UNIVERSITI PUTRA MALAYSIA

DETECTION OF AVIAN POLYOMAVIRUS FROM PSITTACINE BIRDS IN KLANG VALLEY

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TITLE

DETECTION OF AVIAN POLYOMAVIRUS FROM PSITTACINE BIRDS IN KLANG VALLEY

ZAMIR ZANON

A project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfillment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia,

Serdang, Selangor Darul Ehsan.

MARCH 2016

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CERTIFICATION

It is hereby certified that we have read this project paper entitled “Detection of Avian Polyomavirus from Psittacine Birds in Klang Valley”, by Zamir Zanon and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Project

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DEDICATIONS

This project paper is dedicated to the Allah S.W.T., who had created me and made all things possible,

To my family,

Father

Mother

Brother, Sister

And to all my teachers who have committed themselves towards the noble cause of education.

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LIST OF ABBREVIATIONS

%	Percent
µL	Microliter
µM	Micromolar
mm	Millimeter
°C	Degree Celsius
ATPase	Adenosine triphosphatase
bp	Base pairs
kb or kbp	Kilo base pairs
APV	Avian polyomavirus
PBFDV	Psittacine beak and feather disease virus
BFDV	Budgerigar fledging disease virus
GHP	Goose haemorrhagic polyomavirus
BEF	Budgerigar embryonic fibroblast
CEF	Chicken embryonic fibroblast
AVMA	American Veterinary Medicine Association
DNA	Deoxyribonucleic acid
RNase	Ribonuclease
dNTP	Deoxyribonucleotide triphosphate
g	Gram
min	Minutes
ml	Milliliter
ng	Nanogram
nm	Nanometer
no.	Number
VP1	Viral protein 1
VP2	Viral protein 2
ORF	Open reading frame
PCR	Polymerase chain reaction
AGE	Agarose gel electrophoresis
TAE	Tris-acetate EDTA
V	Volt
x g	Relative centrifugal force

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HYB	Hybrid
AGP	African grey parrot
SCC	Sulphur-crested cockatoo
BGM	Blue and gold macaw
SCM	Scarlet macaw
GWM	Green-winged macaw
CFM	Chesnut-fronted macaw
RFM	Red-fronted macaw
BTM	Blue-throated macaw
PQP	Pesquet’s parrot
ECL	Eclectus
YCM	Yellow-collared macaw
HM	Hahn’s macaw
BHP	Blue-headed parrot
RCM	Red-crowned macaw
BPC	Black palm cockatoo
HCM	Hyacinth macaw
MLC	Moluccan cockatoo
AMP	Amazon parrot
CKT	Cockatiel
BD	Budgerigar
LB	Local Budgerigar

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Ilmiah Tahun Akhir

PENGESANAN VIRUS POLIOMA UNGGAS DALAM KALANGAN BURUNG PSITTACI DI SEKITAR LEMBAH KLANG

Oleh

ZAMIR ZANON

2016

Penyelia: Prof. Madya Dr. Jalila Abu

Penyelia bersama: Dr. Mariatulqabtiah Abdul Razak

Virus polioma unggas boleh menjejaskan burung terutamanya anak burung dan boleh menyebabkan kematian dalam kalangan burung psittacine dan bukan burung psittaci. Burung dewasa kebiasannya rintang terhadap jangkitan; pada peingkat ini, ia akan menyimpan virus ini sehingga tempoh 90 hari dan akan bebas daripada sebarang jangkitan. Burung yang menerima jangkitan ini kebiasaannya adalah anak burung yang baru menetas, sebelum mencapai peringkat remaja dengan tanda akut seperti kelesuan, stasis tembolok, dan kematian dalam tempoh 24-48 jam. Tanda-tanda klinikal lain adalah seperti

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pendarahan subkutaneus, perut menggelembung dan keabnormalan bulu pelepah. Burung baji yang berumur lebih daripada tiga minggu kebiasaannya akan menunjukkan distrofi bulu pelepah (*French molt* atau hama bulu pelepah). Satu kajian telah dijalankan untuk mengesan kehadiran APV di kalangan burung psittaci terutama dalam spesies burung kakak tua di sekitar Lembah Klang. Sejumlah 85 spesimen najis telah dipungut daripada empat tempat pembiakbakaan yang berbeza di sekitar Lembah Klang. Spesimen najis telah digabungkan mengikut jenis dan spesis burung dan juga pembiakbaka. DNA telah diasingkan daripada 85 specimen najis burung psittaci yang tidak menunjukkan apa-apa tanda berpenyakit. Kehadiran APV dianalisa dengan melakukan asai tindakbalas berantai polimerase (PCR) dan elektroforesis gel agarosa (AGE) dengan menggunakan primer-primer spesifik yang menyasarkan gene VP1 (5'-CTTATGTGGGAGGCTGCAGTGTT-3' dan 5'-TAC TGAAATAGCGTGGTAGGCCTC-3') dan APV *full length* (5'-ACAATGCCTAACGGAACGCC-3', 5'-CACCGAAGCGGCGATACTATA-3', 5'-GAGGCCTACCACGCTATTTCAGTA-3' dan 5'-GCACTTAGCGCCTGTCCAAT-3'). APV telah ditemui dalam enam daripada 30 spesimen yang digabungkan (20%). Enam spesimen yang dikesan positif APV adalah dari spesis *yellow-collared macaw, blue-headed parrot, red-crowned macaw, sulphur-crested cockatoo, blue-throated macaw* dan *Pesquet's parrot.* Sebagai kesimpulan, kehadiran APV dalam kalangan burung psittaci adalah berjaya dikesan.

Kata kunci: Virus polioma unggas, burung psittaci, PCR.

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ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 – Final Year Project

DETECTION OF AVIAN POLYOMAVIRUS FROM PSITTACINE BIRDS IN KLANG VALLEY

By

ZAMIR ZANON

2016

Supervisor: Assoc. Prof. Dr. Jalila Abu

Co-supervisor: Dr. Mariatulqabtiah Abdul Razak

Avian polyomavirus (APV) primarily affects young birds and can cause mortality in wide range of psittacine and non-psittacine birds. Adult birds typically are resistant to infection; they will seroconvert and shed the virus for up to 90 days, then clear the infection. The typical presentation of APV-infected birds is a well-fleshed juvenile, just before fledgling age, with acute onset of lethargy, crop stasis, and death within 24–48 hour. Other clinical signs are cutaneous hemorrhage, abdominal distention, and feather abnormalities. Surviving budgerigars with age more than three week-old often exhibit feather dystrophy (French molt or feather dusters). This is the first study to detect the presence of APV in

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Malaysia. Therefore, a preliminary study was conducted to detect the presence of avian polyomavirus among psittacine birds especially in parrot species in Klang Valley by using polymerase chain reaction (PCR) technique. A total of 85 faecal samples were collected from psittacines' species of individual pet owners and parrot breeders. DNA was isolated from feces of 85 symptom-free psittacine birds taken from four different breeders from Klang Valley. The presence of APV was analyzed by performing polymerase chain reaction assays (PCR) and agarose gel electrophoresis (AGE) using specific primers targeting VP1 (5'-CTTATGTGGGAGGCTGCAGTGTT-3' and 5'-TACTGAAATAGCGTGGTAGGCCTC-3') and APV full length (5'-ACAATGCCTAACGGAACGCC-3', 5'-CACCGAAGCGGCGATACTATA-3', 5'-GAGGCCTACCACGCTATTTCAGTA-3' and 5'-GCACTTAGCGCCTGTCCAAT-3') genes. Positive results were detected in six out of 30 pooled samples (20%) which were from yellow-collared macaw, blue-headed parrot, red-crowned macaw, sulphur-crested cockatoo, blue-throated macaw and Pesquet's parrot. As a conclusion, presence of APV in psittacine birds has been successfully detected.

Keywords: avian polyomavirus, psittacine birds, PCR

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1.0 INTRODUCTION

1.1 Polyomavirus

The family *Polyomaviridae* contains a single genus, *Polyomavirus*, which includes viruses in: humans; non-human primates (African green monkey, baboon, stump-tail and rhesus macaque); rodents, including mice (polyomavirus and K virus), hamsters (hamster polyomavirus) and rats (rat polyomavirus); rabbits (rabbit kidney vacuolating agent); birds (avian polyomaviruses, including budgerigar fledging disease polyomavirus); cattle (bovine polyomavirus); equine (equine polyomavirus). The avian polyomaviruses segregate independently of the mammalian ones on the basis of genome sequence analyses, indicating long-standing evolutionary divergence that may result in their being included in different genera in the future. Polyomaviruses of veterinary importance occur in laboratory animals and birds (Parrish, 2011).

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1.2 Avian Polyomavirus

Avian polyomavirus have been subclinically identified in numerous species of birds (Parrish, 2011). Disease syndromes associated with polyomavirus infections in birds include increased mortality in a variety of young captive psittacine birds (e.g. lovebirds, macaws, conures, ring-necked parakeets, caiques, Eclectus parrots, Amazon parrots and cockatoos) (Parrish, 2011). Avian polyomavirus is also associated with budgerigar fledging disease (also known as "French molt, a milder disease of budgerigars that results in chronic disorder of feather formation) (Parrish, 2011). Other birds species are also get affected with this virus. Subclinical polyomavirus infection has been described in European raptors, zebra finches, Ross's turaco and a kookaburra. It is believed that only sulphur-crested cockatoo has been infected in natural setting in Australia (Parrish, 2011).

The virus can be shed in the feces for up to six months (Parrish, 2011). Sudden death of the affected birds usually associated with minimal clinical warning, but they briefly manifest weakness, pallor, subcutaneous hemorrhages, anorexia, dehydration and crop stasis (Parrish, 2011).

Currently, the information or status of Avian Polyomavirus (APV) is lacking in Malaysia. No study has been done on the disease status as well as virus detection among psittacine species in Malaysia. Many birds are subclinically infected and shed the virus in respiratory secretions, crop secretions, feather dust and droppings during times of stress such as during the breeding season and juvenile stage in life.

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Despite the significant contribution of exotic birds breeding to the Malaysian pet birds industry, no study has been carried out to determine the presence of APV in Malaysia. Hence, this study was undertaken to fulfill the following objective:

i. To detect the presence of avian polyomavirus among psittacine birds especially in parrots species.

For this research, the following hypothesis was proposed; avian polyomavirus might present in psittacine birds in Klang Valley.

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2.0 LITERATURE REVIEW

2.1 Psittacine birds

Psittaciformes are commonly referred to ‘psittacine birds’ or parrots and are very popular either caged pet birds or aviary birds (Harcourt-Brown, 2009). The order Psittaciformes contains two families which include 6 genera of Cacatuidae (cockatoo) with 21 species and 78 genera of Psittacidae (parrots, macaws, conures, Lories, parakeet) with 332 species (Rowley, 1997; Collar, 1997).

Parrots may be defined by their characteristically large heads and distinctive, powerful hooked rostrum (upper beak) with a prominent cere (the featherless area dorsal to the upper beak); the rostrum is hinged to the skull by a synovial joint in large birds (e.g. macaws) and an elastic zone in small birds (e.g. budgerigars); this feature is unique among birds (Harcourt-Brown, 2009).

The prehensile feet are zygodactyls with digits I and IV directed caudally and digits II and III cranially. This structure together with the beak acting as a third hand, making them very agile arboreal climbers, thus hanging upside down while feeding (Harcourt-Brown, 2005).

Psittacine birds range in size from the hyacinth macaw (*Anodorhynchus hyacinthinus*), which measures 100 cm and weighs 1500 g, down to pygmy parrots (e.g. buff-faced pygmy parrot; *Micropsitta pusio*) at slightly less than 10 cm and weighing 11

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g. The family is mainly vegetarian in which some of its members are specialized feeders, such as Lories and lorikeets that eat only pollen and nectar (Harcourt-Brown, 2009).

2.2 Psittacine birds as pet birds

The earliest known captive pet birds were from the parrot family with the evidence of the records of Alexander the Great bringing ring-necked parakeets with him from India to Europe in 1840, and over the next 40 years many tens of thousands were imported from Australia (Harcourt-Brown, 2009).

The attraction of parrots as companion animals is in their intelligence and potential for taming and training, their ability to mimic vocally, and their rounded faces which most people find it an attractive feature. Psittaciformes (parrots) have the largest avail cerebral hemispheres thus made them appeared to be very ‘intelligent’ (Harcourt-Brown, 2009).

A statistical study made by the American Veterinary Medicine Association (AVMA) recorded 11 to 16 million companion and exotics birds in the United States in 2007.

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2.3 History of Avian Polyomavirus

APV was first isolated from nestling budgerigars obtained from aviaries in the United States and Canada in the early 1980s experiencing a high rate of nestling mortality (Bernier *et al.*, 1981; Davis *et al.*, 1981). APV was subsequently shown to cause disease in parent-raised lovebirds (*Agapornis* sp.) and several species of hand-raised nestling parrots (Jacobson *et al.*, 1993; Graham *et al.*, 1987). A similar and possibly by the same virus has been detected in several species of passerines (Phalen, 1997). It was assumed that this virus was originally present in wild birds, but conclusive evidence of wild bird infection was not shown until 1998 when antibodies to APV were detected in the serum of wild-caught Sulphur-crested Cockatoos (*Cacatua gallerita*) in Australia (Raidal *et al.*, 1998). APV has subsequently been isolated from a small sampling of diurnal raptors in Europe (Johne and Müller, 1998).

A second APV, named goose haemorrhagic polyomavirus (GHP), has recently been isolated and characterized from farm-raised geese (species not provided) in France (Guerin *et al.*, 2000). Sequence analysis of amino acids of the GHP-encoded proteins shows that this virus is most closely related to APV, and it has been suggested that both viruses be included in new subgenus *Avipolyomavirus*.

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2.4 Virus Structure

APV is non-capsulated, approximately 40-50 nm icosahedral virus that has a 4984 bp circular double-stranded DNA genome. The genome of this virus is organized in a similar manner to other polyomaviruses. It contains two early proteins, large T and small t antigens, which interact with cellular proteins and regulate virus replication. There are four structural proteins, the VP 1, 2, 3 and the agno protein. VP1 is the major capsid protein. VP1 is the major capsid protein and the other structural proteins are involved in packaging the viral DNA. Between the open-reading frame for the T antigens and the agno protein is presumed origin of replication and adjacent regulatory domains. A small degree of genetic variation has been found in the open reading frames of all but one of the APVs sequenced to date (Johne and Müller, 2003; Phalen *et al.*, 1999). Partial duplications of some of the regulatory elements have been documented in viruses that have been grown *in-vitro* (Phalen *et al.*, 1999). However, duplication has also been identified in virus DNA amplified directly from tissue, so these duplications may also occur in vivo to a lesser extent (Phalen *et al.*, 2001). In general, there is little evidence to suggest that these genetic variants have mutations that impart host specificity to the virus (Johne and Müller, 1998; Phalen *et al.*, 1999).

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2.5 Epidemiology of Avian Polyomavirus

There is no exact data on the detection of APV around the world. Not many countries had done a study on the existence of APV within their countries. Some of the country only detecting the virus based on the evidence and clinical signs with supportive diagnoses of the disease. In Italy, the study was done with the PCR assay and showing only seven birds out of 877 birds (0.79%) having positive PCR assay of APV (Bert *et al.*, 2004). However, in Germany, there is no detection on the presence of APV from feathers sample taken from 85 symptom-free birds (from 20 different genera; all psittaciformes). The existence of a subpopulation of captive psittacine birds having a persistent APV infection in Germany seems to be relatively low (Rahaus and Wolff, 2004). While in Costa Rica, the first study was conducted from 2005 to 2009, from the total of 269 samples (from veterinary clinics, shelters and rescue centers), 13 birds (4.8%) showing the positive result of APV and 53 birds (19.7%) are positive for PBFDV, while 9 birds (3.3%) have infected with both of the diseases (Dolz *et al.*, 2013). Most of the birds with positive result are come from shelters and rescue centers. According to Dolz *et al.*, 2013, birds with PBFDV had 6.24 times more probability to become infected with APV, than non-infected birds.

Meanwhile in Thailand, there are the occurrences of the cases on one four week-old Eclectus and two three week-old macaws (Mamom *et al.*, 2009).

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2.6 Pathology of Avian Polyomavirus

According to Rahaus and Wolff, 2004, several reports indicate that diseases caused by APV infection vary in their symptoms depending on the avian species as well as on the bird's age at the time of infection. Sudden death without showing any premonitory signs of the disease is reported from fledgings of various psittaciformes; in fledging budgerigars the mortality rate may reach 100% (Krautwald *et al.,* 1989; Müller and Nitschke, 1986). The clinical signs shown by acutely infected birds are polyuria, subcutaneous hemorrhage, dyspnea and depression as well as chronic infections of adult psittacines also been described (Graham and Calnek, 1987). The older group of budgerigars may shed the virus intermittently in the feces without showing an obvious clinical signs (Rahaus and Wolff, 2004).

The infection's outcome depends on the age at which the bird was exposed to the virus, maternal antibody levels, route of infection and the amount of the virus that the bird was exposed to. These factors determine if the animal will raise a good immune response (Gerlach, 1986). Secondary infection (e.g.: bacteria, fungi or other viruses) may become a cause of death of APV infection (Dolz *et al.,* 2013).

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2.7 Detection Methods of Avian Polyomavirus

The clinical specimens used to detect APV include blood (Bert *et al.*, 2004; Dolz *et al.*, 2013), liver (Katoh *et al.*, 2008), and feathers (Rahaus and Wolff, 2004). From these samples, detection methods of APV involve cell culture, immunofluorescence assay, and sequence detection assay such as PCR. GFM-1, a strain of APV was successfully isolated from budgerigar in laboratory by culturing it on budgerigar embryonic fibroblast (BEF) and chicken embryonic fibroblast (CEF) (Katoh *et al.*, 2008).

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3.0 MATERIALS AND METHODS

3.1 Sample collection

A total of 85 birds were sampled from four different breeders located in Klang Valley using the convenience sampling method. Among the samples collected, the birds were classified with different groups based on their type from Psittaciformes group. Fecal samples were collected by using sterile wooden stick and kept in the 15 ml sterile tube with cap.

3.2 DNA Extraction

The sample was thawed at room temperature after kept at -80 °C. The sample then re-suspend with prepared SM buffer with the ratio of 1:1 (weight of sample/g:volume of SM buffer/mL). Homogenizer was used to break the cellular particles in the sample. Then, the mixture was centrifuged at 10000 x g for 20 minutes by using Allegra™ X-22R Centrifuge (Beckman Coulter™, United States). The supernatant of the centrifuged mixture was collected and filtered sequentially by using 0.45 µm and 0.2 µm pore-sized syringe filter. The product of this procedure is cell-free sample that would be used in next step of the procedure.

To preparing the lysate, Purelink® Viral RNA/DNA Mini Kit was used in this method. A 25 µL of proteinase K was added into sterile micro-centrifuge tube followed by 200 µL of cell-free sample and 200 µL of lysis buffer and vortex for 15 seconds. The sample was

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then incubated in water at 56°C for 15 minutes. After that, the sample was centrifuged with a short-spin speed for a minute to removes any drops from inside the lid.

To proceed with binding and washing procedure, 250 µL of 96-100% ethanol was added to lysate and vortexed for 15 seconds. The lysate was then incubated with ethanol for 15 minutes in room temperature. The mixture of the lysate and ethanol was centrifuged with a short-spin speed for a minute before it can be transferred into the viral spin column. Then, it was centrifuged again at 6800 x g for a 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 and proceed with centrifugation at 6800 x g for a minute. The wash tube then was discarded. The spin column was transferred into another 2 mL sterile wash tube and centrifuged at 10000 x g for a minute. The flow-through then was discarded. The viral spin column was transferred in a 1.5 mL sterile recovery tube. A 10-50 µL of sterile, RNAse-free water was added to the center of the column. Then, incubate it at room temperature for a minute. The column was centrifuged at 10000 x g for a minute. The spin column then removed and discarded. The purified DNA sample is produced and stored at -80°C.

3.3 Measurement of DNA Concentration

The final DNA concentration was obtained by performing spectrophotometry. After the extraction process, 2 µL of the DNA extract was pipetted out to a cuvette and placed in a BioSpectrometer™ photometer (Eppendorf, Germany).

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3.4 Primers Selection

PCR amplification relies on two primers that determine the region of sequence to amplify in the forward and reverse direction. The forward primer (F) is designed to amplify the sequence towards the reverse primer (R); while the reverse primer is designed for the complementary strand in the opposing direction. Therefore, primer design is an important consideration for specific amplification with high yield. The primers used in this study are listed in Table 1. The functions of all primers used are described in Table 2.

Name	Sequence (5'-3')	Length	Product	References
12SrDNA-F	GGA TTA GAT ACC CCA CTA TGC	21 bp	436 bp	Bert *et al.*, 2005
12SrDNA-R	AGG GTG ACG GGC GGT ATG TAC G	22 bp	436 bp	
tT-F	CAA GCA TAT GTC CCT TTA TCC C	22 bp	310 bp	John and Müller, 1998
tT-R	CTG TTT AAG GCC TTC CAA GAT G	22 bp	310 bp	Chih-Ming Hsu *et al.,* 2006 Dolz *et al.*, 2013

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VP1-F	CTT ATG TGG GAG GCT GCA GTG TT	23 bp	550 bp	Bert *et al.*, 2005 Rahaus and Wolff, 2005 Phalen *et al.*, 1991
VP1-R	TAC TGA AAT AGC GTG GTA GGC CTC	24 bp	550 bp	
APVfull-AF	ACA ATG CCT AAC GGA ACG CC	20 bp	3 kb	Katoh *et al.*, 2009
APVfull-AR	CAC CGA AGC GGC GAT ACT ATA	21 bp	3 kb	
APVfull-BF	GAG GCC TACC ACG CTA TTT CAG TA	24 bp	3 kb	
APVfull-BR	GCA CTT AGC GCC TGT CCA AT	20 bp	3 kb	

Table 1: List of the primers used in this study with a purpose to detect the presence of avian polyomavirus from psittacine birds.

Name	Functions
12SrDNA	Target on avian mitochondrial DNA
tT	Target t/T antigen coding region of APV
VP1	Target ORF1 coding VP1 protein of APV
APVfull-A APVfull-B	Amplify full length of APV (4971-4981 bp)

Table 2: The function of each primer used in this study.

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3.5 Polymerase Chain Reaction

PCR reaction was carried out using MyTaq™ Red Mix, (Bioline™, United States). The PCR reaction was set up in each tube by adding 10 µL of MyTaq™ Red Mix, (Bioline™, United States), 2x, 0.8 µL of forward primer, 0.8 µL of reverse primer, and 2 µL of DNA template depending on the DNA concentration (200 ng/reaction). Sterile distilled water (ddH_2O) was added to make the final volume in each tube to 20 µL.

The amplification was performed using a Bio-Rad T100™ thermal cycler (Bio-Rad, United States). The cycling conditions were carried out as recommended by the manufacturer as described in Table 3.

Step of PCR	Time	Temperature
Initial heat activation	3 minutes	95°C
Denaturation	30 seconds	95°C
Annealing	30 seconds	54/58°C
Extension	1 minute	72°C
Number of cycles	30 cycles	-
Final Extension	5 minutes	72°C

Table 3: Optimized cycling conditions of PCR assay for detection of avian polyomavirus

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3.6 Agarose Gel Electrophoresis

A commercially prepared agarose (Vivantis, #PC0701 – 500 g) was used to prepare 1.0 % (w/v) agarose gel for electrophoresis. A mixture of 60 mL of electrophoresis buffer (TAE buffer) was mixed with 0.6 g of agarose powder and boiled in a microwave (NN-SM332M, Panasonic, Malaysia) until all the powder had dissolved and the agarose solution was homogenous. The agarose solution was then cooled to about 60ºC and 2 µL of DNA staining solution (GelRed™) was added before poured the mixture into a gel mold with a well forming comb. After the gel solidified, it was removed from the mold and placed into an electrophoresis tank (Bio-Rad, USA). TAE buffer was added into the tank until the buffer immerses the surface of the gel by approximately 5 mm. Next, 5 µL of PCR product from each tube was pipetted out and mixed individually with 2 µL of DNA loading dye (CoralLoad Concentrate, 10x, Qiagen®) and loaded into the wells. Subsequently, 1 kb GeneRuler DNA Ladder (Thermo Fisher Scientific) DNA marker was added into the first well as comparison for the PCR products. Electrophoresis was carried out using PowerPac™ Basic (Bio-Rad, USA) power supply at 80 V and 400 mA for approximately 60 minutes or until the DNA ladder (blue dye) reached the opposite end of the gel. After electrophoresis was complete, the gel was placed in a Gel Doc XR+ UV transilluminator (Bio-Rad, USA) for viewing and image captured.

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4.0 RESULT

4.1 Faecal Sampling

A total of 85 faecal samples collected were combined together into 30 pooled samples. Pooled samples are combined according to the species of the birds and location of sampling. Table 4 to 7 are showing the species with number of samples of psittacine birds according to breeders.

Species of Psittacine birds	Abbreviations	No. of samples
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	HM(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

Table 4: Samples collected from Breeder A

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Species of Psittacine birds	Abbreviations	No. of samples
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(A)	3
Hahn's Macaw	HM(A)	1

Table 5: Samples collected from Breeder B

Species of Psittacine Birds	Abbreviations	No. of samples
Budgerigar	BD(C)	3
Local Budgerigar	LB(C)	5
Cockatiel	CKT(C)	3

Table 6: Samples collected from Breeder C

Species of Psittacine Birds	Abbreviations	No. of samples
Budgerigar	BD(D)	12

Table 7: Samples collected from Breeder D

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4.2 Detection of Positive Samples of APV

Out of the 30 pooled samples for this study, six pooled 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 are mostly from Breeder A which is 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 come from Breeder A which is sulphur-crested cockatoo [SCC(B)].

All of positive samples were amplified by APV full-A primer (5'-ACAATGCCTAACGGAACGCC-3', 5'-CACCGAAGCGGCGATACTATA-3') as shown in **Figure 1**.

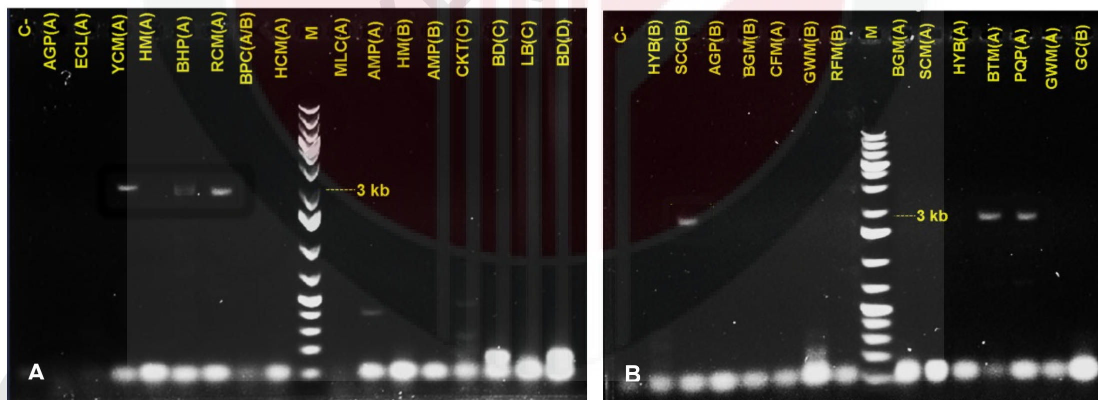

Figure 1: PCR assay for all pooled samples using specific primers targeting the APV full-A gene of avian polyomavirus to produce 3 kbp PCR products. Electrophoresis was carried out on 1.0% agarose gel for an hour. The target bands are observed in six species of psittacine birds. **Figure 1(A):** Lane 4 [yellow-collared macaw (breeder A)], lane 6 [blue-headed parrot (breeder A)], lane 7 [red-crowned macaw (breeder A)]. **Figure 1(B):** Lane 3 [sulphur-crested cockatoo (breeder B)], lane 13 [blue-throated macaw (breeder A)] and lane 14 [Pesquet's parrot (breeder A)]. 1 kb GeneRuler DNA Ladder (Thermo Fisher Scientific) DNA marker was used as reference in lane M.

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5.0 DISCUSSIONS

This study has successfully described APV in psittacine birds for the first time in Malaysia. By using the convenience sampling method to select the various species of psittacine birds, six out of 30 pooled samples of faecal samples were positive for APV by PCR assay, which means a positive detection rate of 20%. This finding met the expected prevalence of 20% as stated by Wayne *et al.,* 2002.

In this study, APV was detected in a sulphur-crested cockatoo. It has been proved by the isolation done by Raidal *et al.,* 1998, and stated that APV has been isolated from wild-caught sulphur-crested cockatoo in Australia. All of the birds involved in this study are apparently healthy and not showing any signs of illness. Many more wild birds will be found to be naturally infected with this virus. Infection in captive-raised birds is widespread. It is likely that all psittacine birds and many other species of birds are susceptible to infection (Phalen, 2007). Most of the APV positive birds were from breeder A. According to the breeder, most of the birds are originated from the wild and it is one of the possible reason why the virus can be easily found in breeder A compared to the others.

According to Merck Veterinary Manual (10th edition), adult birds typically are resistant to infection; they will seroconvert and shed the virus for up to 90 days, then clear the infection. The typical presentation of APV-infected birds is a well-fleshed juvenile and just before fledgling age. Most of adult birds act as a carrier of APV without exhibit any clinical warning to other susceptible birds around them.

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During the process of agarose gel electrophoresis, there was a problem occurred caused by the material used in DNA staining process. A DNA staining solution (GelRed™) used was added before gel was poured into a gel mold with a well forming comb. However, the use of GelRed™ as DNA staining solution sometimes may cause smeared or smiling DNA band or discrepant DNA migration. However, because GelRed™ is high affinity dyes designed to be larger dyes to improve their safety, they can affect the migration of DNA in precast gels. Some samples, such as restriction digested DNA may migrate abnormally in GelRed™ precast gels. The following modifications as recommended by the manufacturer may improve band resolution in precast gels such as reduce the amount of DNA loaded. Smearing and smiling is often caused by overloading of DNA. The recommended loading amount for ladders and samples of known concentration is 50-200 ng/lane. Or reduce the amount of GelRed™ in the gel, for example use 0.5X instead of 1X final concentration of GelRed™.

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6.0 CONCLUSION

In conclusion, the presence of avian polyomavirus (APV) in psittacine birds has been successfully detected. APV was successfully detected using PCR assay. Further study on bioinformatics analysis should be done for APV to identify the characteristics of the virus based on whole genome sequence and phylogenetic tree.

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7.0 RECOMMENDATIONS

Firstly, as avian polyomavirus (APV) has been detected in Malaysia, a prevalence study should be carried out in order to better understand the distribution of this virus in psittacine birds in Malaysia. Optimally, more pet bird breeders and more pet bird owners from more states should be involved. In addition, birds from different age and gender groups should be included to cover all birds in a farm such as hatchlings, juveniles, and adults.

Secondly, the virus should also be isolated and cultured in a suitable cell culture for confirmatory description of the virus through methods such as electron microscopy and immunohistochemistry. Furthermore, cell cultures would also allow study into the cytopathic effects of APV which may increase understanding of its pathogenesis. To take a leap further, once a susceptible cell culture is established and the virus is adequately adapted in the cell culture for cloning, virus inoculation into animal models such as healthy birds may be performed for transmission studies. Other type of samples can be used such as blood, feathers, liver, and spleen.

Lastly, bioinformatics analysis can be done such as whole APV genome sequence and construction of phylogenetic tree and identification of complete genomes of local isolates through next generation sequencing would be ideal for genotyping and further understanding protein expressions of APV.

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APPENDICES

Appendix 1: List of the psittacine birds involved in this study

Birds	Scientific Name
Moluccan cockatoo	*Cacatua moluccensis*
Green-winged macaw	*Ara chloropterus*
Timneh African grey parrot	*Psittacus erithacus timneh*
Congo African grey parrot	*Psittacus erithacus congo*
Scarlet macaw	*Ara macao*
Blue and gold macaw	*Ara ararauna*
Red-fronted macaw	*Ara rubrogenys*
Yellow-collared macaw	*Primolius auricollis*
Chestnut-fronted macaw	*Ara severus*
Eclectus	*Eclectus roratus*
Amazon parrot	*Amazona ochrocephala complex*
Hahn's macaw	*Diopsittaca nobilis nobilis*
Red-crowned macaw	*Ara rubrogenys*
Blue-headed parrot	*Pionus menstruus*
Blue-throated macaw	*Ara glaucogularis*
Pesquet's macaw	*Psittrichas fulgidus*
Hyacinth macaw	*Anodorhynchus hyacinthinus*
Black palm cockatoo	*Probosciger aterrimus*
Sulphur-crested cockatoo	*Cacatua sulphurea*
Galah cockatoo	*Eolophus roseicapillus*
Budgerigar	*Melopsittacus undulatus*
Local budgerigar	*Melopsittacus undulatus*
Cockatiels	*Nymphicus hollandicus*
Hybrid	N/A

Appendix 2: Faecal sampling on the ground in bird's captivity

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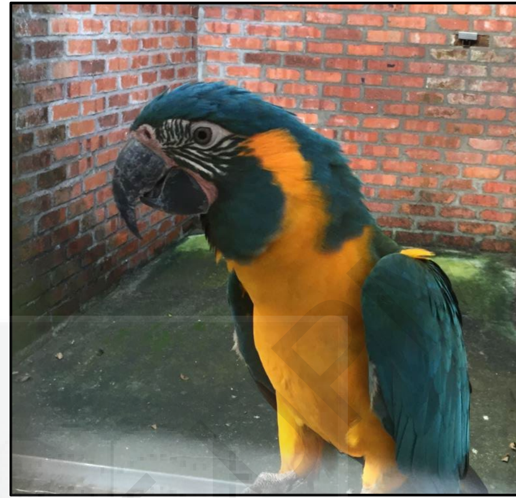
(a)



(b)

(c)

(d)

Appendix 3: Photos of various psittacine species used in the study. (a) Sulphur-crested cockatoo. (b) Blue-throated macaw. (c) Cockatiels (d) Black palm cockatoo

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