
neg.: negative
y.: year
m.: month
w.: week

Cells

Spodoptera frugiperda (Sf9) cells (Invitrogen, Merelbeke, Belgium) were used to propagate baculovirus and were cultured in Sf 900 medium (Gibco, Invitrogen, Merelbeke, Belgium) at 27° C.

Production of PiCV ORF C1 structural gene

Primers 5'-GCC ACC ATG AGA AGG AGG AGA TTC CGC-3' and 5'-TTC AGA ATC CAC AGC TGA GTC-3' were designed for amplification of PiCV open reading frame (ORF C1). ORF C1 (an 825 base pairs (bp) fragment) was amplified from the genome clone of PiCV isolate 9030 (accession number AJ298229). PCR was performed on a Biometra TGradient DNA thermal cycler (Westburg, Leusden, The Netherlands). An initial denaturation step of 10 min at 94 °C, was followed by 40 thermocycles each comprising 45 sec at 94 °C, 60 sec at 62 °C, 90 sec at 72 °C and a final elongation step for 10 min at 72 °C. The PCR amplicon was visualised by electrophoresis in 1.2 % agarose gel containing ethidium bromide under ultraviolet transillumination.

Molecular cloning and expression of PiCV ORF C1 protein

The vector pBlueBac4.5/V5-His-Topo® (Invitrogen, Merelbeke, Belgium) was used as baculovirus transfer vector according to the manufacturer's instruction and the recombinant vector was transformed into TOP10 *Escherichia coli* (Invitrogen, Merelbeke, Belgium). Ten colonies were analyzed by PCR with polyhedrin forward (5'-AAA TGA TAA CCA TCT CGC-3') and V5 C-term reverse (5'-ACC GAG GAG AGG GTT AGG GAT-3'). An initial denaturation step (10 min at 94° C) was followed by 30 thermocycles each comprising 60 sec at 94.0 ° C, 60 sec at 55° C, 60 sec at 72.0° C and a final elongation step for 10 min at 72.0° C. The PCR amplicon was visualised by electrophoresis in 1.2 % agarose gel containing ethidium bromide under ultraviolet transillumination and a positive clone containing the gene of interest was selected.

The recombinant pBlueBac4.5/V5-His-Topo was transfected into Sf9 cells with Bac-N-Blue™ DNA in presence of Cellfectin® (Invitrogen, Merelbeke, Belgium) to generate a recombinant baculovirus expressing ORF C1 protein (Ac. PiCVcap.ORF C1). Selection of the transfected baculovirus was performed by purification of recombinant virus by plaque assay. Sf9 cells that had been infected with virus dilutions were overlaid with Grace's insect medium containing agar (1%) and X-Gal (50 mg/ml). Blue plaques were selected and amplified. To verify the purity of the recombinant virus, viral DNA was extracted from the infected cells pellet with QIAmp® DNA Mini Kit (Qiagen, Benelux b. v., Venlo, The Netherlands) and PCR was performed with forward primer: 5'-TTT ACT GTT TTC GTA ACA GTT TTG-3' and reverse primer: 5'-CAA CAA CGC ACA GAA TCT AGC-3'. An initial denaturation step (2 min at 94° C) was followed by 30 thermocycles each comprising 1 min at 94.0 ° C, 2 min at 55° C, 3 min at 72.0° C and a final elongation step for 7 min at 72.0° C. The PCR amplicon was visualised by electrophoresis in 1.2 % agarose gel containing ethidium bromide under ultraviolet transillumination.

Conventional PCR

Conventional PCR assays were performed using a protocol fully described previously (20). Briefly, the primer set (forward, 5'- GCA TAA GGT GCC CGT GAA AGG-3' ; reverse, 5'-ATT CGC GGT CGC TCC GCT-3') was used at 0.5 µM in 50 µl reaction mixtures, each comprising 25 µl *Taq* Master Mix (Qiagen *Taq* PCR Master Mix Kit), 10 µl Q solution, 5 µl forward primer, 5 µl reverse primer and 5 µl template. An initial denaturation step (2 min at 94° C) was followed by 45 thermocycles each comprising 45 sec at 94.0 ° C, 60 sec at 64.5° C, 60 sec at 72.0° C and a final elongation step for 7 min at 72.0° C. The PCR amplicon (331 bp; nucleotides 787 to 1117 within the sequence of the full PiCV genome clone 9030; accession number AJ298229) was visualised by electrophoresis in 2 % agarose gel containing ethidium bromide under ultraviolet transillumination. This amplicon encompassed the 3' terminus of the ORF V1 encoding the Rep and part of the 3' intergenic region of the PiCV genome. The PCR test was capable of detecting 4 attograms of target DNA.

SYBR Green real time PCR

The SYBR green real time PCR was performed using a method which has been fully described elsewhere (23). Briefly, a primer set (forward YP09, 5'-GGT ACC CGC ATA AGG TGC CCG T-3'; reverse YP10 5'-TTG ATC CGC CGG AAG AGC GCC T-3') was used and all amplification reactions were performed in a total volume of 25 µl using a mini Opticon (Bio-Rad Laboratories, Nazareth, Belgium) with 48-microwell plates. Each well contained 2.5 µl of the extracted DNA sample or positive standard control, 12.5 µl of iQ SYBR® Green Supermix (Bio-Rad, Laboratories, Nazareth, Belgium), 2.5 µl of each diluted primer (2 µM) and 5 µl of distilled water. Samples with copy numbers greater than 10^8 were diluted in water so that they generated values within the standard curve. The thermal profile consisted of a first step of denaturation of 95° C for 15 min and 45 cycles of 3 steps: 95° C, 64.3° C and 72° C each for 30 sec. The melting curve was performed from 45° C to 95° C in 0.5° C/10 sec increments. Each reaction was performed in duplicate. Absolute quantification of copies was carried out by comparing the cycle threshold (Ct) values obtained with the standard curve using the Thermal cycler software (Bio-Rad). Each analysis included a positive control (10^3 copies) and a blank control consisting of distilled water. PCR efficiency (E) was calculated from standard curves using the following formula: % E = [10-1/slope-1] x 100 that gives the percentage of template molecules that was doubled during each cycle. Only runs within the range 95-105 % and with a coefficient of determination (R^2) > 0.98 were analysed.

Indirect immunofluorescence assay

Pigeon sera were diluted in two-fold increments starting at 1:16 in PBS with 0.05% tween 20 containing 10 % calf serum. Sf9 cells were cultured in 24-well microtiter plates and monolayers were infected with the recombinant baculovirus Ac.PiCVcap.ORF C1. For control purposes, sera were tested against both infected and untreated cells. After 3 days the overlaying medium was removed and cells rinsed with PBS. Then the cell monolayer was fixed with paraformaldehyde and permeabilization of cells was performed as described elsewhere (26). Subsequently, an immunofluorescent staining was performed. In brief, 150 µl of dilutions of the pigeon sera were added for 1 h at 37°C. After 3 washing steps, conjugated rabbit anti-pigeon Ig/FITC (Nordic Immunology, Tilburg, The Netherlands) were used as secondary conjugate. All analyses were made in duplicate. Epifluorescence microscopy analysis was performed and the last dilution giving clear fluorescence was considered as the end titre.

Western immunoblot analysis

The Sf9 cells infected with recombinant baculovirus (MOI 5) were harvested 72 h post inoculation. Cells were washed twice with PBS and were lysed by freezing at – 80°C and thawing. Total proteins were separated on a 12 % SDS-PAGE (NuPAGE® Gel, Invitrogen, Merelbeke, Belgium), transferred onto a nitrocellulose membrane and identified with specific mouse monoclonal anti-histidine antibody (Invitrogen, Merelbeke, Belgium) and a chemiluminescence peroxidase substrate kit (Sigma-Aldrich, Bornem, Belgium).

Statistical analysis

For the statistical analysis, data were transferred to Microsoft EXCEL, where the means, standard deviation were calculated. A permutation test was used to determine the statistical significance of the means. Differences were considered significant when $p<0.05$.

RESULTS

Expression of the PiCV ORF C1 protein

PiCV ORF C1 gene was successfully amplified, inserted into the baculovirus transfer vector pBlueBac4.5/V5-His-Topo® and by recombination with Bac-N-Blue™ baculovirus DNA resulted in the construct of a recombinant baculovirus expressing recombinant PiCV capsid protein in Sf9 insect cells harvested 72 h post infection. The western immunoblot analysis with mouse monoclonal anti-histidine antibody demonstrated the presence of a protein of approximately 30 kDA (Figure 1). This protein was not present in the control uninoculated insect cells.
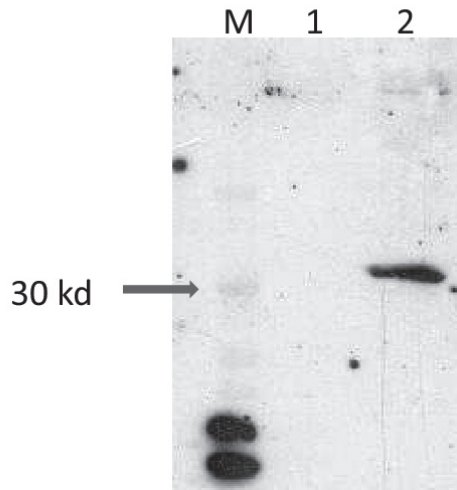

Figure 1: Western blotting analysis of PiCV ORF C1 protein expressed in insect cells Sf9. Lane M is a protein molecular weight marker standard. Total lysate of Sf9 cells infected with Ac.PiCVcap.ORF C1 (lane 1) or not infected (lane 2) was loaded.

Histological studies

Botryoid inclusion bodies were seen in macrophages of the BF of 8 pigeons (Table 1, # 13, 18, 20, 23, 24, 25, 26 and 27). BFs from 3 (Table 1, #: 20, 24 and 27) of the four sick pigeons contained the greatest number of inclusion bodies. No bursitis, necrosis or lymphoid depletion was observed. The BF of pigeon 28 was noted to be smaller than for the age of the bird.

Immunofluorescence assay

The results are presented in Table 1. With the exception of pigeon number 27, serum samples from all the birds were positive for antibodies to PiCV antigen present in insect cells infected with the recombinant baculovirus Ac.PiCVcap.ORF C1. Indirect immunofluorescence (IIF) test $\log_2$ titers varied from 4 to 8 (Figure 2). There were no significant differences between different age groups or between sick and healthy pigeons and pigeons with or without bursal inclusions.
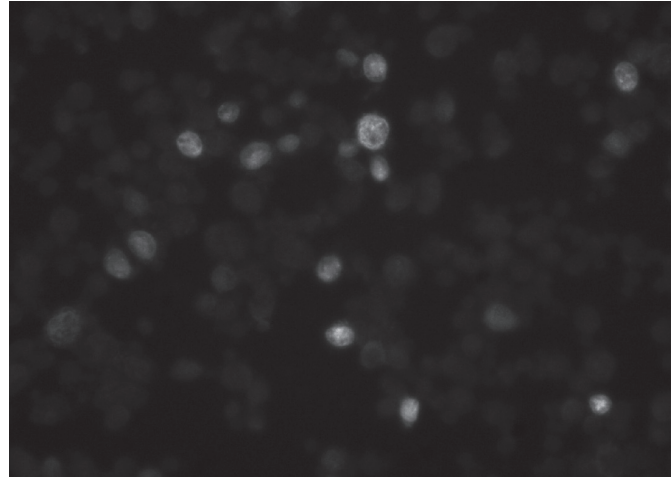

Figure 2: Expression of PiCV capsid protein in Sf 9 cells fixed 72 h post-infection and detected using an IIF assay with pigeon sera and rabbit anti-pigeon-FITC.

PCR and SYBR Green Real Time PCR assays

Results are presented in Table 1. All sera of adult pigeons (1 year or older) were negative by the conventional and SYBR real time PCR assays. Among the 6 eight- month-old pigeons tested, 3 birds (Table 1, # 7, 8 and 9,) were found positive both by PCR and real time PCR with weak viral loads (up to 3.3×10^3 copies per µl of serum). In contrast, a high viral load has been demonstrated in all the young pigeons in both sera (up to 1.84×10^9 copies genome/µl) and bursa (up to 9.6×10^{10} copies genome/ milligram tissue) (Table 1, # 13 to 28).

Viral loads in the sera of sick young pigeons (Table 1, # 20, 24, 25 and 27) were significantly higher when compared to those of clinically healthy ones (Table 1, # 13, 15, 16, 17, 18, 19, 21, 22, 23, 26, 28) when results were submitted to a permutation test (10000 runs; $p = 0.01$). Significant differences (permutation test; 10000 runs; $p = 0,026$) were also observed when the viral loads in sera of pigeons (Table 1, # 13, 18, 20, 23, 24, 25, 26 and 27) with classical botryoid inclusions in BF were compared with the viral loads of pigeons (Table 1, # 15, 16, 17, 19, 21, 22 and 28) without BF inclusions.

BF viral loads in sick pigeons (Table 1, # 20, 24, 25 and 27) were significantly higher (permutation test (10000 runs; $p = 0.015$) when compared to the viral loads of clinically healthy pigeons (Table 1, # 13, 15, 16, 17, 18, 19, 21, 22, 23, 26, 28). Similarly the BF viral loads of pigeons (Table 1, # 13, 18, 20, 23, 24, 25, 26 and 27) with bursal botryoid inclusions were significantly higher (permutation test; 10000 runs; $p= 0.017$) than those of pigeons (Table 1, # 15, 16, 17, 19, 21, 22 and 28) without BF inclusions.

DISCUSSION

To date, avian circoviruses have not been grown in cell culture and detection of specific antibodies has only been performed in a few cases. An IIF assay that utilized goose circovirus capsid protein antigen produced within baby hamster kidney cells by the eukaryotic Semliki forest virus expression vector has been described for geese (27) and a hemagglutination inhibition assay was developed for psittacine beak and feather disease (PBFD) virus serum antibody (28). Baculovirus expression of PBFD virus capsid protein was a suitable replacement antigen for serological detection of the psittacine circovirus by hemagglutination inhibition assay (29). An indirect enzyme-linked immunosorbent assay for the detection of PiCV-specific serum antibody is now available. By testing 118 field sera collected in the years 1989, 1991, 1994 and 2008, virus-specific antibody was detected in 75% of the sera, without correlation for the disease status of the birds (25).

Serological tests for detecting virus-specific antibodies are important tools for the diagnosis of infection and epidemiological studies, and as in the present study, to investigate if there is a relationship between disease status and antibody levels. The IIF assay described here is a fast, specific and sensitive method for the detection and titration of circulating antibodies to PiCV in pigeon serum. In the present study, the antibody titers were relatively low; only 4 pigeons out of 28 had a titre of 8 expressed in $\log_2$ IIF titres. One four-week-old pigeon (Table 1, # 27) presenting with the signs of YPDS was serologically negative. This could be due to early infection leading to immunotolerance or to the direct effect of the virus on the immune system, or it could be that maternal derived antibodies has waned, and the bird has not yet developed its own antibodies. The three other affected birds (Table 1, # 20, 24 and 25) had serum $\log_2$ titres between 8 to 4. There was no significant difference between the titers in sick and clinically normal birds. This is in contrast to the findings in psittacine birds where a hemagglutination inhibition assay showed that antibody titers could not be detected in serum from 42 PBFD-affected birds whereas serum titers from 64 normal psittacine birds ranged from less than $\log_2$ 1 to $\log_2$ 8 (29) and the observation that birds with active PBFD virus infections had lower antibody value than birds that had been exposed to the virus, but remained clinically normal (28). For CAV, maternal antibodies provide complete protection of young chicks against the virus for approximately the first 3 weeks of life (31, 32). Although the adult pigeons have antibodies as measured by IIF, infection and the disease seem to reoccur in young pigeons annually. It may be that either maternally derived antibody to PiCV is not protective or that the majority of the young pigeons are infected in the rearing loft after 3 weeks of age (12). In this scenario, it may be relevant that the primary effect of CAV is depletion of the T cells in the thymus with minimal effect on the B cells in the BF, while PiCV appears to deplete both the B and T cells populations which may impair severely the ability to mount an antibody response. This difference may influence the pathogenesis (16).

These preliminary results suggest that the antibodies measured by IIF play a minor role as far as infection and disease expression are concerned or possibly that some young birds do not have the time or ability to develop a suitable antibody response.

The present study also describes the quantification of viral load by real time PCR, in samples of serum and BF collected from clinically healthy or naturally affected pigeons. The results show that in sick pigeons viral load is significantly higher in BF and serum than in clinically healthy pigeons. This suggests that the detection of high levels of virus could be associated with the clinical status of the birds.

In pigs, PMWS is associated with PCV2 infection. In this species, the capsid protein was found to be a major immunogen able to protect pigs against a PCV2 challenge (33). A recombinant PBFD virus capsid protein assessed as a vaccine for psittacine beak and feather disease protected vaccinated birds from feather lesions and, reduced viral replication in vaccinated birds compared with non-vaccinated control birds (34). Thus the capsid protein of PiCV expressed by recombinant baculovirus in insect cells could be considered as a potential vaccine candidate in three week-old unprotected pigeons and for vaccination before mating to increase maternal derived antibody levels.

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