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Effect of a Commercial Paratyphus Vaccine on the Development of Pigeon Circovirus Infection in Young Pigeons (*Columba livia domestica*)

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Abstract: Infection with pigeon circovirus (PiCV) has been associated with young pigeon disease syndrome (YPDS), which is considered to be a multifactorial disease. The factors that determine whether birds succumb to clinical disease are not known. To evaluate the potential effect of vaccination with a commercial paratyphus vaccine on the progression of PiCV infection in young pigeons, forty 6-week-old pigeons naturally infected with PiCV were randomly assigned to two equal groups. The pigeons of one group were vaccinated at 6 and 9 weeks of age, and pigeons of the second group were unvaccinated controls. Cloacal swab and blood samples collected from all the birds were tested for PiCV by polymerase chain reaction (PCR) analysis. Three weeks after the second vaccination, all pigeons were euthanatized, and tissues were collected for PiCV PCR analysis and histopathologic evaluation. No significant difference in the number of PCR-positive cloacal swab and blood samples was found between the vaccinated and control pigeons. Positive PCR results in tissue samples also were not significantly different between the groups, with 18 positive samples in vaccinated birds (90%) and 16 in control birds (80%). Characteristic botryoid inclusions were detected in more vaccinated than control pigeons, but this difference was not significant. In this study, vaccination with a commercial paratyphus vaccine was not a risk factor for development of young pigeon disease syndrome.

Key words: pigeon circovirus, infection, vaccination, paratyphus, avian, pigeon, *Columba livia domestica*

Introduction

Pigeon circovirus (PiCV) infection is an emerging problem that has been reported worldwide in both young racing and meat pigeons.^{1–10} This infection has been associated with young pigeon disease syndrome (YPDS), also known as young pigeon sickness.^{11–13} Pigeon circovirus infection has been identified in pigeons (*Columba livia*

domestica) between 1 day and 6 months of age, and is characterized by a broad range of nonspecific clinical signs, including lethargy, anorexia, weight loss, ruffled feathers, respiratory distress, vomiting, diarrhea, polyuria, fluid-filled crop, poor racing performance, and mortality.^{11,13,14} The predisposition to secondary infections is suggestive of immunosuppression.¹⁵ The genome of PiCV has been sequenced,^{16,17} and, on the basis of similarities in their genomes, PiCV is classified, together with psittacine beak and feather disease virus, porcine circovirus types 1 and 2, goose circovirus, canary circovirus, and duck circovirus, as a member of the genus *Circovirus* in the family *Circoviridae*.^{3,17–20}

To date, no reports have described the isolation and propagation of PiCV in cultured cells. In the

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absence of virus-specific antisera for antigen detection, infection with PiCV was previously based on histologic and electron microscopic findings. 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, which can sensitively detect viral DNA.^{21–26} On histologic examination, lymphocyte depletion and the presence of characteristic intracytoplasmic botryoid inclusion bodies within lymphoid tissues, commonly in macrophages and particularly in the bursa of Fabricius, have frequently been reported either singly or together in infected birds. However, because lymphocyte depletion is not always present and can sometimes be caused by other agents, the only pathognomonic lesion of PiCV is the inclusion body composed of arrays of tightly packed virus particles. In situ hybridization has shown that PiCV in naturally infected young birds can be detected in a wide range of tissues, including the bursa, thymus, liver, kidney, brain, crop, and intestine, even when microscopic lesions are not obvious.²¹ By using PCR, PiCV was also detected in the bursa of a high proportion of young pigeons, including clinically and histologically normal birds, which suggests that many PiCV infections may be subclinical.^{24,27,28} Used semiquantitatively, dot blot hybridization has shown that tissue from the bursa of some birds contains considerable amounts of viral DNA; this finding has led to speculation that clinical effects may depend on viral load.^{21,24}

Although YPDS is considered to be a multifactorial disease, the factors that determine whether birds succumb to clinical disease are not known. An opinion held by some pigeon fanciers is that administration of a commercially available paratyphus vaccine exacerbates the severity of clinical signs attributed to YPDS. Experimental investigations demonstrated the pathogenic effects caused by porcine circovirus type 2, which were exacerbated by the administration of oil-adjuvanted mycoplasma vaccines.^{29,30} This has led to speculation that the pathogenicity of PiCV may be similarly affected.

The objective of this study was to evaluate the effect of vaccination of 6-week-old pigeons (an age at which YPDS is usually observed under field conditions) with a commercially available paratyphus vaccine on the progression of PiCV infection and detection of PiCV in cloacal swab, blood, and tissue samples. PCR assays were used to investigate the occurrence

of PiCV in tissue samples from vaccinated and nonvaccinated pigeons from a single pigeon loft in which PiCV infections had been previously diagnosed.

Materials and Methods

Pigeons

Forty 6-week-old pigeons were used in the study. The birds were obtained from a loft in which infection with PiCV had been previously diagnosed in young pigeons by results of histologic and electron microscopic examination and PCR testing. As pigeons arrived in the loft, fecal examinations were performed and cloacal swab samples were collected for aerobic culture to screen for *Salmonella* species. Once the birds were found to be free of infection with parasites or salmonella, they were randomly assigned to two equal groups: V (vaccinates) and C (controls). All the birds were housed together in a large cage inside a clean building. The protocol was approved by the animal care and use committee of the University of Liège.

Experimental design

A commercially available inactivated paratyphus vaccine (Colombovac Paratyphus, Fort Dodge Animal Health, Weesp, Netherlands) was used in the study. The vaccine was formulated by using a strain *Salmonella typhimurium* var. Copenhagen isolated from pigeons and was suspended in an aqueous Carbomer adjuvant. Pigeons in group V (V1–V20) were injected at 6 weeks of age with 0.2 mL of vaccine administered subcutaneously in the neck. A booster dose (0.2 mL) was injected 3 weeks later according to the manufacturer's instructions. Pigeons in group C (C1–C20) were not vaccinated and were kept as controls. Before the first and second vaccinations and euthanasia, cloacal swabs were collected from all the pigeons. In addition, approximately 0.2 mL of blood was collected from the medial metatarsal vein of each bird and was stored at -20°C (-4°F) before analysis.

Three weeks after the second vaccination, all the pigeons were euthanatized by barbiturate overdose (86 mg/kg administered intraperitoneally) and submitted for necropsy. Samples of liver, spleen, intestine, thymus, and bursa were submitted for PiCV PCR analysis, and additional tissue samples were collected and fixed in 10% neutral buffered formalin. Formalin-fixed samples were dehydrated, embedded in paraffin wax, and

sectioned at 4 μ m for examination by light microscopy. All sections were stained with hematoxylin and eosin.

Extraction of viral DNA

Viral DNA was extracted from unfixed samples with a commercially available nucleic acid purification kit by following the manufacturer's instructions (Qiaamp DNA Mini Kit, Qiagen, Venlo, Netherlands). Cloacal swab samples were resuspended by washing in 0.5 mL sterile, distilled, deionized water, and a volume of 400 μ L was used for DNA extraction. The same methodology was used to extract DNA from swabs that contained dried blood. Similar extractions were performed with 10 mg of tissue from the spleen as well as 50 mg of tissue from each of the other organs collected at necropsy. Extracted DNA was eluted with 100 μ L elution buffer and stored at -20°C (-4°F) until the time of analysis.

Polymerase chain reaction

A PCR assay was performed by using an adaptation of a test previously described.²⁶ Briefly, primers 5'-GCA TAA GGT GCC CGT GAA AGG-3' (forward) and 5'-ATT CGC GGT CGC TCC GCT-3' (reverse) were used in 50 μ L reaction mixtures, each composed of 25 μ L Taq Master Mix, 10 μ L Q Solution (Taq PCR Master Mix Kit, Qiagen), 5 μ L forward primer, 5 μ L reverse primer, and 5 μ L template. An initial denaturation step (2 minutes at 94.0°C [201.2°F]) was followed by 20 thermocycles, each comprising 45 seconds at 94.0°C (201.2°F), 1 minute at 64.5°C (148.1°F), 1 minute at 72.0°C (161.6°F), and a final elongation step for 7 minutes at 72.0°C (161.6°F). By reducing the number of cycles from 45 to 20, the sensitivity of the test is reduced by a factor of 10^3 , such that only the samples with viral DNA in excess of 10 femtograms (fg) (1300 copies) are detected as positive (D. T., unpublished data, May 2005). The PCR amplicon (330 base pairs) was visualized by electrophoresis in 2% agarose gel that contained ethidium bromide under ultraviolet transillumination.

Statistical analyses

The Fisher exact test for contingency tables (2×2) was applied to assess whether the number of positive tests was different between vaccinated and unvaccinated groups. Results were considered significant at $P \leq .05$.

Results

No clinical signs were observed in any of the pigeons during the study period, and no significant gross lesions were observed at necropsy. Most microscopic lesions were seen in the bursa, with the exception of those in 2 control pigeons. In one of these birds (C6), characteristic botryoid inclusions were detected in the thymus, and the other (C7) exhibited nodular lymphoid hyperplasia and a small number of cytoplasmic inclusion body-like structures in the villous intestinal cells. Botryoid inclusion bodies were seen in macrophages within the bursa in most vaccinates (16/20) and control pigeons (11/20), but the difference was not significant (Table 1). No inflammation, necrosis, or calcification was seen in the bursa of any vaccinated pigeons, and only one bird (V20) demonstrated mild lymphoid depletion. In contrast, moderate-to-severe diffuse acute bursitis was seen in 5 control pigeons (Fig 1), and 4 other control birds demonstrated multifocal to medullary anhistiocytic calcification. Mild lymphoid depletion was also seen in 2 control pigeons. The difference between the numbers of birds with bursal lesions detected in the control group (9) and the vaccinated group (0) was highly significant ($P < .01$).

The difference between the number of PCR-positive cloacal swab and blood samples from vaccinated and control pigeons was not significant (Table 1). Tissue samples retrieved at necropsy yielded positive PCR results in 18 vaccinates (90%) and 16 control birds (80%). Results of PCR testing of individual tissues were not significantly different between vaccinated and control groups (Table 2). All tissues analyzed were PCR positive in 8 control birds (C2, C4, C5, C6, C7, C10, C13, and C17) compared with 4 birds in the vaccinated group (V4, V10, V15, and V18); however, this difference was not significant. No inclusions were detected in the bursa of control pigeons C4 and C10 and only a few were observed in pigeon C7. Three control birds (C4, C5, and C13) and 2 vaccinates (V10 and V15) were PCR positive for the 3 cloacal swab and blood samples as well as all organ tissues tested.

Discussion

In this study, administration of Colombovac Paratyphus vaccine to 6-week-old pigeons naturally infected with PiCV did not affect the course of the infection nor did it induce signs of young pigeon disease syndrome. These results are similar to those obtained when commercial adjuvant was

Table 1. Histologic lesions in the bursa of Fabricius and results of polymerase chain reaction (PCR) assay for pigeon circovirus (PiCV) in naturally infected pigeons. Pigeons C1–C20 were controls and pigeons V1–V20 were vaccinated with a commercially available inactivated paratyphus vaccine (Colombovac Paratyphus, Fort Dodge Animal Health, Weesp, Netherlands).

Pigeon	No. of positive/total PCR tests			Histologic lesions/bursa	Lymphoid depletion
	Cloaca	Blood	Organs		
C1	0/3	0/3	1/5	IB (+++) necrotizing bursitis	—
C2	2/3	3/3	5/5	IB (+++) necrotizing bursitis	Mild
C3	0/3	1/3	0/5	—	—
C4	3/3	3/3	5/5	Anhistic calcification	—
C5	3/3	3/3	5/5	IB (+++)	—
C6	2/3	2/3	5/5	IB (+++) anhistic calcification	—
C7	0/3	0/3	5/5	IB (+)	—
C8	0/3	0/3	3/5	—	—
C9	0/3	0/3	4/5	IB (+) anhistic calcification	—
C10	1/3	0/3	5/5	Anhistic calcification	—
C11	0/3	0/3	2/5	—	—
C12	0/3	0/3	0/5	—	—
C13	3/3	3/3	5/5	IB (+++) bursitis	—
C14	0/3	0/3	0/5	Necrotizing bursitis	—
C15	0/3	0/3	3/5	IB (+)	—
C16	0/3	1/3	2/5	IB (+)	—
C17	1/3	0/3	5/5	IB (++)	—
C18	1/3	0/3	4/5	Necrotizing bursitis	Mild
C19	0/3	0/3	1/5	IB (+)	—
C20	0/3	0/3	0/5	—	—
V1	0/3	0/3	1/5	IB (+)	—
V2	1/3	2/3	4/5	—	—
V3	0/3	0/3	3/5	IB (+)	—
V4	2/3	2/3	5/5	IB (+++)	—
V5	0/3	0/3	2/5	—	—
V6	0/3	0/3	0/5	—	—
V7	0/3	0/3	3/5	IB (+)	—
V8	1/3	0/3	3/5	IB (+)	—
V9	2/3	1/3	2/5	IB (++)	—
V10	3/3	3/3	5/5	IB (+++)	—
V11	3/3	0/3	2/5	IB (+)	—
V12	0/3	0/3	1/5	IB (++)	—
V13	2/3	1/3	4/5	IB (+)	—
V14	0/3	0/3	2/5	IB (+)	—
V15	3/3	3/3	5/5	IB (+++)	—
V16	1/3	1/3	2/5	IB (++)	—
V17	0/3	0/3	0/5	—	—
V18	1/3	2/3	5/5	IB (+++)	—
V19	0/3	0/3	3/5	IB (+)	—
V20	1/3	0/3	4/5	IB (+++)	Mild

Abbreviations: IB indicates botryoid inclusions of PiCV; +, few; ++, moderate; +++, many; —, negative or none seen.

administered to conventional pigs experimentally infected with porcine circovirus type 2 and subsequently observed for the development of postweaning multisystemic wasting syndrome.³¹

Although histopathologic examination indicated that the botryoid inclusions characteristic of circovirus infection were detected in more vaccinated pigeons (16/20) than in control pigeons (11/

20), the difference was not significant. Based on the significant differences in histopathologic lesions (bursitis, necrosis, and anhistic calcification) observed between the vaccinated and control groups of pigeons, results suggest that vaccination with Colombovac Paratyphus vaccine may have had a protective effect. Previous reports indicate that the aqueous Carbomer adjuvant is

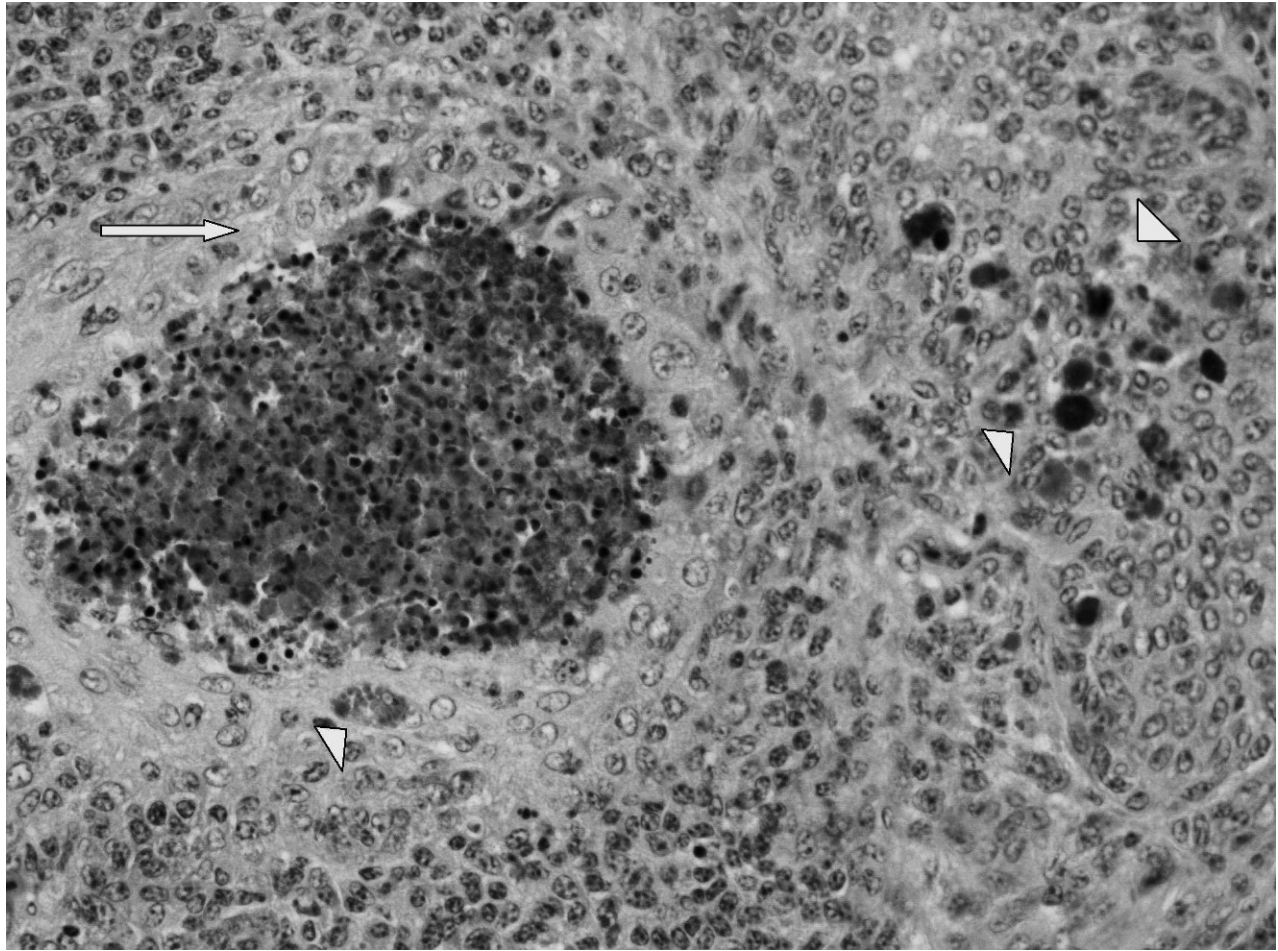


Figure 1. Acute inflammation (arrow) and botryoid inclusions (arrowheads) in the bursa of Fabricius of a pigeon from a loft in which pigeon circovirus infection had been previously diagnosed in pigeons (hematoxylin and eosin, $\times 400$).

particularly well tolerated and markedly enhances the immune response when compared with the oil-emulsion vaccines.^{32,33} We can hypothesize that vaccination by means of the aqueous adjuvant stimulates the production of interferon, which is known to play an important role in the recovery of parrots infected with psittacine circovirus.³⁴

Our experimental approach involved using a PCR assay to investigate the presence of PiCV DNA in tissues from vaccinated and unvaccinated pigeons. The PCR test originally developed used 45 thermocycles and was capable of detecting as little as 0.01 fg DNA under experimental conditions.²⁶ By reducing the number of thermocycles from 45 to 20 in this study, the threshold of detection was increased to approximately 10 fg; therefore, larger amounts of viral DNA were required for detection of a positive PCR signal. Earlier work showed that

PCR could detect PiCV DNA in a high proportion of tissues from young infected pigeons.^{22,24} On this basis, it was likely that most of the tissue samples from the experimental pigeons would have been positive when using the 45-cycle PCR test. Use of the less-sensitive 20-cycle test was designed to provide greater differentiation. In this investigation, the choice of tissues analyzed by PCR was based on previous studies in which PiCV was detected in the spleen, liver, intestine, and primarily in the bursa of Fabricius.^{21,26} In our study, results of PCR testing on tissues were not significantly different between the vaccine and control groups. In both groups, PiCV DNA was most commonly detected in the bursa, followed by the spleen, intestines, liver, and thymus. The numbers of PCR positive results obtained from blood and cloacal swab samples were also not significantly different between the two groups.

Table 2. Results (+/–) of a polymerase chain reaction assay for pigeon circovirus in tissues harvested at necropsy from 40 pigeons naturally infected with pigeon circovirus described in Table 1.

Pigeon	Bursa of Fabricius	Spleen	Liver	Thymus	Intestine	Total +/total tissues tested
C1	+	–	–	–	–	1/5
C2	+	+	+	+	+	5/5
C3	–	–	–	–	–	0/5
C4	+	+	+	+	+	5/5
C5	+	+	+	+	+	5/5
C6	+	+	+	+	+	5/5
C7	+	+	+	+	+	5/5
C8	+	+	–	–	+	3/5
C9	+	+	+	–	+	4/5
C10	+	+	+	+	+	5/5
C11	+	+	–	–	–	2/5
C12	–	–	–	–	–	0/5
C13	+	+	+	+	+	5/5
C14	–	–	–	–	–	0/5
C15	+	+	–	–	+	3/5
C16	+	–	–	+	–	2/5
C17	+	+	+	+	+	5/5
C18	+	+	+	–	+	4/5
C19	+	–	–	–	–	1/5
C20	–	–	–	–	–	0/5
TOTAL	16	13	10	9	12	60/100
V1	+	–	–	–	–	1/5
V2	+	+	+	–	+	4/5
V3	+	+	–	–	+	3/5
V4	+	+	+	+	+	5/5
V5	+	+	–	–	–	2/5
V6	–	–	–	–	–	0/5
V7	+	+	–	–	+	3/5
V8	+	+	–	–	+	3/5
V9	+	+	–	–	–	2/5
V10	+	+	+	+	+	5/5
V11	+	–	–	–	+	2/5
V12	+	–	–	–	–	1/5
V13	+	+	+	+	–	4/5
V14	+	+	–	–	–	2/5
V15	+	+	+	+	+	5/5
V16	+	–	–	–	+	2/5
V17	–	–	–	–	–	0/5
V18	+	+	+	+	+	5/5
V19	+	+	–	–	+	3/5
V20	+	–	+	+	+	4/5
TOTAL	18	13	7	6	12	56/100

Some birds in both the control and vaccinate groups experienced viremia, and PiCV DNA was detected in tissues of most of the pigeons in both groups, although signs of disease were not apparent. This may be explained by the fact that pigeons used in the study were housed under ideal conditions and were at low risk for secondary infections. These results are similar to the observations made in a study of pigs observed for the development of postweaning multisystem-

ic wasting syndrome.³¹ This same observation was made in a recent study in which experimental infection of domestic pigeons with PiCV in combination with vaccination against paramyxovirus failed to reproduce the clinical signs of YPDS, leading the authors to suggest that stressful conditions may serve as a trigger for development of YPDS.³⁵

The results of our study are encouraging, and the role of the Carbomer adjuvant must be tested

in future work along with evaluating the viral load of PCR-positive organs by quantitative PCR. Studies are also required to measure the effect of vaccines and the timing of administration on viral synergism and the development of lesions associated with pigeon circovirus infection.

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