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Short Communication

Prevalence of Avian Polyomavirus in Psittacine Birds in the Klang Valley

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ABSTRACT

Avian polyomavirus (APV) primarily affects young birds and can cause mortality in a wide range of psittacine birds. This is the first study to detect the presence of APV in Malaysia. A total of 85 faecal samples were collected from symptom-free psittacines species from four different breeders in the Klang Valley. Upon genomic DNA extraction, the presence of APV was analysed by PCR using primers APVfull-AF (5'- ACAATGCCTAACGGAACGCC-3') and APVfull-AR (5'-CACCGAAGCGGCGATACTATA-3'). Positive results of 3 kbp PCR amplicon were detected in six out of the 30 samples (20%), which were from yellow-collared macaws, blue-headed parrots, red-crowned macaws, sulphur-crested cockatoos, blue-throated macaws, and Pesquet's parrots. As a conclusion, the prevalence of APV was clearly indicated.

Keywords: PCR, polyomavirus, psittacine birds

INTRODUCTION

Avian polyomavirus (APV) has been subclinically identified in numerous species of birds and is categorised under the family of Polyomaviridae. In 2016, the taxonomy developed by the International Committee on Taxonomy of Virus documented four genera within this family: Alphapolyomavirus

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(37 species), Betapolyomavirus (29 species), Deltapolyomavirus (4 species), Gammapolyomavirus (7 species), with three species still unassigned. APV, which is also known as *Aves polyomavirus 1,* is classified under the Gammapolyomavirus genus. Molecularly, it is a non-enveloped virus with icosahedral viral capsid containing circular double-stranded DNA, with a genome size of 4981 bp (Hsu et al., 2006; Johne, Jungmann, & Muller, 2000). The APV genomes consist of two regions - an early region which codes for tumour (T) antigen, and a late region which encodes for structural proteins; major structural virus protein 1 (VP1) and minor structural proteins VP2, VP3 and VP4 (Halami et al., 2010; Katoh et al., 2009). Increased mortality associated with polyomavirus infections in birds can be seen in a variety of young captive psittacine birds, namely, lovebirds, macaws, conures, ring-necked parakeets, caiques, Eclectus parrots, Amazon parrots and cockatoos (Parrish, 2011). Clinical signs and symptoms due to APV infection include cutaneous haemorrhage, abdominal distension, and feather abnormalities. APV is also associated with budgerigar fledging disease (also known as French molt, a milder disease of budgerigars that results in a chronic disorder of feather formation). Other bird species may also get affected by this virus. Subclinical APV infection was found in European raptors, zebra finches, Ross's turaco and a kookaburra (Parrish, 2011). It is believed that only sulphur-crested cockatoos are infected in natural settings in Australia. The virus can be stored in faeces for up to six months. Sudden death of the affected birds is usually associated with minimal clinical warnings, but weakness, pallor, subcutaneous haemorrhages, anorexia, dehydration and crop stasis are briefly manifested (Parrish, 2011). Currently, APV is still not prevalent in Malaysia and information pertaining to APV is still lacking. No study has been carried out on the disease status and virus detection among psittacine species in Malaysia. However, many birds are subclinically infected and possibly store the virus in respiratory secretions, crop secretions, feather dust and droppings in times of stress, such as during the breeding season and juvenile stage in life. Despite significant contributions by exotic birds to the Malaysian pet bird industry due to its breeding activities, to date, no study to determine the presence and/or prevalence of APV for exotic birds population in Malaysia is available. Hence, this study was undertaken to screen the presence of APV among psittacine birds, especially in parrot species, in the Klang Valley, Malaysia.

MATERIALS AND METHODS

Samples Acquisition and Preparation

A total of 85 faecal bird samples were obtained from four different breeders (Breeder A, B, C, and D) located in the Klang Valley. All the birds were classified into different groups based on their species in the Psittaciformes order. Samples which were collected from different birds of the same species from the same breeder

were pooled into one, making up the total number of 30 samples (Table 1). Faecal samples were collected with a sterile wooden stick and transferred into a 15 ml sterile tube with cap before being stored in -80°C freezer until further use. The sample was thawed at room temperature and re-suspended with prepared SM buffer with a ratio of 1:1 (weight of sample/g: volume of SM buffer/ml). A homogeniser was used to break the cellular particles in the sample. Then, the mixture was centrifuged at 10,000 x g for 20 minutes by using Allegra™ X-22R Centrifuge (Beckman Coulter™, United States). The supernatant was collected and filtered through 0.45 µm and 0.2 µm pore-sized syringe filter. The filtrate (cell-free sample) was used in the subsequent experiment to extract the viral RNA/DNA.

Table 1
Data on sampling strategy

Species of psittacine birds	*Abbreviations*	*No. of tube samples*
Samples collected from breeder A		
Moluccan Cockatoo	MLC(A)	1
Green-winged Macaw	GWM(A)	2
Timneh African Grey Parrot	AGP(A)	1
Scarlet Macaw	SCM(A)	2
Hybrid	HYB(A)	1
Blue and Gold Macaw	BGM(A)	1
Red-fronted Macaw (RFM)	RFM(A)	1
Amazon Parrot	AMP(A)	2
Eclectus	ECL(A)	2
Yellow-collared Macaw	YCM(A)	1
Chestnut-fronted Macaw	CFM(A)	1
Hahn's Macaw	HM(A)	2
Blue-headed Parrot	BHP(A)	2
Red-crowned Macaw	RCM(A)	4
Blue-throated Macaw	BTM(A)	1
Pesquet's Parrot	PQP(A)	1
Black Palm Cockatoo	BPC(A)	1
Hyacinth Macaw	HCM(A)	1
Samples collected from breeder B		
Hybrid	HYB(B)	1
Sulphur-crested Cockatoo	SCC(B)	7
Congo African Grey Parrot	AGP(B)	11
Blue & Gold Macaw	BGM(B)	6
Galah Cockatoo	GC(B)	3
Green-winged Macaw	GWM(B)	2
Amazon Parrot	AMP(B)	3
Hahn's Macaw	HM(B)	1
Samples collected from breeder C		
Budgerigar	BD(C)	3
Local Budgerigar	LB(C)	5
Cockatiel	CKT(C)	3
Samples collected from breeder D		
Budgerigar	BD(D)	12
Total	30	85

Note: Samples collected from different birds of the same species from the same breeder / location were pooled into one, making the total number of 30 samples.

Viral DNA Extraction and Purification

To prepare the lysate, Purelink® Viral RNA/DNA Mini Kit (Invitrogen) was used. Briefly, a 25 µl of proteinase K was added into a sterile micro-centrifuge tube followed by 200 µl of cell-free sample and 200 µl of lysis buffer. This mixture was vortexed for 15 seconds. The sample was then incubated in a water bath at 56°C for 15 minutes. After that, the sample was centrifuged at a short-spin speed for one minute. To proceed with the binding and washing procedure, 250 µl of 96-100% ethanol was added to the lysate and vortexed for 15 seconds. The lysate was then incubated with ethanol for 15 minutes at room temperature. The mixture of the lysate and ethanol was centrifuged with a short-spin speed for one minute before it was transferred into a viral spin column. The loaded-column was centrifuged again at 6800 x g for one minute. The spin column was placed in a clean wash tube and 500 µl of wash buffer (W5) was added with ethanol to the spin column before centrifugation at 6800 x g for one minute. The flow-through in the wash tube was then discarded and the spin column was transferred into another 2 ml sterile tube before being centrifuged at 10,000 x g for one minute. The flow-through was discarded. The viral spin column was transferred into a 1.5 ml sterile recovery tube. A 10-50 µl of sterile, RNAse-free water was added to the centre of the column before incubation at room temperature for one minute. The column was centrifuged at 10,000 x g for one minute. The spin column was then removed and discarded. The collected-purified DNA sample produced was stored at -80°C until further use. Two(2) µl of the DNA extract was pipetted out into a cuvette and placed in a BioSpectrometer™ photometer (Eppendorf, Germany) for quantification.

Polymerase Chain Reaction (PCR) of Viral DNA

Information regarding the primers used in this study are listed in Table 2. PCR reaction was set in each tube by adding 10 µl of 2X MyTaq™ Red Mix, (Bioline™, United States), 0.4 µM of forward primer, 0.4 µM of reverse primer, and 200 ng of DNA template. Sterile distilled water (ddH_2O) was added to a final volume of 20 µl. About 30 cycles of PCR amplification were performed using a Bio-Rad T100™ thermal cycler (Bio-Rad, United States) with the following conditions: denaturation at 95^{o}C for 30 seconds, annealing at 54 ^{o}C or 58^{o}C for 30 seconds, and elongation at 72^{o}C for one minute. PCR products were resolved in agarose 1 % (w/v) gel electrophoresis.

Table 2
Information on the primer set used in the study

Name/Type	*Sequence (5'-3')*	*Length*	*Targeted nucleotide (nt) position in APV genome*	**Amplicon *size***
APVfull-AF/ Forward	ACAATGCCTAACGGAACGCC	20 bp	nt 376 - 395	3.2 kb
APVfull-AR/ Reverse	CACCGAAGCGGCGATACTATA	21 bp	nt 3604 - 3624	

Note. Primers were adapted from Katoh, Ohya, Une, Yamaguchi, & Fukushi, 2009.

RESULTS AND DISCUSSION

A total of 85 faecal samples collected were further categorised into 30 samples according to the bird species and location of sampling (data not shown). Out of the 30 samples, six samples were found to be positive for APV by conventional PCR (20%) [Figure 1 (A) and (B)]. The species of the birds tested as positive were mostly from Breeder A: yellow-collared macaw [YCM(A)], blue-headed parrot [BHP(A)], red-crowned macaw [RCM(A)], blue-throated macaw [BTM(A)], and Pesquet's parrot[PQP(A)]. One species came from Breeder B: sulphur-crested cockatoo [SCC(B)].

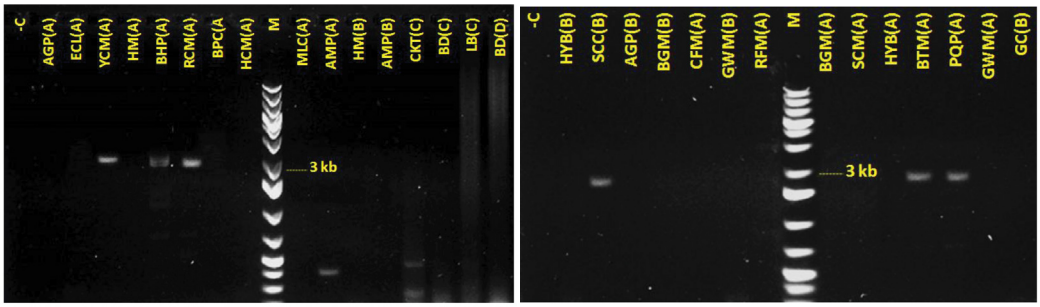

Figure 1. PCR assay for 30 faecal samples from different species and breeders (using specific primers, APVfull-AF and APVfull-AR, with an expected size of 3 kbp)

Electrophoresis was carried out on 1.0% agarose gel. Positive bands were observed in six species of psittacine birds; (A): Lane 4 [yellow-collared macaw (Breeder A)], lane 6 [blue-headed parrot (Breeder A)], lane 7 [red-crowned macaw (Breeder A)]; (B): Lane 3 [sulphur-crested cockatoo (Breeder B)], lane 13 [blue-throated macaw (Breeder A)] and lane 14 [Pesquet's parrot (breeder A)]; C was a no-template negative control, while M indicates 1 kb GeneRuler DNA ladder marker (Thermo Fisher Scientific).

By using convenience sampling as a component of the methodology, various species of psittacine birds, six out of 30 faecal samples were found to be positive for APV by PCR assay, which means a positive detection rate of 20%. This finding corresponds to the expected prevalence of 22% in Poland (Piasecki & Wieliczko, 2010), but not to a prevalence study in Costa Rica with only 4.8% occurrence of APV infection based on 269 feather or blood samples (Dolz et al., 2013). In this study, APV DNA was detected in six different types of psittacine birds, including a sulphur-crested cockatoo. DNA of APV was also been detected from wild-caught sulphur-crested cockatoos in Australia by Raidal and colleagues (Raidal et al., 1998). All the birds involved in this study were healthy and did not show any signs of illness (asymptomatic). Most of the APV positive birds were from Breeder A. According to the breeder, most of the birds acquired originated from the wild environment. This might be the foundation of such an observation. Many wild birds can be found naturally infected with APV. However, infection in captive-raised birds is also widespread. According to Kahn and Line (2010), adult birds typically are resistant to infection, they are able to seroconvert and shed the virus for up to 90 days, then clear the infection. The typical presentation of APV-infected birds are well-fleshed juveniles and just before fledgling age. Most adult birds act as carriers of APV without exhibiting any clinical signs to other susceptible birds around them. There are a few limitations in this study: i) some of the birds were kept together in a cage, such as budgerigars, or with their mating pair, for example, green-winged macaw, hence, individual faecal sample was difficult to obtain; ii) individual blood or cloacal swab sampling might offer a better surveillance analysis for APV infection. However, faecal sampling is considerably a fast, non-invasive screening technique which avoids physical contact and stress to the exotic birds. Future recommendation would be to increase the sample size and expand the prevalence parameters to specific species, breeders or location.

CONCLUSIONS

This study successfully described the prevalence of APV in psittacine birds for the first time in Malaysia. Further studies on bioinformatics analysis should be conducted to study the characteristics of locally isolated APV based on whole genome sequence and phylogenetic tree.

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