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SHORT COMMUNICATION

Evaluation of beak and feather disease virus, avian polyomavirus and avian papillomavirus of captives psittacine birds in Seri Kembangan, Selangor, Malaysia

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ABSTRACT

Aims: Psittacine birds such as parrots, macaws, cockatoos, lovebirds and parakeets, are widely reared as household pets or at aviary due to their attractive features. However, the status of virus-causing diseases of psittacine species in Malaysia is fairly under-documented. Therefore, this study was aimed to detect the presence of three common avian viruses that infect psittacine birds, i.e. beak and feather disease virus (BFDV), avian polyomavirus and avian papillomavirus.
Methodology and results: Faecal samples from twelve asymptomatic captive psittacine birds of different species were collected from an undisclosed animal garden in Serdang, Selangor, Malaysia. Briefly, the sample was homogenised and resuspended with SM buffer with the ratio 1:1 (weight of sample/g: volume of SM buffer/mL) before centrifugation at 1,000 × *g* for 20 min. The supernatant was collected and filtered before subjected to genomic DNA extraction using a commercialised kit. Polymerase chain reaction (PCR) technique was used to screen the V1, VP1 and L1 genes of beak and feather disease virus (BFDV), avian polyomavirus and avian papillomavirus, respectively. Findings revealed that the samples were negative for BFDV and avian polyomavirus. However, positive results of 1.5 kbp PCR amplicon were detected for avian papillomavirus in four out of the 12 samples (33.33%), which was from the white-crested cockatoo, African grey parrot, yellow-collared macaw and Senegal parrot. Sequence analysis of the L1 gene from the Senegal parrot *Poicephalus senegalus* revealed 93% identity to a reference *Psittacus erithacus timneh* avian papillomavirus.
Conclusion, significance and impact of study: This study added to the limited prevalence data of three important avian viruses which infect captive psittacines in Seri Kembangan, Selangor, Malaysia. Avian papillomavirus, but not BFDV and avian polyomavirus, was detected in the collected captive psittacine birds. Therefore, a routine screening can be performed to monitor the health status of birds despite their asymptomatic manifestation, in order to prevent possible virus transmission.

Keywords: Avian viruses, beak and feather disease virus (BFDV), avian polyomavirus, avian papillomavirus, psittacine

INTRODUCTION

Psittacines, including parrots, macaws, cockatoos, lovebirds, and parakeets are a popular order of tropical birds being reared in aviary centres and kept as pet animal due to their attractive appearances. Parrots, for example, possess a distinctive, well-developed, and hooked rostrum (upper beak), with various bright coloured-feathers and showed intelligence for taming, training and mimicking vocals (Harcourt-Brown, 2009). To date, several avian viral diseases are found to be the cause of health problem among the captive psittacine species. The causative agents are varying, which include both DNA and RNA viruses. Examples of avian DNA

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viruses are beak and feather disease virus (BFDV), avian polyomavirus, psittacid herpesvirus, psittacine adenovirus, poxvirus and avian papillomavirus, whereas avian RNA viruses may cause reovirus, coronavirus, paramyxovirus, influenza virus and bornavirus infections (Katoh *et al*., 2010).

BFDV is a non-enveloped, isometric or spherical symmetry virus with a circular, single-stranded DNA (ssDNA) with a length of 1992–2018 nucleotides (Hsu *et al.*, 2006). It is originated and evolved in psittacine birds. The infected psittacine commonly presented with chronic, progressive loss of feathers and, in some species, deformities of the beak and claws. The virus may threaten psittacine populations as it causes very high mortality in nestlings of both in captivity and the wild (Fogell *et al*., 2016). Avian polyomavirus is a non-enveloped, icosahedral virus with a circular, double-stranded DNA (dsDNA) with 4981 bp genome. Avian polyomavirus is an acute disease affecting young Budgerigars (*Melopsittacus undulatus*) in which mortality rates almost 100% (Hsu *et al*., 2006). Information pertaining to avian polyomavirus status in Malaysia is fairly limited, whereby a recent study using only 30 samples of psittacine birds indicated the prevalence rate of 20% (Zanon *et al.*, 2018). Vaccination against avian polyomavirus is possible with a cost of between USD40-60 per bird. However, there is still a risk for the younger birds to encounter avian polyomavirus infection before the primary immunisation at 5 weeks of age (Padzil *et al.*, 2017).

Avian papillomaviruses have circular dsDNA genomes of ~8 kb that encode at least six conserved open reading frames. The virus causes the development of papillomas along the length of the gastrointestinal tract (Girling, 2003). Most papillomaviruses appear to be species-specific or at least restricted to infection of closely related animals within the same genus (Tachezy *et al*., 2002). Although virus-induced papillomas are initially benign, some may develop into malignancies over time. Infectious papillomatosis (warts) is contagious and the virus reproduction is typically tied to the differentiation of skin (Bian and Wilson, 2010).

Compared to other avian species, treatments of virus infections in psittacine birds are not known while efficient vaccines are limited (Padzil *et al*., 2017). Thus, early and rapid detection of the causative agents is important to prevent disease transmission. In the present study, we reported a pilot study for the prevalence of BFDV, avian polyomavirus and avian papillomavirus in captive psittacine birds in Seri Kembangan, Selangor, Malaysia, using polymerase chain reaction (PCR) technique.

MATERIALS AND METHODS

Sample attainment and preparation

A total of 12 birds of different species were obtained from an undisclosed animal garden in Seri Kembangan, Selangor, Malaysia. All the birds were classified into different groups based on their species in the Psittaciformes order (Table 1). Faecal samples were collected by using sterile wooden sticks and transferred into 15 mL tube. The sample was homogenised with SM buffer [0.1 M NaCl, 50 mM Tris/HCl (pH 7.4), 10 mM $MgSO_4$] with the ratio 1:1 (weight of sample/g: volume of SM buffer/mL). A rod was used to break the cellular particles in the samples. Then the homogenised mixture was centrifuged (Allegra^{TM} X-22R model from Beckman Coulter^{TM}, United States) at 1,000 × *g* for 20 min. The collected supernatant was filtered sequentially by using a nylon 0.45 µm and 0.2 µm pore-sized syringe filter.

Table 1: List of species samples collected from an undisclosed animal garden in Serdang, Selangor.

Common name of psittacine birds	Scientific name	Abbreviation
Double yellow-headed amazon parrot	*Amazona oratrix*	DYA
White-crested cockatoo	*Cacatua alba*	WCC
Congo African grey parrot	*Psittacus erithacus*	CAG
Blue-fronted amazon parrot	*Amazona aestiva*	BFA
Blue angel macaw	*Anodorhynchus hyacinthinus*	BAM
Greater sulphur-crested cockatoo	*Cacatua galerita*	SCC
Harlequin macaw (Hybrid)	*Ara chloropterus* x *Ara ararauna*	HM
Green-winged macaw	*Ara chloropterus*	GWM
Electus parrot	*Eclectus roratus*	ELP
Black-headed caique	*Pionites melanocephalus*	BHC
Yellow-collared macaw	*Primolius auricollis*	YCM
Senegal parrot	*Poicephalus senegalus*	SGP

Isolation and purification of viral DNA

The filtrate was used for viral DNA isolation using Purelink Viral DNA/RNA Mini Kit (Invitrogen, United States), by following manufacturer's protocol. Briefly, 25 µL of proteinase K, 200 µL of cell-free sample and 200 µL of lysis buffer were mixed well in a sterile microcentrifuge tube. After vortexing for 15 sec, the tube sample was incubated at 56 °C for 15 min. Then, a volume of 250 µL ethanol (96-100%) was added into the lysate tube. After vortexing again for 15 sec, the lysate in the presence of the ethanol, was incubated at room temperature for 15 min. Then, the mixture was transferred into spin column, followed by centrifugation at 6,800 × *g* for one minute. The eluent was discarded, and a clean wash tube was placed under the spin column. A total of 500 µL of washing buffer was added to the spin column before centrifugation again at the same speed and duration. The eluent was discarded, and a new clean 2 mL tube was placed under the spin column. Subsequent centrifugation was occurred

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Table 2: List of primers used in this study.

Target genes	Primer	Sequences (5' – 3')	Amplicon size	Sources
V1 gene (Beak and Feather Disease Virus)	BFDV – F BFDV – R	GCCCACGTGACTTCAAGACT ACGGAGCATTTCGCAATAAG	194 bp	Hakimuddin *et al.*, 2016
VP1 gene (Avian Polyomavirus)	APV – F APV – R	ATGTCCCAAAAAGGA GCGGGGAGCTTTGGGG	1032 bp	Katoh *et al.*, 2009; Zanon *et al.*, 2018
L1 gene (Avian Papillomavirus)	PV – F PV – R	ATGAGTGCTGCTGGGCCTG CCTCCGCCTACGCTTCGCA	1578 bp	Tachezy *et al.*, 2002

at 10,000 × *g* for 1 min, and the eluent was discarded. For final recovery, a 1.5 mL sterile tube was placed under the spin column. A 30 µL of sterile, RNAse-free water was added to the column and incubated for one min at room temperature. Next, the column was centrifuged at 10,000 × *g* for one min. The spin column was then discarded. The eluted, purified DNA sample was quantified using a photometer (model BioSpectrometer™ from Eppendorf, Germany) and stored at ─80 °C until use.

Polymerase chain reaction (PCR) of viral DNA

All the primers used for PCR were listed in Table 2. The amplification was carried out in a final volume of 50 µL containing 1× of MytaqTM Red Mix (Bioline™, United states), 0.4 µM of forward primer, 0.4 µM of reverse primer and 60 ng of isolated DNA template. PCR reaction was performed by using a thermal cycler (model Bio-Rad T100™ from Bio-Rad, United States) with initial denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 sec, 56 °C for 30 sec and 72 °C for one min; and a final extension at 72 °C for 5 min. The product was subjected to gel electrophoresis in 1% agarose gel stained with SYBR™ Safe Stain (Invitrogen, United States), and electrophoretically run under 90 volts for 40 min, followed by visualisation using a UV transilluminator (model Gel Doc XR+ from Bio-Rad, USA). The positive PCR products were subjected for DNA sequencing by outsourcing to Apical Scientific (Malaysia). Sequences derived from this study were analysed by using basic local alignment search tool (BLAST) and aligned by Clustal Omega (Multiple Sequence Alignment).

RESULTS AND DISCUSSION

A total of 12 faecal samples were acquired from 12 different species of psittacine in the same animal garden. Based on the observation, all of the birds were asymptomatic. The PCR screening showed that all collected samples were negative for BFDV (expected size was 194 bp) and avian polyomavirus (expected size was 1032 bp), whilst four out of twelve samples (33.33%) were positive for avian papillomavirus (expected size was 1578 bp). Referring to Figure 1c, the species that were tested positive for avian papillomavirus were white-crested cockatoo (lane 2), Congo African grey (lane 3), yellow-collared macaw (lane 11) and Senegal parrot (lane 12). There were also non-specific bands appeared on the positive samples, but do not indicate any contamination

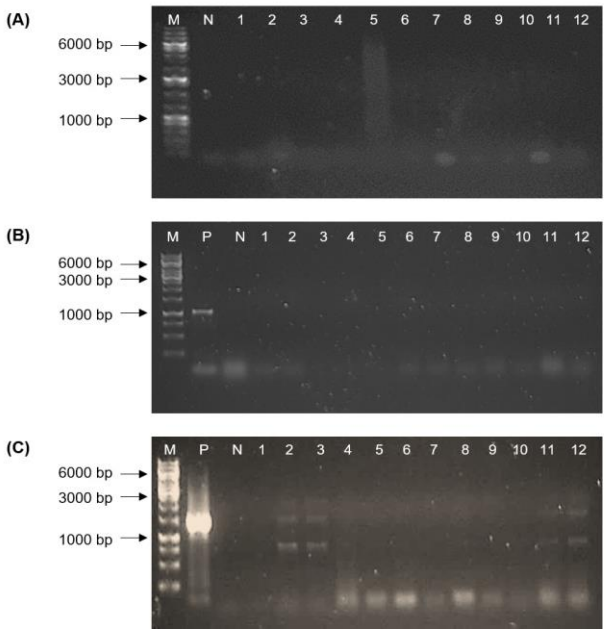

Figure 1: PCR detection of V1 gene of BFDV (A), VP1 gene of avian polyomavirus (B) and L1 gene of avian papillomavirus (C). Lane M, 1kb GeneRuler DNA ladder; Lane P, positive control; Lane N, blank; Lane 1, DYA, Lane 2, WCC; Lane 3, CAG; Lane 4, BFA; Lane 5, BAM; Lane 6, SCC; Lane 7, HM; Lane 8, GWM; Lane 9, ELP; Lane 10, BHC; Lane 11, YCM; Lane 12, SGP.

since no PCR products were amplified from any of the negative control reactions. The additional bands may be due to the non-specific binding of the universal primer set targeting the L1 gene from different bird species. The 1578 bp amplicon of the Senegal parrot *Poicephalus senegalus* avian papillomavirus L1 gene was further purified and sent for DNA sequencing. The sequence analysis of the L1 gene (GenBank accession number: MW732642) revealed 93% identity to a reference *Psittacus erithacus timneh* papillomavirus (GenBank accession number: NC_003973.1) (Supplementary Figure 1).

Psittacine birds are among the most threatened bird groups, which are found mostly in the tropical parts of the Southern Hemisphere (Raidal *et al*., 2015). With 28% (111 out of 398) of extant species classified as threatened under International Union Conservation of Nature (IUCN)

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Red List criteria, parrot populations face the risk of continued decline (Olah *et al*., 2016). In Malaysia, some species of psittacine such as blue-naped parrot and red-breasted parakeet are described as near-threatened species (Lepage, 2020), which sparks the conservation concern. It has been reported that viral disease is an important threat to bird populations globally due to sudden death. In Australia, all threatened bird species are susceptible to BFDV (Raidal *et al*., 2015). The identification of BFDV in orange-bellied parrot population in Australia become conservation concern because their populations are critically endangered (Peters *et al*., 2014). Here, we screened three viruses that are significant conservation threat to psittacine globally, which are BFDV, avian polyomavirus and avian papillomavirus, in asymptomatic psittacine birds in Seri Kembangan, Selangor, Malaysia.

Knowledge about avian papillomaviruses is fairly lacking, while most of the currently known papillomavirus infects human. Tachezy *et al*. (2002) had described the first complete avian papillomavirus genome from an African grey parrot (*Psittacus erithacus timneh*), however, thus far, prevalence study of avian papillomavirus particularly in psittacine, particularly in Malaysia, is still very limited. Canuti *et al*. (2019) had identified twelve novel avian papillomaviruses in wild birds that could represent five distinct species, which were mallard, American black duck, puffin, American herring gull and black-legged kittiwake (Canuti *et al*., 2019). It was suggested that cross-species transmission may occur between genetically closely related hosts. BFDV, on the other hand, is a highly prevalent virus among all species within the order Psittaciformes. A prevalence study conducted in Australia reported 29 out of 84 wild crimson rosellas were infected by BFDV, which indicated 34.5% occurrence of BFDV (Eastwood *et al*., 2015). Hakimuddin *et al*. (2016) reported higher BFDV prevalence in psittacine birds, which were 58.33% in African grey parrots, 34.42% in cockatoos, 31.8% in amazon parrots and 25.53% in macaws. Surprisingly, there was no BFDV detected in all collected samples in the present study. However, the BFDV-PCR assay in this study has lacked a positive control, a limitation which makes the result a doubtful true negative. Nevertheless, the primer set used for BFDV detection was adopted from a study conducted by Hakimuddin *et al*. (2016), which reported high sensitivity and specificity in BFDV detection. Avian polyomavirus is also one of the most important viruses in psittacine birds. It is a highly infectious virus with almost 100% infection rates in indoor aviaries (Philadelpho *et al*., 2015). A recent local study conducted in Klang Valley, Malaysia, has shown 20% occurrence of avian polyomavirus in 30 pooled samples of psittacine birds from four different breeders (Zanon *et al.*, 2018), whereas in Thailand, the prevalence of avian polyomavirus was 8.1% (10 out of 124 samples) (Fungwitaya *et al*., 2009). The previous prevalence studies have shown high incidence of avian polyomavirus, whereas no bird sample, in this study, was positive for avian polyomavirus. Therefore, increasing the sample size is likely to provide better surveillance of the avian polyomavirus incidence.

The finding of the present study suggested that the occurrence of infection is not always presented in clinical symptoms of the disease. All psittacine birds were not showing any clinical signs but 33.33% of them were found to be shedding avian papillomavirus. Avian papillomavirus infection is commonly characterised as a tumour disease with progressive development of papillomas in the oral and cloacal mucosa, which leads to anorexia and chronic weight loss due to the obstruction of food movement through oral cavity or esophagus (Katoh *et al*., 2010). However, the papillomas development differs in different species. As being reported by Latimer *et al.* (1997), papillomas may only developed in African grey parrots, but apparently no similar lesions were observed in unrelated species of psittacine birds. Besides, a very high viral load is required to cause the development of feather disease in the susceptible species (Sarker *et al*., 2015). Therefore, if the virus is once detected in any species in the population, virus screening and clinical signs assessment for the whole birds in the population should be implemented comprehensively due to different symptoms occurrence among species. Also, it is suggested that screening of the pathogens in the captive birds should not only be done for the birds when they are sick, but also upon their arrival.

Selection of appropriate host biological samples are important in detecting the presence and the viral load of pathogens. Eastwood *et al.* (2015) had conducted a study to compare the rate of BFDV detection between blood, muscle tissue and feather samples from 13 wild crimson rosellas, and it was found that feather samples provided discordant results when compared with muscle tissue and blood samples. BFDV DNA was reported to be detected most commonly in feather samples, followed by cloacal swabs, and least frequently from blood samples (Hess *et al.*, 2004). It is supported with a study conducted by Hsu *et al.* (2006), by which the detection rate of avian polyomavirus and BFDV in feather samples were higher than in faecal samples. However, in the present study, faecal sample is still preferable to reduce physical contact and stress to the birds. Besides, the prevalence of virus in cloacal swabs was significantly higher than in blood samples, which indicated high level virus shedding in faeces (Martens *et al*., 2020). BFDV, particularly, is largely shed in faeces and feather dander as it is described as many as one billion virus particles per microlitre are found in the feather dust (Sarker *et al*., 2015). Most importantly, transmission of BFDV, avian polyomavirus and avian papillomavirus is commonly occurred via faecal-oral route (Girling 2003; Amery-Gale *et al*., 2017), which suggest that virus can be found copiously in faeces.

There are few limitations in this study: i) the sample type is limited to faeces only, hence, varying in sample types, such as blood, feather and cloacal swab might offer a better virus detection; ii) increasing the sample size at different aviary sites would provide assured evidence or prevalence of each virus occurrence among

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psittacines; iii) the BFDV-PCR assay would be more certain in the presence of a positive control, even when the primer used was adopted from previous work which reported its high sensitivity and specificity in detecting BFDV in psittacine birds in the United Arab Emirates (UAE).

CONCLUSION

As a conclusion, this study added to the limited prevalence data of three important avian viruses which infect captive psittacines in Seri Kembangan, Selangor, Malaysia. Avian papillomavirus, but not BFDV and avian polyomavirus, was detected in the collected captive psittacine birds, with the prevalence rate of 33.33%. Therefore, a routine screening can be performed to monitor the health status of birds despite their asymptomatic manifestation, in order to prevent possible virus transmission.

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SUPPLEMENTARY INFORMATION

Score	Expect	Identities	Gaps	Strand
854 bits(462)	0.0	555/600(93%)	9/600(1%)	Plus/Plus

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              |||||||||||||| |||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  6117  CGAGTGTTTATCTTAATACCTCGTTTACGAGTACTAATAACTGTGCTTACATGTGTGTTC  6176

Query  301   CTTCCGGGTCTGTATACACCTCTGATACCCAGTTGTTTAATCGGCTGTACTGGCTGTCCA  360
             |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  6177  CTTCCGGGTCTGTATACACCTCTGATACCCAGTTGTTTAATCGGCCGTACTGGCTGTCCA  6236

Query  361   AGGCGCAAGGTCCTAACAACGGCGTTCGTTGGGGTGATGATCTGTTCATTACTAAGTTGG  420
             |||||||||||||||||||||||| |||||||||||||||||||||||  |||||
Sbjct  6237  AGGCGCAAGGTCCTAACAACGGCGTTTGTTGGGGTGATGATCTGTTCATTACTGTGTTGG  6296

Query  421   ACAATACGCGGGGTGGGGTCATGAACACTTCTACGAAACCTACGGATAGTGGGGATGTGT  480
             |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
Sbjct  6297  ACAATACGCGGGGTGGGGTCATGAACATTTCTACGAAACCTACGGATAGTGGGGATGTGT  6356

Query  481   ATAAACCTTCGGACTTCCGTGAATATGTCCGACATGTAGAGGAATACGAATTATCCTGTG  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6357  ATAAACCTTCGGACTTCCGTGAATATGTCCGACATGTAGAGGAATACGAATTATCCTGTG  6416

Query  541   TGTTACGGCTATGTAAAGTGCCCCTCTCCCCAGATGTTTTTGCCTCTCTCTACCGAGCTG  600
             |||||||||||||||||||||||||||||||||||||||| ||||||||||||||| ||||
Sbjct  6417  TGTTACGGCTATGTAAAGTGCCCCTCTCCCCAGATGTTCTTGCCTCTCTCTACCGTGCTG  6476
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Supplementary Figure 1: Sequence analysis of the L1 gene from the Senegal parrot *Poicephalus senegalus* (GenBank accession number: MW732642) revealed 93% identity to a reference *Psittacus erithacus timneh* avian papillomavirus (GenBank accession number: NC_003973.1).

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