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A Longitudinal Study on Avian Polyomavirus-specific Antibodies in Captive Spix's Macaws (*Cyanopsitta spixii*)

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Abstract: Avian polyomavirus (APV) causes a range of disease syndromes in psittacine birds, from acute fatal disease to subclinical infections, depending on age, species, and other unidentified risk factors. To determine the prevalence of APV-specific antibodies in a captive population of Spix's macaws (*Cyanopsitta spixii*), 54 birds were tested by blocking enzyme-linked immunosorbent assay. A prevalence of 48.1% for APV antibodies, which indicates viral exposure, was found. Of 36 Spix's macaws that were serially tested over a period of 4 years, 50.0% were consistently positive, 36.1% were consistently negative, 5.5% had permanently declining antibody levels, and 2.8% showed variable results. By using polymerase chain reaction testing on whole blood samples, an apparent viremia was detected in 1 of 44 birds (2.3%), although contamination provides a likely explanation for this isolated positive result in a hand-reared chick. The white blood cell count was significantly higher in antibody-positive birds compared with antibody-negative birds ($P < .05$). Because antibody-positive and antibody-negative birds were housed together without a change in their respective antibody status, transmission of APV within the adult breeding population appeared to be a rare event.

Key words: avian polyoma virus, antibodies, blocking enzyme-linked immunosorbent assay, avian, Spix's macaws, *Cyanopsitta spixii*

Introduction

Avian polyomavirus (APV) was first described as the causative agent of budgerigar fledgling disease, an acute fatal disease of budgerigars (*Melopsittacus undulatus*) that causes up to 100% mortality in neonates and was subsequently designated as budgerigar fledgling disease virus (BFDV).[1–4] Thereafter, APV has been detected repeatedly in other psittacine birds[5–9] and sporadically in nonpsittacine bird species.[10–12]

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In nonbudgerigar psittacine species, APV infection of hand-reared nestlings can lead to either sudden death, without any premonitory signs, or to acute fatal disease characterized by a brief period of lassitude; anorexia; delayed crop emptying; and extensive subcutaneous, epicardial, and serosal hemorrhages before death.[5,6] Among parrots, macaws, conures, Eclectus parrots (*Eclectus roratus*), and lovebirds (*Agapornis* species) display a particular susceptibility to APV disease.[13,14] Conversely, infection of parent-raised chicks and adult birds, in most cases, is not synonymous with disease.[11,13,15] Asymptomatic, juvenile, and young adult budgerigars are suggested as being responsible for transmission of the virus, through droppings and skin and feather dander, to neonatal birds, which are at a substantially higher risk of fatal disease.[14–17] In the case of nonbudgerigar psittacine birds, however, the lack of consistent epidemiologic data and the variation observed among species precludes a definite conclusion regarding persis-

tence of antibody titers, duration of viremia, and virus shedding in natural APV infections.[8,9,18–21]

The APV is a polyomavirus known to be highly stable in the environment.[2,3,22,23] All APV strains characterized so far belong to 1 genotype and 1 serotype.[10,24–26] Diagnosis of APV infection in live birds can be done by a polymerase chain reaction (PCR) on blood, feathers, and cloacal swab samples.[8,10,27] In addition, detection of APV-specific antibodies can be performed by using either a serum neutralization test or a blocking enzyme-linked immunosorbent assay (BELISA); the results of both tests have been shown to correlate well with each other.[9,17,24,28]

The Spix's macaw (*Cyanopsitta spixii*) is often considered to be the most threatened parrot in the world and was duly classified as "critically endangered (possibly extinct in the wild)" after the last known male disappeared from its native habitat in late 2000.[29,30] Of a captive population of 78 Spix's macaws, which are part of an international recovery effort, 50 birds, which represent almost 65% of the extant population, are kept at Al Wabra Wildlife Preservation (AWWP), Qatar. In total, AWWP has been home to 62 birds up until 2007.

Materials and Methods

The 54 Spix's macaws in this study included 23 males and 31 females. Most of the birds had been acquired between 2000 and 2004 by AWWP from captive populations in the Philippines and Switzerland. Unfortunately, no reliable health or management records existed for these birds before their transfer to AWWP. Between 2004 and 2007, 16 chicks were hatched at AWWP, and all the chicks were hand reared and housed in isolation from the adult birds. The Spix's macaws at AWWP are maintained in aviary complexes separate from the other avian species kept at the facility.

The age of the birds tested ranged from 1 to 22 years. All the birds were housed in aviaries with inside air-conditioned rooms and outdoor flights with artificial rainfall systems. They were usually kept either individually or in pairs, except in some cases in which juveniles were flocked. The birds had been subjected to annual health checks since 2004, during which they were tested for APV among other pathogens. The facility had no history of APV vaccination, clinical APV infection, or evidence of APV-associated mortality in the Spix's macaw population.

For blood sample collection, each bird was anesthetized with isoflurane, and approximately 2.5 mL of blood was collected by jugular venipuncture and then stored in lithium-heparin Microtainer tubes. A fresh thin blood smear was prepared immediately from each bird by using the slide-to-slide method. Plasma was separated within 4 hours of collection and frozen (−20°C) until dispatched to the laboratory for detection of APV antibodies. For detection of APV DNA, approximately 0.2 mL of blood was also stored immediately after collection in microtubes of 1.3-mL capacity that contained potassium ~~EDTA~~ and frozen (−20°C) until dispatched to the laboratory. Feather samples for APV DNA analysis were stored in resealable plastic bags.

The presence of APV-specific antibodies was tested by BELISA.[24] The test uses a monoclonal antibody directed against the major structural protein VP1 of APV in an enzyme-linked immunosorbent assay (ELISA) format with purified APV particles as antigen and measures the inhibition of color reaction of a test serum compared with a defined negative control serum. According to results of a serum neutralization test, more than 50% inhibition is considered positive, whereas less than 50% inhibition is considered negative.[24] The BELISA has been validated for several psittacine bird species.[24,31] In subsequent years after the initial sampling, 36 Spix's macaw individuals were resampled. Testing intervals for an individual ranged from 1 to 28 months, with a median of 10 months between consecutive tests.

Testing for the presence of the APV genome in blood or feathers was performed by PCR. The DNA was isolated from blood samples by using the DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany) as recommended by the supplier. For DNA preparation from feather samples, a proteinase K digestion, followed by phenol-chloroform-isoamylalcohol extraction and ethanol precipitation, was performed as described previously.[32] The PCR analysis was done by amplification of a 310-bp fragment of the APV genome, as described previously,[10,33,34] but by addition of SYBR Green I dye (Invitrogen BV, NV Leek, The Netherlands) and PCR product melting curve analysis in a Rotorgene 2000 Thermal Cycler (Corbett Research Australia, Mortlake, NSW, Australia). Precautions were taken to prevent laboratory contaminations, and all reactions were controlled by the use of negative DNA isolation controls as well as negative PCR reaction controls. The PCR reactions were first analyzed by melting curve analysis, and samples that showed a melting

peak between 80°C and 85°C were further analyzed by electrophoresis on ethidium bromide–stained 2% agarose gels. The PCR products with a length of 310 bp were considered positive.

Fresh thin blood smears stained with Diff-Quick stain (Medion Diagnostics AG, Duedingen, Switzerland) were evaluated for total white blood cell counts (WBC) and differential leukocyte counts by following methods described previously.[35] Plasma biochemical analyses were processed on a dry automated analyzer (Spotchem EZ SP-4430, A. Menarini Diagnostics, Neuss, Germany).

The hematologic and biochemical data from 2004 to 2006 were evaluated separately for juveniles (birds less than 4 years; n = 16 testing occasions) and adults (n = 89 testing occasions). Only decreased or increased parameters, compared with reference ranges recently established for the species,[35] were examined. Differences between birds with positive and negative APV titers were assessed by the *t* test for parameters with normal distribution. Because the percentage of monocytes was not normally distributed, differences in this parameter were tested ~~by *U* test~~. Statistical analyses were performed by using the statistical package SPSS 14.0 (SPSS Inc, Chicago, IL, USA), with $P < .05$ as the significance threshold.

Results

All the birds were found to be normal on clinical examination for physical parameters such as body condition, general appearance, heart and respiratory rate, palpation of abdominal organs, and appearance of choanal and cloacal mucous membranes.

From 2004 to 2007, a total of 144 plasma samples from 54 individuals were tested for APV-specific antibodies. Of these, 48.1% of the birds (26/54) were found to be positive on at least 1 sampling occasion in the BELISA, whereas 51.9% (28/54) always tested negative. Of 36 Spix's macaws that were tested again in subsequent years for APV, 91.7% (33/36) displayed a consistent APV-BELISA result, with 18 birds showing consistently positive and 15 showing consistently negative results (Table 1). Only 3 birds showed changing APV-BELISA results in the study period: 2 had initial positive results followed by a gradual decline to negative results, and 1 showed high percentages of inhibition (positive results) in 3 tests interspersed with 1 negative result (0% inhibition).

A closer examination of the BELISA percentages of inhibition revealed some fluctuations for each individual over the time period; however, they remained well above 70% in all of the consistently seropositive birds. The mean percentage of inhibition (with 95% confidence interval) of the APV-BELISA positive subpopulation ranged from 86.8% (82.2%–91.4%, n = 13) in 2005 to 98.8% (98.2%–99.7%, n = 16) in 2007. In case of the APV-BELISA negative birds, mean percentage of inhibition remained consistently between 16.7% (9.5%–23.9%, n = 10) and 13.3% (9.4%–17.3%, n = 14) in 2004 and 2006, respectively. To date, all AWWP hatched and hand-reared chicks tested (n = 10) have always shown negative APV-BELISA results in repeated samples. However, this was not the case with juveniles imported from Switzerland or the Philippines, with the youngest bird showing a positive APV-BELISA result at the age of 1 year (Table 1; bird 35). In addition, investigation into the origin of all APV-antibody positive birds indicated that, with the exception of 1 bird of Swiss origin, all seropositive birds originated from the Philippines.

Between 2004 and 2006, whole blood or feather samples or both were also tested for the presence of APV DNA by PCR. In total, 81 samples (69 blood only, 11 combined blood and feather, 1 feather only) from 44 birds were tested over the period of 3 years. Of these, 2.3% birds (1/44) tested positive for APV-specific DNA in whole blood samples. All feather samples were negative. A comparison of results of PCR testing with APV-BELISA results of individual birds revealed that the PCR-positive bird (bird 31) tested negative in BELISA (Table 1).

A summary of mean, SD, and range of hematologic values and aspartate aminotransferase (AST) concentrations is shown in Table 2. No statistically significant differences were found between APV-BELISA positive and negative juvenile birds for total WBCs, percentage of lymphocytes, percentage of monocytes, or AST concentrations (data not shown). However, the total WBC was significantly higher ($P = .02$) in APV positive birds than in APV negative birds, and AST concentrations were significantly higher ($P = .014$) in APV negative birds than in APV positive birds.

Discussion

The purpose of this study was to perform a retrospective evaluation of data recording clinical

Table 1. Summary of serologic results by BELISA of Spix's macaws (N = 36) tested for APV-specific antibodies between 2004 and 2007. The percentage of inhibition is shown; percentages >50% are considered positive, percentages <50% are considered negative; APV-PCR positive results are indicated with (*).

	Persistent BELISA: positive results (%) by year (n = 18)				
Bird no.	2004	2005	2006	2006 (retest)	2007
1	89[4]	85[4]	—	—	100[4]
2	89[36]	73[36]	96[36]	—	100[29]
3	95[5]	79[5]	—	—	100[5]
4	98	90[1]	—	—	100[1]
5	95[3]	89[3]	—	—	100[3]
6	95	88	—	—	—
7	90[28]	—	100[29]	90[29]	—
8	95	97[15]	—	—	100[15]
9	90[10]	96[10]	100[10]	90[10]	100[14]
10	87[9]	94[9]	100[9]	89[9]	100[27]
11	—	—	—	100	100
12	93	—	—	—	99[13]
13	92[14]	—	—	—	100[12]
14	94[13]	—	100[18]	—	99[9]
15	—	90[8]	—	—	100[8]
16	95[34]	76[34]	97[34]	—	88[34]
17	88	83	100[32]	90[32]	100[32]
18	—	90[14]	100[14]	—	97
Persistent BELISA: negative results (%) by year (n = 15)					
19	—	13	20	—	—
20	1[21]	—	16[28]	16[28]	—
21	15[20]	—	11[25]	13[25]	47[25]
22	16[23]	—	13[23]	—	—
23	21[22]	—	24[22]	—	—
24	16[26]	23[26]	—	—	—
25	20[27]	—	15[21]	22[21]	16[21]
26	19[24]	15[24]	11	—	0
27	26[25]	26[25]	13	—	0[10]
28	1[7]	—	21[20]	—	—
29	33	—	19[7]	—	0[2]
30	—	19	13	—	0[31]
31	—	—	12(*)	14[30]	0[30]
32	—	0	1[17]	—	—
33	—	16	0	—	—
Changing results (%) by year (n = 3)					
34	67[16]	50[16]	23[16]	—	9[16]
35	—	78	36	26	14
36	75[2]	0[2]	94[2]	—	64[7]

Abbreviations: BELISA indicates blocking enzyme-linked immunosorbent assay; APV, avian polyomavirus; PCR, polymerase chain reaction.

The cage mate at the time of testing, when available, is indicated in superscript and corresponds to the "Bird no." column (eg, bird 7 was housed with birds 28 and 29 in 2004 and 2006, respectively).

health status, APV-specific antibody titers, and PCR results for APV-DNA in a closed captive population of unvaccinated adult Spix's macaws over a period of 4 years. These results should contribute to the understanding of the nature and course of natural APV infection within this population at AWWP and, possibly, to augment recent efforts in determining the duration of persistence of antibodies after naturally acquired APV infections in macaw species.

Attempts have been made to characterize the persistence of antibodies and the duration of cloacal virus shedding for APV in several psittacine bird species. The variation observed

Table 2. Mean, SD, and range of hematologic values, as well as AST concentrations for adult Spix's macaws with regard to positive or negative results in APV-BELISA testing from 2004 to 2006.

Parameter	Positive APV titer, mean ± SD (range), n = 53	Negative APV titer, mean ± SD (range), n = 36
White blood cells ($\times 10^3/\mu L$)	10.8 ± 4.4 (3–25)[a]	8.6 ± 4 (2.8–17.2)[a]
Heterophils (%)	59.9 ± 15.9 (26–83)	61.7 ± 13.2 (32–85)
Lymphocytes (%)	37 ± 16.5 (8–73)	35.2 ± 13.1 (14–67)
Monocytes (%)	3.2 ± 2.6 (1–12)	3.5 ± 2.4 (1–13)
AST (U/L)	136 ± 49.9 (21–364)[b]	172.9 ± 77.1 (82.8–471)[b]

Abbreviations: AST indicates aspartate aminotransferase; APV, avian polyomavirus; BELISA, blocking enzyme-linked immunosorbent assay.

[a] Differences between means are significant ($P = .02$).

[b] differences between means are significant ($P = .014$).

has been attributed to the species; age at the time of exposure; concurrent infections, such as psittacine beak and feather disease (PBFD); and certain unidentified individual factors.[5,6,8,9,14,19] The susceptibility of macaws to APV infections has been previously noted,[5,9,13,21,36] and this study presents evidence that the Spix's macaw was no exception. Because most of the birds that tested positive were acquired as adults, the age of exposure or the history of clinical illness cannot be elucidated. Notably, since 2004, PBFD has never been detected during routine annual testing for the virus. In addition, this study confirmed the conclusion of several investigators that antibodies to APV can be detected in adult nonbudgerigar psittacine birds in the absence of clinical symptoms.[8,13,19,21,31,37]

In the case of budgerigars, antibody titers are sustained for periods of up to 5 years and are presumed to be lifelong, and viral shedding from the cloaca ceases at the onset of sexual maturity.[13,15,17,21,38] Recent investigations have contributed to the hitherto scarce data on the course of infection in nonbudgerigar psittacine bird species. In macaws, the susceptibility to potentially fatal clinical disease is between 4 and 14 weeks of age; thereafter, exposure leads to inapparent infection.[5,6,13] Initial serosurveys in nonbudgerigar psittacine birds suggested that antibody titers are transient after APV infection and decline within weeks of infection.[6,9] Results of subsequent research demonstrated that antibodies can persist for up to 2 years and that viremia, when present, is always detected and does not necessarily correlate with antibody status. Also, cloacal shedding of virus can occur irregularly for up to a year.[8,14]

Our retrospective analysis of testing results for APV-specific antibodies in the Spix's macaw population at AWWP revealed that most of the birds showed consistently positive or negative results over the period of 4 years when sampled repeatedly. Only 3 birds demonstrated an observable changing pattern of results, of which 2 had declining antibody levels. In the third bird, a deterioration of 1 sample during storage or shipment provides a likely explanation for the result of 0% inhibition at 1 time point and 3 strong positive results at the remaining time points of analysis.

In general, the extremely low detection rate of the APV genome suggests that the isolated positive PCR result was a result of contamination, either at the time of sampling or at the laboratory, and is supported by our observation that 3 pairs comprising 1 APV-antibody positive and 1 negative bird were housed together with no change in antibody status during the period of 4 years, which indicated that virus transmission did not occur. This drawback of the extremely high sensitivity of the PCR test has been previously acknowledged.[8] Although viremia cannot be completely ruled out, it is highly unlikely given that the bird (bird 31) was a young hand-reared bird that had not been exposed to the adult population, compounded by the fact that the bird was seronegative at the time of sampling and did not seroconvert in subsequent tests (Table 1). In this case, sequencing of the PCR products would have been invaluable to arrive at a conclusion; however, the retrospective nature of this study precluded this possibility.

Blood and plasma biochemical parameters for APV-infected birds have only been described for nonbudgerigar psittacine birds with active and fatal infections.[8,36] In our study, significantly higher total WBCs were found in the APV seropositive adult birds than in APV seronegative birds. The distinct reasons for this finding are not known; nevertheless, we can speculate that it reflects a stimulation of the immune system by

APV infection. In apparently healthy budgerigars, APV had a higher detection rate in tissues than in serum,[17] and the possibility that the virus persists in other tissues of the Spix's macaws as well remains a hypothesis that cannot be substantiated at this point. The finding of lower AST concentrations in APV-antibody positive birds compared with APV-antibody negative birds is in marked contrast to previously reported studies.[8] However, these studies were performed on acutely diseased birds, and, despite the statistical significance recorded here, the probability of it being an unrelated incidental finding is high.

The Spix's macaw population at AWWP plays a significant role in the international species recovery and rehabilitation program. As determined in this study, the likelihood of virus transmission within the adult population is low. However, because cloacal shedding can continue after viremia ceases in macaws,[8,14,39] PCR screening of cloacal swab samples from these birds in the future would be an essential step in bridging the gap necessary to gauge the potential risk of infection of Spix's macaw chicks.

At present, all eggs from Spix's macaws are pulled from the nest and chicks are hand-reared before introduction to the breeding population. A possibility remains that some of the adult birds might be shedding the virus. In the absence of these data, however, precautions to ensure that the chicks are not exposed to the breeding population between 4 and 14 weeks should be adequate to protect them against clinical APV disease. In conclusion, results of this study demonstrated the persistence of antibodies to APV for up to 4 years in Spix's macaws, with little evidence of viremia or virus transmission between seropositive and seronegative birds. This indicates a low level of risk for hand-reared juvenile Spix's macaws, a valuable conservation resource.

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5	Author: Correct as edited: ''In subsequent years after the initial sampling, 36 Spix's macaw individuals were resampled.'' ? Editor	

6	Author: Please verify. Is ''*U* test'' okay as is or ''Mann-Whitney *U* test'': ''Because the percentage of monocytes was not normally distributed, differences in this parameter were tested by *U* test.'' Copy editor	
7	Author: Table 1 reformatted per journal style. OK? Copy editor	
8	Author: Table 2: Footnotes to Table 2 have been modified per journal style. Okay? Copy editor	
9	Author: Please note. Original reference 34 was a duplicate of reference 10. Original reference 34 was deleted; original references 35,36,37,38,39,40 were renumbered in the list and in the text. Please carefully check that all references are correctly cited in the text. Copy editor	
10	Author: Ref. 19 does not match 1991 AAV Proceedings. Please check. Editor	
11	Author: Please verify reference 29. Editor	
12	Author: Reference 30: Please verify. Is ''2006'' okay as is? Copy editor	
13	Author: Please verify reference 36. Editor	
14	Author: Please verify reference 39. Editor	

AUTHORS: THE FOLLOWING QUERIES/COMMENTS ARE FOR EDITORIAL STAFF FROM PROOFREADERS. The editorial staff will contact you if they have any questions regarding these queries and comments.

EDITORIAL STAFF: Query markers appear in boxes in the margins as a question mark followed by a number. Please respond to each query in the margin of the corresponding proof page. Comment markers are suggested corrections that will also appear in the margin, but as a "C" followed by a number. If you wish to implement the suggested correction, please transfer the correction to the margin and text of the corresponding proof page.

C1: In the title, set the parentheses in roman, and make sure this does not cause any text to be touching.
C2: In list of authors, delete "and" from before Hermann Muller, and insert "and" before Sven Hammer.
C3: In affiliation footnote, replace "BOX" with "Box".
C4: In Table 1 footnote a, replace the space after the superscript "a" with a thin space.
C5: In Table 2 footnotes a and b, replace spaces after the superscripts with thin spaces (x2).