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Molecular detection, risk factors and public awareness of avian bornavirus among captive and non-captive birds in Peninsular Malaysia

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Abstract

Introduction: Proventricular dilatation disease (PDD) is caused by avian bornavirus (ABV) has been identified in psittacine, non-psittacine birds and waterfowl. Birds may show signs of gastrointestinal tract deficit or neurological dysfunction or even both. The objectives of this study were to determine the molecular prevalence, risk factors and public awareness of ABV and PDD among captive and non-captive birds in Peninsular Malaysia. **Material and Methods:** A total of 344 cloacal swabs or faeces were collected and subjected to detection using the RT-PCR assay. Meanwhile, KAP questionnaires were distributed by using the Google forms platform. **Results:** Molecular prevalence studies revealed that 4.5% (9/201) of the pet birds were ABV-positive, whereas 0% (0/143) in waterfowl. Nine positive pet birds were identified to be PaBV-2, which is closest to ABV isolates EU781967 (USA). Among the risk factors analysed, category, age and, location, were found to show an association with the ABV positivity. The KAP survey result showed: the respondents have low knowledge (32.9%), however, they showed positive attitude (60.8%) and good practice (94.9%). The association between knowledge, attitude and practice showed that there was a significant association between knowledge-attitude and also attitude-practice ($P < 0.05$). **Conclusion:** This study proved that avian bornavirus (ABV) causes proventricular dilatation disease (PDD) among a group of pet birds of *Psittaciformes,* but it is present in Peninsular Malaysia with a low prevalence rate. Furthermore, in addition to the useful databases obtained from this study, the level of public awareness on the importance of avian bornavirus that causes fatal disorders among a wide range of bird species is satisfactorily raised.

Keywords: avian bornavirus, proventricular dilatation disease, RT-PCR, knowledge, attitude, practice survey, Peninsular Malaysia.

Introduction

Avian bornavirus (ABV), which belongs to the Bornaviridae family, is a major threat to captive and wild birds, causing a disease known as proventricular dilatation disease (PDD) (11). This disease has been reported in more than 80 species of birds, mainly in the Psittacidae family, where it affects lovebirds, macaws (*Ara* spp.), African grey parrots (*Psittacus erithacus*), Afrotropical parrots (*Poicephalus* spp.), Amazon parrots (*Amazona* spp.), and conures (*Aratinga* spp.), but also in the Cacatuidae family, where cockatoos (*Cacatua* spp.) and cockatiels (*Nymphicus hollandicus*) fall prey to PDD (4, 5, 18, 20). Among these species of birds, the most commonly affected by ABV were blue and gold macaws (*Ara ararauna*), African grey parrots, Afrotropical parrots, conures, and cockatoos (20). Moreover, PDD was also found in waterfowl such as swans, geese, gulls and ducks as well as free-ranging birds of five different orders, which were canaries (*Serinus canaria*), greenfinches (*Carduelis chloris*), and long-wattled umbrella birds (*Cephalopterus penduliger*) in the *Passeriformes* order; Canada geese (*Branta canadensis*) in the *Anseriformes* order; roseate spoonbills (*Ajaja ajaja*) in the *Pelecaniformes* order; peregrine falcons (*Falco peregrinus*) in the *Falconiformes* order; and lastly toucans (*Ramphastos* spp.) and bearded barbets (*Lybius dubius*) in the *Piciformes* order (5, 9).

Birds with PDD may show signs of gastrointestinal tract deficit or neurological dysfunction or both (5, 21). Birds with gastrointestinal tract dysfunction may show signs of weight loss, pectoral muscle atrophy, lethargy, reduced appetite, constant or intermittent regurgitation, delayed crop emptying, passage of undigested seed in

faeces and proventricular dilatation with the presence of undigested food (4, 6). Infection of the central nervous system with ABV may manifest in ataxia, abnormal head movements, proprioceptive deficits, seizures, and blindness (6, 15). This virus can be present in groups of captives as well as free-living birds that are clinically diseased or healthy (8, 16). Avian bornavirus was believed to be transmitted horizontally. In many reported cases, ABV was detected in the faeces of affected birds; therefore, the researchers believed that the route of transmission of this virus was faecal-oral (5, 13, 16). Avian bornavirus has a variable incubation period and its survival time in the environment is still unknown (7). Several birds may be affected as early as 11 days or as late as up to 1 month after viral exposure (5). In other studies, it was possible to theorise that it had taken years for the birds to be affected by this virus and to show clinical signs (5, 12). This was because some birds could shed the virus almost at the same interval, while others could shed infrequently (12). The greatest concern is that some healthy birds infected with ABV shed the virus in their droppings without showing any clinical signs (7).

Several potential risk factors have previously been listed modifying the propensity of this disease to spread: the most crowded indoor aviaries were at the highest risk of PDD outbreaks (5). Birds that ingest the droppings shed by infected birds acquire infection, which most birds that are housed together do easily (16). The virus can infect groups of adult birds as well as birds as young as 5 weeks old, but adult birds were more highly susceptible according to previous research. Male and female birds were found to be equally susceptible to PDD (5). In live birds, ABV infection can be easily diagnosed by submitting faeces, blood or feathers samples to molecular of this virus through reverse transcription polymerase chain reaction (RT-PCR) (8, 28). The matrix (M), nucleocapsid (N), phosphoprotein (P) or RNA-dependent RNA polymerase (L) genes of the conserved regions were used as RT-PCR primers which successfully detected the presence of this virus (28).

Avian bornavirus infections can be found in various countries with prevalence assessed through survey detection and reported cases ranging from low to high percentages in both categories of birds. Some of the countries that had ABV-positive birds were Japan with 4.3% of pet cockatoos (Cacatuidae family) recorded in 2013 (23), Brazil with 30% of free-ranging Psittacidae and 28.6% of pet psittacines reported in 2014 (17,19), and the country closest to Malaysia, Thailand, with 54.1% ABV positive in 2019 (22). Three blue and gold macaws were also positive for ABV in South Africa as detected by RT-PCR, histology and immunohistochemistry (14). Moreover, avian bornavirus has also been detected in non-psittacine species. In Germany, from 2010 to 2013, avian bornavirus was detected in two different families, Fringillidae and Estrildidae in the *Passeriformes* order. A total of 12 of the 30 tested canary flocks (40%) were positive for ABV and 3 of 189 samples (1.6%) collected from captive birds were ABV-positive by RT-PCR and virus isolation (21). The three ABV-positive birds were estrildid finches (21). Waterfowl also have documented diagnoses: several countries have reported ABV-positive geese, swans, ducks and gulls after assaying cloacal swabs or brain tissue in real-time PCR or RT-PCR. The list of countries reported were Canada, with 3.4% and 9.3% prevalence detected in geese and mute swans, respectively (3); Denmark, with 2.1% of geese (26); and the USA, with 23% of swans in the north-eastern region (7) and 14.4% of ducks in the state of Oklahoma that tested positive for this virus (9). In North America, 439 gull brain samples were tested and 9 were positive for ABV by RT-PCR (11). Until now, there has been no study of ABV reported in captive and wild birds in Malaysia.

Given that ABV infection prevalence is reported most in psittacine birds and that the owners of such usually keep them as pets rather than professionally, surveillance and control of PDD will fail to be fully effective if it takes no account of the actions of the ordinary individual with an ornamental bird pet. A knowledge, attitude and practice (KAP) survey are defined as a representative study of a target population to collect data from respondents regarding what they know, believe and practise in association with a specific topic (1). Surveys of this kind were selected principally because they are simple to design, conduct, analyse, and interpret by virtue of having a structured questionnaire format. The main objective of conducting a KAP survey is to find the foci for subsequent accurate information provision to the public, especially on the related issues and effective precautionary measures (2). The data from KAP surveys are mainly collected orally by the questioner using an organised and systematic questionnaire (1). It is important to define the objectives and know the purpose of the survey to be conducted. A pre-test is needed before the exact questionnaire is distributed to ensure that the questions are interpreted correctly by respondents (1).

The increasing interest in captive birds as well as the absence of a research study on ABV in Malaysia recommend the conduct of molecular detection through epidemiological surveys, risk analysis and KAP surveys among bird owners as constructive initial actions, as the literature findings regarding ABV infection are still unknown.

Material and Methods

Study area and sample collection. Between January 2019 and March 2021, a cross-sectional sampling study was conducted in which 143 waterfowl (non-captive) and 201 pet bird (captive) samples were randomly collected from Peninsular Malaysia's northern, central, and southern regions and its east coast. The samples taken depended on the availability and permission from the pet owners or farmers or animal centres, because there was no report on this disease in Malaysia yet and consequently no precedent for sampling. Sampling locations for waterfowl and pet birds are mapped in Fig. 1.

A total of 201 pet bird and 143 waterfowl samples were collected. The date, location, species and number of faeces samples collected for each bird are-shown in Tables 1 and 2.

All waterfowl samples were collected by cloacal swabs, while pet birds were fresh faecal material. Cloacal swabs were collected by opening the cloaca, inserting a sterile cotton tip and swabbing the mucosa, whereas faeces were taken from the ground. The swab was then placed in a virus transport medium and kept at −80°C in a freezer.

Ribonucleic acid (RNA) extraction. The presence of the nucleic acid of ABV was evaluated in 344 cloacal swabs and recently collected faecal material samples using an RT-PCR assay. Viral RNA was extracted from cloacal swab or fresh faeces using TRIsure reagent (Bioline, London, UK). Five phases of extraction were conducted, which were homogenisation, phase separation, RNA precipitation, RNA wash and re-dissolution of the RNA. Briefly, in the homogenisation phase, 250 µL of the sample was added to 800 µL of TRIsure. The solution was incubated at room temperature for 5 min before 200 µL of chloroform was added for phase separation. The mixture was vortexed for 30 s before being left at room temperature for 5 min and was then centrifuged at 12,000 × *g* at 4°C for 15 min. The solution was separated into three phases: an aqueous phase (upper), organic phase (lower) and interphase. Approximately 600 µL of the clear aqueous phase was carefully transferred to a new tube without disturbing the interphase. Then, 500 µL of isopropanol was added for RNA precipitation and incubated at room temperature for 10 min before centrifuging at 12,000 × g at 4°C for 10 min. The supernatant was removed, and the pellet was washed with 1,000 µL of 75% ethanol and centrifuged again at 7,500 × *g* at 4°C for 5 min. The supernatant was removed again, and the pellet was air-dried before being dissolved in 15–30 µL of deionised distilled water (ddH_2O) depending on the pellet size. Lastly, the solution was incubated for 10 min at 60°C and stored at −80°C in a freezer.

Complimentary DNA synthesis. The master mix for synthesis of complimentary DNA (Bioline) was prepared by mixing 4 µL of total RNA, 1 µL of primer (oligo), 1 µL of 10Mm deoxyribose nucleotide triphosphate mix, 4 µL of 5× RT buffer, 1 µL of RiboSafe RNase inhibitor, 1 µL of Tetro reverse transcriptase and 8 µL of diethyl pyrocarbonate treated water in a 0.2 mL PCR tube for each synthesis. The solutions were gently mixed by pipetting. The PCR thermal cycler was then programmed for the following synthesis conditions: 45°C for 30 min, 85°C for 5 min for termination, and 4°C for holding.

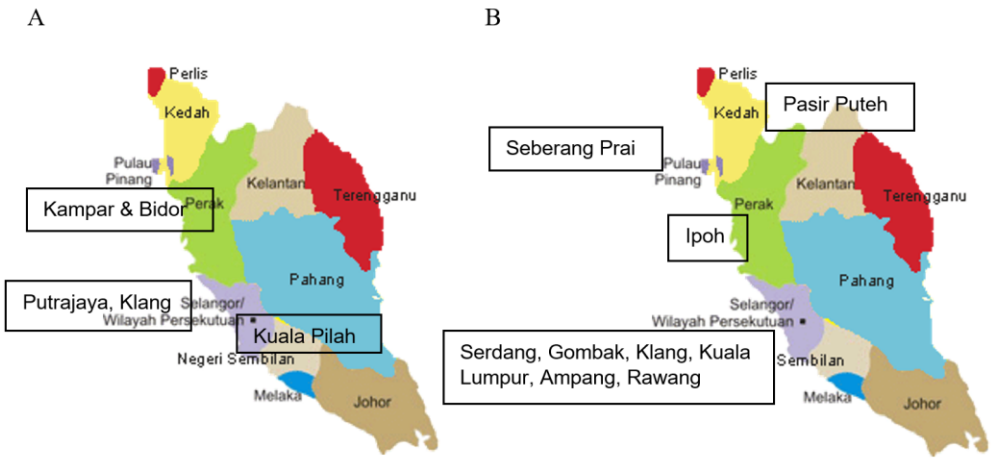

Fig. 1. Peninsular Malaysia sampling locations for waterfowl (A) and pet birds (B) where cloacal swabs or faeces were taken for molecular detection by RT-PCR

Table 1. Waterfowl species and number of swabs collected in different locations from 2019 to 2021

Date	Location	Waterfowl species	Age	Management (group or individual/pair)	Number of swabs collected
2 February 2019	Kampar, Perak	*Anas platyrhynchos* (Pekin duck)	Adult	Group	35
	Bidor, Perak	*Anas platyrhynchos* (Pekin duck)	Adult	Group	37
12 October 2019	Kuala Pilah, Negeri Sembilan	*Anas platyrhynchos* (Khaki Campbell)	Adult	Group	30
9 December 2020	Putrajaya	*Anas platyrhynchos* (domestic duck)	Adult	Group	14
		Cairina moschata (Muscovy duck)	Adult	Group	2
		Cygnus atratus (black swan)	Adult	Group	3
		Alopochen aegyptiaca (Egypt goose)	Adult	Group	8
12 March 2021	Klang, Selangor	*Anser anser domesticus* (Domestic goose)	Adult	Group	9
		Cygus cygus (White swan)	Adult	Group	2
		Anas platyrhynchos (Domestic duck)	Adult	Group	3
		Total			143

Table 2. Pet bird species and number of faeces samples collected in different locations from 2019 to 2021

Date	Location	Pet bird species	Age	Management (group or individual/pair)	Number of faeces samples collected
27 November 2019	Serdang, Selangor	*Ara ararauna* (Blue and gold macaw)	Juvenile	Individual/pair	1
		Aratinga solstitialis (Sun conure)	Juvenile	Individual/pair	1
		Chalcopsitta atra (Black lory)	Adult	Individual/pair	1
		Pyrrhura molinae (Green-cheeked conure)	Juvenile	Individual/pair	1
		Total			4
28 November 2019	Serdang, Selangor	*Taeniopygia guttata* (Zebra finch)	Adult	Group	18
		Chloebia gouldiae (Gouldian finch)	Adult	Individual/pair	1
		Nymphicus hollandicus (Cockatiel)	Juvenile	Individual/pair	1
		Nymphicus hollandicus (Cockatiel)	Adult	Group	7
		Serinus canaria f. domestica (Domestic canary)	Adult	Individual/pair	8
		Melopsittacus undulatus (Budgerigar)	Adult	Individual/pair	1
		Copsychus saularis (Magpie-robin)	Adult	Individual/pair	4
		Pycnonotus jocosus (Red-whiskered bulbul)	Adult	Group	4
		Total			44
20 January 2020	Klang, Selangor	*Pionus menstruus* (Blue-headed pionus)	Adult	Individual/pair	1
		Eclectus roratus (Eclectus parrot)	Adult	Individual/pair	1
		Cacatua galerita (Sulphur crusted cockatoo)	Adult	Individual/pair	3
		Taeniopygia guttata (Zebra finch)	Adult	Group	6
		Lonchura oryzivora (Java finch)	Adult	Group	3
		Chloebia gouldiae (Gouldian finch)	Adult	Group	2
		Total			16
18 August 2020	Ampang, Selangor	*Nymphicus hollandicus* (Cockatiel)	Adult	Group & indiv/pair	2
		Melopsittacus undulatus (Budgerigar)	Juvenile	Group	6
		Melopsittacus undulatus (Budgerigar)	Adult	Group & indiv/pair	8
		Total			16
12 September 2020	Seberang Prai, Pulau Pinang	*Cacatua galerita* (Sulphur-crested cockatoo)	Juvenile	Individual/pair	2
		Pyrrhura molinae (Green-cheeked conure)	Adult	Individual/pair	6
		Aratinga solstitialis (Sun conure)	Adult	Individual/pair	1
		*Trichoglossus moluccanus (*Rainbow lorikeet*)*	Adult	Individual/pair	4
		Eclectus roratus (Eclectus parrot)	Juvenile	Individual/pair	1
		Agapornis personatus (Yellow-collared lovebird)	Adult	Group & indiv/pair	10
		Psittacula krameria (Ring-necked parakeet)	Adult	Individual/pair	8
		Total			32
24 September 2020	Kuala Lumpur	*Ara ararauna* (Blue and gold macaw)	Adult	Individual/pair	7
		Cacatua galerita (Sulphur-crested cockatoo)	Adult	Individual/pair	6
		Cacatua alba (Umbrella cockatoo)	Adult	Individual/pair	1
		Cacatua moluccensis (Salmon-crested cockatoo)	Adult	Individual/pair	1
		*Psittacus erithacus (*African grey parrot*)*	Adult	Individual/pair	4
		Amazona sp. *(*Amazon parrot)	Adult	Individual/pair	2
		Aratinga solstitialis (Sun conure)	Adult	Group & indiv/pair	5
		Aratinga solstitialis (Sun conure)	Juvenile	Group	1
		Eclectus roratus (Eclectus parrot)	Adult	Individual/pair	4
		Trichoglossus moluccanus (Rainbow lorikeet*)*	Adult	Individual/pair	1
		Acridotheres tristis (Indian mynah)	Adult	Group	1
		Pycnonotus jocosus (Red-whiskered bulbul)	Adult	Group	3
		Total			36
28 September 2020	Ipoh, Perak	*Ara ararauna* (Blue and gold macaw)	Juvenile	Individual/pair	1
		Total			1
2 October 2020	Pasir Puteh, Kelantan	*Pyrrhura molinae* (Green-cheeked conure)	Adult	Group & indiv/pair	16
		Pyrrhura molinae (Green-cheeked conure)	Juvenile	Individual/pair	7
		Trichoglossus moluccanus (Rainbow lorikeet)	Adult	Individual/pair	4
		Psittacus erithacus (African grey parrot)	Adult	Individual/pair	2
		Amazona sp. (Amazon parrot)	Adult	Individual/pair	1
		Psittacula krameria (Ring-neck parakeet)	Adult		2
		Total			32
16 November 2020	Serdang, Selangor	*Ara ararauna* (Blue and gold macaw)	Adult	Individual/pair	1
		Ara ararauna (Blue and gold macaw)	Juvenile	Individual/pair	1
		Total			2
25 November 2020	Gombak, Selangor	*Ara ararauna* (Blue and gold macaw)	Adult	Individual/pair	1
		Probosciger aterrimus (Black palm cockatoo)	Adult	Individual/pair	1
		Cacatua galerita (Sulphur crested cockatoo)	Adult	Individual/pair	3
		Psittacus erithacus (African grey parrot)	Adult	Individual/pair	1
		Eclectus roratus (Eclectus parrot)	Adult	Individual/pair	3
		Eclectus roratus (Eclectus parrot)	Juvenile	Individual/pair	1
		Psittacula krameria (Ring-necked parakeet)	Adult	Individual/pair	2
		Aratinga solstitialis (Sun conure)	Adult	Individual/pair	2
		Total			14
9 March 2021	Rawang, Selangor	*Ara ararauna* (Blue and gold macaw)	Adult	Individual/pair	1
		Nymphicus hollandicus (Cockatiel)	Adult	Individual/pair	2
		Serinus canaria f. domestica (Domestic canary)	Adult	Individual/pair	1
		Total			4
		All-location total			201

PCR reaction. Cloacal swab or faeces were the material in which the detection of M antigen by RT-PCR was attempted as described by Guo *et al.* (8). An ABV M forward primer (5′-GGTAATTGTTCCTGG ATGG-3′) and ABV M reverse primer (5′ACA CCAATGTTCCGAAGACG 3′) were used to yield an expected ABV M product of 350 base pair in length. The master mix was prepared on ice. The solutions were vortexed and centrifuged before use. The PCR reaction master mix (Bioline) was prepared by mixing 0.5 µL primer, 12.5 µL myTaq red mix and 8 µL ddH_20 in a 0.2 mL PCR tube. A 4 µL volume of template was then added to the master mix, giving a total volume of 25 µL of solution for each reaction. The thermal cycler started with an initial denaturation step at 95°C for 1 min, there followed 30 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 15 sec, and extension at 72°C for 10 sec, and the cycler ended at 4°C for holding.

Agarose gel electrophoresis. The final step was the preparation of 1.5% agarose gel in Tris acetate ethylenediaminetetraacetic acid (TAE) buffer containing 3 µL red gel stain (Vivantis, Shah Alam, Malaysia). The gel was then placed in the electrophoresis tank, and TAE buffer was slowly added. Then 3 µL DNA ladder (Bioline) was loaded into the first well, followed by 10 µL positive and negative controls and PCR product for each well of the gel. The gel was run at 120 V for approximately 30 min. The products were then visualised under ultraviolet light.

DNA sequencing. The PCR product or gel was sent for sequencing, where the M gene was targeted. The PCR products were directly sequenced using the Sanger technique by a commercial company (Apical Scientific, Seri Kembangan, Malaysia). The sequencing result was then initially screened with nucleotide BLAST (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and a phylogenetic tree was constructed using MEGA X software by selecting the maximum-likelihood method with the Tamura-3 parameter model and 1,000 bootstrap replicates after multiple sequence alignment *via* MUSCLE (Multiple Sample Comparison by Log-Expectation) (26).

Statistical analysis. The chi-squared test is a statistical test which measures the relation between two categorical variables (27). In this study, the test was used to determine the association between ABV infection and risk factor for ABV-positivity. Each data category was determined in SPSS software version 25.0 (IBM, Armonk, NY, USA) by selecting descriptive statistics, crosstabs and chi-squared analysis, where a P-value of less than or equal to 0.05 was considered significant.

Odds ratio and confidence intervals for positive samples. The odds ratio (OR) is a measure of the association between odd yields exposed and odd yields unexposed. It is the ratio of the probability of positive samples to the probability of negative samples. Odds ratios are most commonly used in case-control studies as well as cross-sectional and cohort study designs. The 95% confidence interval (CI) is used to measure the accuracy of the OR (24). The OR was calculated with a 95% CI using binary logistic regression in SPSS version 25.0 to study the association between risk factors. In the logistic regression analysis, the result was recorded as dependent, and the covariates were listed as possible risk factors. The birds were categorised by category, family, species, location, age and management.

Knowledge, attitude and practice survey. A cross-sectional study was conducted by evaluating the knowledge of, attitude towards and practice when encountering avian bornavirus among the pet owners or farmers in Peninsular Malaysia. This KAP survey was performed with the permission of the Ethics Committee for Research Involving Human Subjects of the Universiti Putra Malaysia (JKEUPM) and the birds'owners (No: JKEUPM-2020-362).

Survey sampling. The study was conducted in Peninsular Malaysia targeting farmers, pet owners and animal centres randomly. The sample size was calculated using the formula:

$$\text{Sample size} = \left(\frac{z^2 p(1-p)}{e^2}\right)/1 + \left(\frac{z^2 p(1-p)}{e^2 N}\right)$$

where N is the population size, e is the margin of error (5%), and z is the statistic for the level of confidence (95% CI, 1.962). The population size was based on total samples collected for molecular detection. A total of 181 respondents were asked about their knowledge of, attitude towards and practices when confronting this virus. The KAP survey was distributed to the respondents online *via* Google Forms (Google, Mountain View, CA, USA).

Sampling method. The respondents' KAP regarding ABV were evaluated using a self-administered questionnaire and convenience sampling. The survey took around 10 to 15 min to complete. The respondents were required to answer the questions completely from their own point of view. The inclusion and exclusion criteria are listed below.:

i. Inclusion criteria:
   - Respondents consist of farmers or pet owners
   - Respondents are over 18 years old

ii. Exclusion criteria:
   - Respondents do not understand the Malay or English language
   - Respondents do not own any birds

Study instrumentation (questionnaire). The questionnaire comprised three parts:

Part A consisted of socio-demographic characteristics of the respondents;

Part B consisted of general questions on ABV;

Part C consisted of 3 subsections:

- Knowledge about ABV,
- Attitude of the respondents towards ABV,
- Practices regarding ABV.

Part A (six questions) obtained the respondents' age, gender, race, educational level and occupation. Part B (four questions) probed for general knowledge of ABV. This part aimed to ascertain whether the

respondents knew about the existence of this virus. Lastly, part C (three main questions) gauged the respondents' knowledge, and attitude and sought information about their practices. There were nine questions in the first subsection related to knowledge on ABV which broached the aetiology, signs and symptoms, species affected, transmission and management. The answer choices were "Yes", "No", and "I do not know". The second subsection was about the attitude of the subjects towards ABV. Eight questions were asked, to answer which the subjects chose from the three alternatives. The final subsection was about practices when ABV is known to circulate. There were nine questions, and the responses were "Agree", "Disagree" or "Not sure".

Statistical analysis. The collected data were analysed using SPSS software version 25.0. The socio-demographic characteristics and each question on KAP were analysed using frequency and percentage and each answer in the subsection of part C was scored accordingly. The total score for each subsection in part C was analysed with descriptive statistics using the chi-squared test to identify correlations between KAP, in which the significance level was $\alpha = 0.05$ with 95% CI, and a P-value of $P < 0.05$ was considered statistically significant.

Results

Molecular detection of ABV in pet birds and waterfowl by RT-PCR assay. The gel electrophoresis result showed that 4.5% (9/201) of the pet birds, of the *Psittaciformes* order were positive but no ABV nucleic acid was detected in waterfowl (0/143). Of nine ABV-positive samples, two were from blue and gold macaws (*Ara ararauna*), one from an Amazon parrot (*Amazona* sp.), four from green-cheeked conures (*Pyrrhura molinae*), one from a cockatiel (*Nymphicus hollandicus*) and one from a rainbow lorikeet (*Trichoglossus moluccanus*). The two virus-positive blue and gold macaws showed clinical signs of PDD such as lethargy, crop dilatation and undigested seeds in faeces whereas the other seven birds were healthy with good body condition and had no outward clinical signs of PDD. The summary of the RT-PCR results for both categories of birds is presented in Table 3. Representative bands of gel electrophoresis are shown in the figures below (Figs 2 and 3).

Table 3. Prevalence (%) of avian bornavirus in pet birds and waterfowl

Category	Order	Family	Genus/species	Common name	Number of testedbirds	RT-PCR positive birds	RT-PCR negative birds
Pet birds	Psittaciformes	Psittacidae	*Ara ararauna*	Blue and gold macaw	13	2	11
			Psittacus erithacus	African grey parrot	7	0	7
			Amazona sp.	Amazon parrot	3	1	2
			Aratinga solstitialis	Sun conure	10	0	10
			Pyrrhura molinae	Green-cheeked conure	30	4	26
			Pionus menstruus	Blue-headed pionus	1	0	1
		Cacatuidae	*Nymphicus hollandicus*	Cockatiel	12	1	11
			Cacatua galerita	Sulphur-crested cockatoo	14	0	14
			Cacatua alba	Umbrella cockatoo	1	0	1
			Cacatua moluccensis	Salmon-crested cockatoo	1	0	1
			Probosciger aterrimus	Black palm cockatoo	1	0	1
		Psittaculidae	*Chalcopsitta atra*	Black lory	1	0	1
			Melopsittacus undulatus	Budgerigar	15	0	15
			Eclectus roratus	Eclectus parrot	10	0	10
			Trichoglossus moluccanus	Rainbow lorikeet	9	1	8
			Agapornis personatus	Yellow-collared lovebird	10	0	10
			Psittacula krameria	Ring-necked parakeet	12	0	12
	Passeriformes	Estrildidae	*Taeniopygia guttata*	Zebra finch	24	0	24
			Chloebia gouldiae	Gouldian finch	3	0	3
			Lonchura oryzivora	Java finch	3	0	3
		Fringillidae	*Serinus canaria f. domestica*	Domestic canary	9	0	9
		Corvidae	*Copsychus saularis*	Oriental magpie-robin	4	0	4
		Pycnonotidae	*Pycnonotus jocosus*	Red-whiskered bulbul	7	0	7
		Sturnidae	*Acridotheres tristis*	Indian mynah	1	0	1
			Total		201	9 (4.5%)	192
Waterfowl	Anseriformes	Anatidae	*Anas platyrhynchos*	Pekin duck	72	0	72
			Anas platyrhynchos	Khaki Campbell	30	0	30
			Anas platyrhynchos	Domestic duck	17	0	17
			Cairina moschata	Muscovy duck	2	0	2
			Cygnus atratus	Black swan	3	0	3
			Alopochen aegyptiaca	Egypt goose	8	0	8
			Anser domesticus	Domestic goose	9	0	9
			Cygnus cygnus	Whooper swan	2	0	2
			Total		143	0 (0%)	143

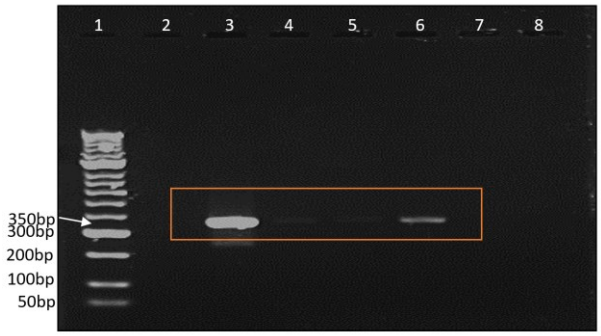

Fig. 2. Agarose gel electrophoresis result in pet birds using avian bornavirus (ABV) M primers yielding an expected PCR product of 350 bp. Lane 1 – 50 bp DNA ladder; Lane 2 – negative control; Lane 3 – positive control; Lanes 4-6 – ABV-positive; Lanes 7 and 8 – ABV-negative

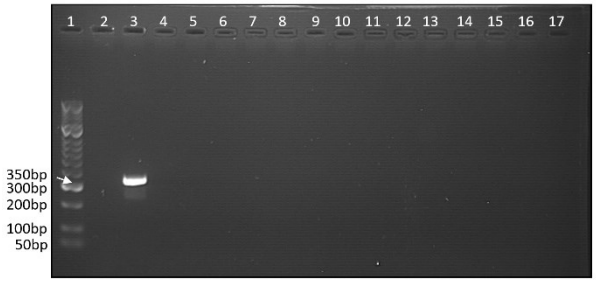

Fig. 3. Agarose gel electrophoresis result in waterfowl using avian bornavirus (ABV) M primers yielding an expected PCR product of 350 bp. Lane 1 – 50 bp DNA; Lane 2 – negative control; Lane 3 – positive control; Lanes 4-17 – ABV-negative

DNA sequencing analysis. The nine bornavirus positive samples from pet birds were sent to Apical laboratory for DNA sequencing to confirm the identification of ABV. The sequence results obtained were analysed using nucleotide BLAST at the National Centre for Biotechnology Information in the USA to compare the new sequence with existing reference sequences obtained from GenBank. The result showed that the nine samples from pet birds shared an overall 88 to 99% nucleotide sequence identity with parrot bornavirus (PaBV)-2, one sample (cockatiel) being closest to the EU781967 ABV isolate that originated from the USA. Figure 4 is the phylogenetic tree showing the relatedness of the isolates from the present study to previously sequenced ABV isolates.

Risk factors associated with ABV infection. The binary logistic regression results showed the association between several potential risk factors with ABV positivity (Table 2). Overall higher prevalences were shown in pet birds (4.5%), the Psittacidae family (10.6%), the *Pyrrhura molinae* species (6.1%), adult age (3%), the east coast region (3%) and the birds reared or kept in a group (2.5%). There was a significant association between ABV-positivity and each of category (pet birds), age (adult) and region (east coast), indicated by the P-value of less than 0.05.

Socio-demographic characteristics. A total of 158 bird owners responded out of the 181 who received a questionnaire, giving a response rate of 87%. Briefly, the largest part of the study population was in the age group of 31–40 years old (62%), men dominated in the respondents (86.1%) and almost all the participants were Malay (82.3%). It emerged additionally, that 46.8% of the respondents had a university education and 40.5% worked in the private sector. Only 62 and 103 respondents had previously heard respectively about ABV and PDD, mainly getting the information from social media, this being the case for 45.2% (28/62) of those cognisant of ABV and for 68% (70/103), of those with some awareness of PDD.

Assessment of knowledge of avian bornavirus and proventricular dilatation disease. Table 6 shows the responses of the participants regarding their knowledge of ABV and PDD. Nine questions were asked to gauge the respondents' knowledge. The evaluation of the knowledge was focused on aetiology, clinical signs, species, transmission and management. The choice for the responses was "Yes", "No" or "I don't know", and the correct answer to all the questions was "Yes". The respondents got 1 point for an affirmative answer and 0 points for a negative or unknowing answer. The range of scores was between 0 and 9 points. An overall score of ≤5 was considered to evince little knowledge, whereas one of >5 was considered to indicate extensive knowledge about this disease. Of the 158 respondents, only 32.9% (52/158) of the participants showed adequate knowledge of ABV and PDD, whereas 67.1% had scant knowledge. Based on each questionnaire analysis, most of the respondents were aware of the disease, with 65.2% having previously heard of it. Although the respondents had heard about the disease, its aetiology was still unfamiliar, with affirmative answers from only 35.4%. In addition, low knowledge was also apparent from the responses to questions relating to the common species liable to be affected, the disease's main signs and symptoms, the phenomenon of intermittent shedding, and the possibility of ABV being transmitted by healthy birds: 43%, 35.4%, 22.8% and 29.1% of respondents answered "Yes", respectively. The "I don't know" answer was mostly selected in the knowledge criteria. Meanwhile, the percentages of affirmative answers and unknowing answers to questions regarding the body system affected, and the preventive measures to take were in almost the same range for the answer 'Yes' and the answer 'I don't know'.

Assessment of attitude towards avian bornavirus and proventricular dilatation disease. Table 7 shows the responses of the participants on their attitude towards ABV and PDD. The criteria were encoded as eight questions, as shown in the table below. The choice for the responses was again "Yes", "No" or "I don't know" and the correct answer for all the questions was "Yes". Points were allocated as noted above and gave a score in the range of 0 to 8. A score of ≤4 was interpreted as an obstructive attitude whereas one of >4 was interpreted an amenable attitude towards the control of this virus. Unlike the knowledge assessment scores, the scores of the majority of the respondents were sufficiently high to indicate an engaged attitude toward ABV and PDD

prevention, with 60.8% (96/158) of the participants scoring >4. The majority of them gave affirmative responses to all attitude criteria questions except for questions number 2 ("Can PDD be prevented?") and number 3 ("Do you believe that healthy birds may have ABV but not show any signs and symptoms?") to which the percentages of the respondents that agreed were 30.4% and 45.6%, respectively.

Assessment of practices to minimise avian bornavirus infection and proventricular dilatation disease outbreaks. Table 8 shows the responses of the participants on their practices to combat ABV and PDD. Nine questions were asked to assess these practices. The answer choices were "Agree", "Disagree", and "Not sure", and the correct answer for all the questions was "Agree". A score of 1 was given for the "Agree" answer while one of 0 was given for the "Disagree", and "Not sure" answers, and scores cumulated to a value in the range of 0 to 9. The scale classified respondent practices as good at >5 and poor at ≤5. Most of the respondents applied good practices to impede ABV spread as well as PDD outbreaks, with almost 94.9% of the respondents agreeing with the recommended practices stated in the questionnaire.

Association between socio-demographic data and knowledge, attitude and practices towards avian bornavirus proventricular dilatation disease. Table 9 presents the association between demographic characteristics and total KAP scores. The relationship between the total KAP score and demographic information was analysed using the chi-squared test. Among the socio-demographic characteristics, age was the only aspect that was associated with all KAP scores, with a $P < 0.05$. There were no significant associations evident between the other characteristics, except for one between education and respondents' knowledge of ABV and PDD ($P = 0.037$) and one between occupation and the preventive practices score ($P = 0.000$).

Association between knowledge, attitude and practice scores. Table 10 shows the association between knowledge, attitude and practice scores. The correlation revealed significant positive linear correlations between knowledge and attitude ($P = 0.000$) as well as attitude and practice ($P = 0.034$) with $P < 0.05$. Thus, the result confirms the relationship between the knowledge and attitude pair and that of attitude and practice regarding ABV and PDD.

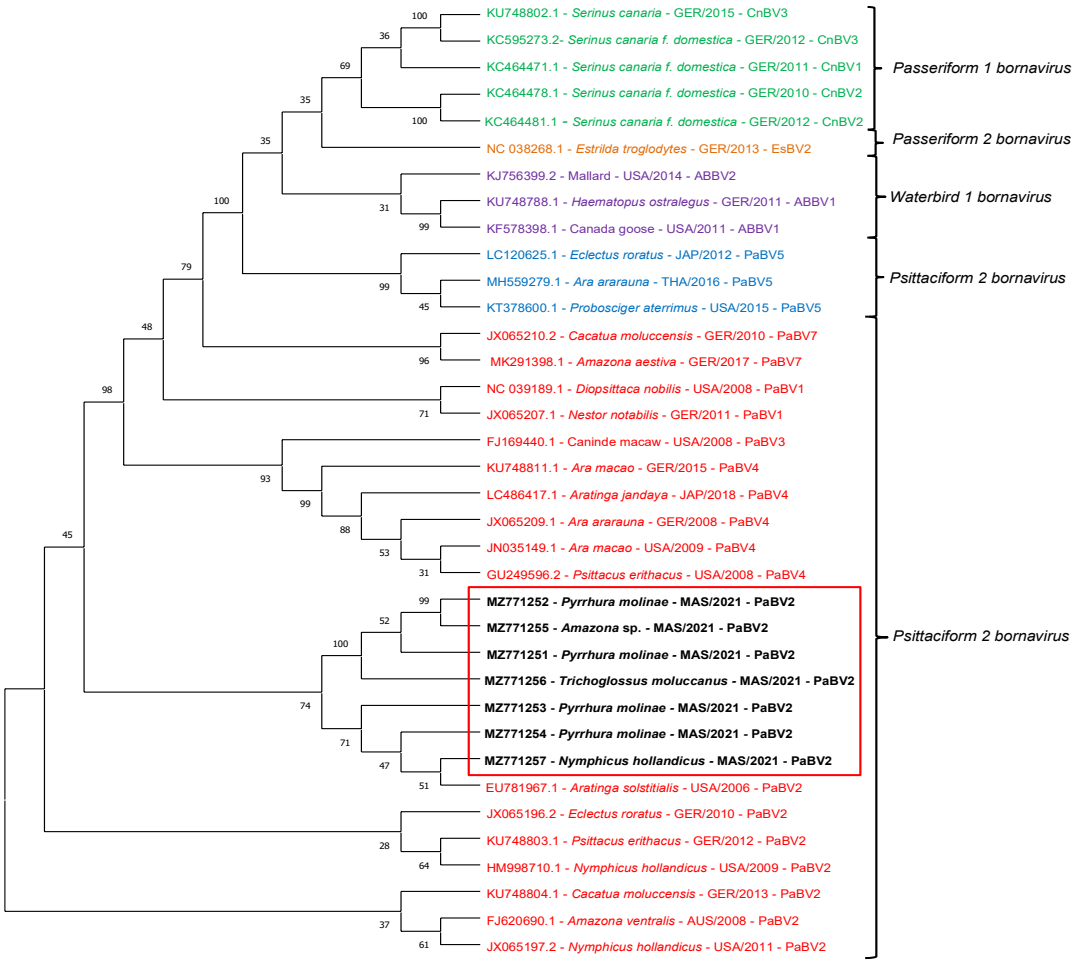

Fig. 4. Phylogenetic tree using maximum likelihood tree building based on the partial M gene (350 bp). The tree was constructed based on reference sequences stored in the GenBank database. Sequences emphasised in the box were this study's ABV-positive samples

Table 4. Association between ABV-positivity and potential risk factors estimated by binary logistic regression

			Total	Prevalence of ABV (%)	P-value	Odd ratio	95% confidence interval
Factor	Category	Pet birds	201	4.5 (9/201)	0.010	1.7	1.591–1.914
		Waterfowl	143	0 (0/143)		NA	NA
	Family	Psittacidae	46	10.6 (7/66)	0.844	0.7	0.075–6.471
		Cacatuidae	11	1.5 (1/66)		1.3	0.067–23.259
		Psittaculidae	9	1.5 (1/66)		Ref	Ref
	Species	*Ara ararauna*	13	3 (2/66)	0.867	0.7	0.053–8.960
		Amazona sp.	3	1.5 (1/66)		0.3	0.010–5.985
		Pyrrhura molinae	30	6.1 (4/66)		0.8	0.079–8.352
		Nymphicus hollandicus	11	1.5 (1/66)		1.3	0.067–23.259
		Trichoglossus moluccanus	9	1.5 (1/66)		Ref	Ref
	Age	Adult	177	3 (6/201)	0.043	4.1	0.947–17.499
		Juvenile	24	1.5 (3/201)		Ref	Ref
	Region	East coast	32	3 (6/201)	0.000	0.1	0.012–0.388
		North	33	0.5 (1/201)		0.5	0.042–5.432
		Central	136	1 (2/201)		Ref	Ref
	Management	Group	75	2.5 (5/201)	0.247	0.5	0.119–1.766
		Individual/pair	126	2 (4/201)		Ref	Ref

Ref–reference categories

Table 5. Socio-demographic characteristics of study population

			n (/158)	Percentage (%)
Characteristic	Age	18-30	36	22.8
		31-40	98	62
		41-50	20	12.7
		51-60	4	2.5
	Gender	Male	136	86.1
		Female	22	13.9
	Race	Malay	130	82.3
		Chinese	16	10.1
		Indian	10	6.3
		Others	2	1.3
	Education	No formal education	0	0
		Primary school	4	2.5
		Secondary school	44	27.8
		Matriculation/foundation/college	28	17.7
		University (bachelor, masters, doctorate)	74	46.8
		Others	8	5.1
	Occupation	Government	38	24.1
		Private	64	40.5
		Self-employed	44	27.8
		Others	12	7.6
			n (/62)	
	Source of ABV information	Veterinarian	16	25.8
		Brochures, posters and other printed materials	0	0
		Internet, blog, website, social, media, Facebook	28	45.2
		Family, friends, neighbours and colleagues	18	29
		Others	0	0
			n (103)	
	Source of PDD information	Veterinarian	19	18.4
		Brochures, posters and other printed materials	0	0
		Internet, blog, website, social, media, Facebook	70	68
		Family, friends, neighbours and colleagues	14	13.6
		Others	0	0

Table 6. Knowledge of the participants about avian bornavirus (ABV) and proventricular dilatation disease (PDD)

ABV and PDD knowledge items	Yes	No	I don't know
	n (%)	n (%)	n (%)
Had you ever heard about ABV before?	62 (39.2)	96 (60.8)	0 (0)
Had you ever heard about PDD before?	103 (65.2)	55 (34.8)	0 (0)
PDD is caused by ABV	56 (35.4)	0 (0)	102 (64.6)
It is a fatal disease that can affect mainly the neurological and digestive systems of the bird	82 (51.9)	0 (0)	76 (48.1)
Common species affected by ABV are domestic birds (*e.g.*: macaws, cockatoos, and cockatiels) and waterfowl (*e.g.*: ducks, swans, and geese)	68 (43)	4 (2.5)	86 (54.4)
The main signs and symptoms of birds affected by ABV are a reduced appetite, dilatation of the proventriculus, ventriculus or intestines, passage of undigested seeds in the faeces and seizures	56 (35.4)	2 (1.3)	100 (63.3)
ABV is intermittently shed in faeces and urates	36 (22.8)	4 (2.5)	118 (74.7)
ABV can be transmitted by healthy birds without the birds showing any clinical signs	46 (29.1)	0 (0)	112 (70.9)
ABV can be prevented by minimising stress through providing good nutrition and practising good husbandry	78 (49.4)	4 (2.5)	76 (48.1)

Table 7. Attitude of the participants toward ABV and PDD

ABV and PDD attitude criterion	Yes	No	I don't know
	N (%)	N (%)	N (%)
Do you think PDD is a fatal bird disease?	90 (57)	4 (2.5)	64 (40.5)
Can PDD be prevented?	48 (30.4)	6 (3.8)	104 (65.8)
Do you believe that healthy birds may have ABV but not show any signs or symptoms?	72 (45.6)	2 (1.3)	84 (53.2)
Do you believe that healthy birds with ABV without showing clinical signs may infect other birds?	82 (51.9)	2 (1.3)	74 (46.8)
Should a bird suspected of PDD or having PDD-like symptoms be isolated or quarantined from others?	120 (75.9)	4 (2.5)	34 (21.5)
Will you seek the advice of a veterinarian if your bird has PDD-like symptoms?	126 (79.7)	12 (7.6)	20 (12.7)
Do you carry out regular health checks on your bird?	108 (68.4)	44 (27.8)	6 (3.8)
Should you carry out a health check on a new bird?	140 (88.6)	8 (5.1)	10 (6.3)

Table 8. Practices of the participants with respect to avian bornavirus and proventricular dilatation disease

ABV and PDD practices	Agree	Disagree	Not sure
	n (%)	n (%)	n (%)
Provide good nutrition with an adequate amount of feed	148 (93.7)	0 (0)	10 (6.3)
Provide good husbandry and adequate ventilation	152 (96.2)	0 (0)	6 (3.8)
Wash hands between handlings of birds	150 (94.9)	0 (0)	8 (5.1)
Use a footbath or disinfect shoes when going from one place to another	138 (87.3)	2 (1.3)	18 (11.4)
Properly clean cages and dispose of bird faeces	154 (97.5)	0 (0)	4 (2.5)
Carry out regular health checks on birds	140 (88.6)	0 (0)	18 (11.4)
Quarantine or isolate if a bird shows signs inviting suspect of PDD	154 (97.5)	0 (0)	4 (2.5)
Quarantine and carry out a health check on a new bird	152 (96.2)	0 (0)	6 (3.8)
Seek veterinarian advice if bird is suspected of having PDD	150 (94.9)	2 (1.3)	6 (3.8)

Table 9. Comparison of Demographic characteristics and knowledge, attitude and practice scores

			n (158)	Knowledge score	Attitude score	Practice score
				P-value	P-value	P-value
Characteristic	Age	18–30	36	0.005	0.009	0.000
		31–40	98			
		41–50	20			
		51–60	4			
	Gender	Male	136	0.273	0.766	0.243
		Female	22			
	Race	Malay	130	0.634	0.369	0.469
		Chinese	16			
		Indian	10			
		Others	2			
	Education	No formal education	0	0.037	0.084	0.920
		Primary school	4			
		Secondary school	44			
		Matriculation/Foundation/College	28			
		University (Bachelors/Masters/Doctorate)	74			
		Other	8			
	Occupation	Government	38	0.322	0.529	0.000
		Private	64			
		Self-employed	44			
		Other	12			

Table 10. Association between knowledge, attitude and practice scores

Variable	Correlation coefficient	P-value
Knowledge-attitude	50.058	0.000
Knowledge-practice	0.239	0.625
Attitude-practice	4.519	0.034

Discussion

Proventricular dilatation disease caused by ABV is a fatal infectious disease reported mostly in psittacine birds as well as waterfowl such as swans, geese, gulls and ducks (4, 9, 18). In this study, there was 4.5% occurrence of this virus among pet birds from the *Psittaciformes* order, indicating the first ABV occurrence in Malaysia. Lower and higher percentages of detection can be seen when making a comparison with other countries. A higher prevalence was found in Thailand with 54% ABV-positive cases, while a lower one was detected in Japan with 4.3% (22, 23). The results from this study indicated a low percentage of ABV infection among pet birds. Out of nine pet bird's positives for ABV, two blue and gold macaws showed clinical signs of PDD. Based on previous research, PDD was first detected in macaws imported from Bolivia to the United States (13). In fact, a previous study showed that blue and gold macaws are one of the most common species affected by ABV (20). All nine samples of pet birds were identified to harbour PaBV-2 by sequencing;

according to previous research, PaBV-2 and PaBV-4 were found to be responsible for most of the disease cases (10).

All 143 waterfowl samples collected were ABV negative. Avian bornavirus was first detected in waterfowl in 2009 in free-ranging Canada geese (*Branta canadensis*) and trumpeter swans (*Cygnus buccinator*) in Ontario, Canada, followed by the finding in 2014 when ABV was isolated from ducks in North America (26). Different prevalence has previously been reported from several countries, the north-eastern USA being an example, where a higher rate of 23% was found in swans but a lower one in gulls, with 2% testing positive for ABV (7, 11). The overall prevalence in waterfowl were between 2 and 23% in various countries, indicating low infection levels among water birds. In this study, different waterfowl species' samples were collected for molecular detection by taking cloacal swabs. So far, no case has been reported in any species of waterfowl in Malaysia; therefore, breed disposition to infection might be one of the factors that causes results to trend to the negative. The particularities of the molecular method employed may be discounted with regard to inadequate sensivity. Furthermore, there is no issue regarding the primer used, as ABV has previously been detected in duck using the same primer (9). Moreover, ABV has been proven to be detectable in faeces as well as cloacal swabs; therefore, the method of collecting the samples would not affect the result because the presence of ABV may be detected by molecular detection through RT-PCR either directly from a cloacal swab or from the droppings (12).

Some ABV-infected birds may remain healthy while shedding the virus in their droppings. These birds may shed the virus either constantly or intermittently (12). Hence, the collection of faecal samples just once is not sufficient to prove the absence of ABV infection, because some of the birds may become carriers and intermittently shed the virus. It was recommended that faecal samples be collected more than three times several days apart in order to detect infection reliably in birds (8). Since the investigated waterfowl were free-ranging birds and some of the pet bird samples were collected from pet stores, pooling of multiple droppings was rather difficult. Thus, it was recommended to collect choanal swabs or feather calami to increase the detection rate. To mitigate the limitation of using a one-off PCR test as described above, ABV infection in live birds may also be diagnosed using serologic assays such as ELISA or Western blot. Since some birds may shed the virus continually, ABV-specific antibodies can be detected by serology techniques using avian serum or plasma. It is recommended to perform both methods since serogical analysis is usually undertaken as a complimentary step for comparison with the results of RT-PCR in order for an optimum result to be obtained (8).

In this study, the samples used were taken from pet birds mainly bred in captivity and from commercial waterfowl. Regarding geographical aspects of the pathogen, the obtainment of each sample from pet birds and waterfowl in a different location was not conducive to their elucidation. The possible transmission routes from waterfowl to pet birds or in the opposite direction are still unknown and need further investigation. Nevertheless, the possible transmission routes or other potential carriers need to be highlighted and further discussed to find better preventive measures against the pathogen.

In this study, several factors have been listed as possibly modifying the risk of ABV infection, such as category, family, species, age, place and management. This is the first study to seek associations between possible risk factors and ABV infection in both pet birds and waterfowl. In this study, the risk factors associated with ABV infection were category (pet birds), age (adult) and location (east coast). The association between risk and category may be explained by the selectiveness of PDD as a disease that primarily occurred in psittacine birds until 2009, when it was found in waterfowl (26). In this study, the result showed that ABV was detected in pet birds rather than in waterfowl. A previous study showed that PDD caused by ABV was reported more frequently in groups of pet birds compared to waterfowl with a clear difference in percentage, thus the category might be significantly associated with the risk. The association of ABV infection with age has also been previously described. Most ABV infection cases reported were in groups of adult birds; however, young birds at the age of 5 weeks could also get infected (5). The association between location and ABV infection was not explainable as no other similar studies have been conducted and this study is the first in Malaysia. Nevertheless, location is suggested to be a possible risk factor for ABV infection. Further investigations are needed to verify the influence of difference in location on ABV infection prevalence.

The KAP survey was the first conducted among bird owners in Malaysia, and so far, there has been no survey conducted in other countries either. This survey is important because it can help educate people in managing animal welfare with respect this disease, and as interest in captive birds is slowly increasing, so should the dissemination be broader of knowledge about diseases to which they are prone. In this study, of the 158 participants, several were found to have basic knowledge or to have at least heard of the disease before, but to not be aware of the causative agent. In fact, most of the respondents got their information mainly from social media, which sometimes adds or pares down its informational content. Proventricular dilatation disease is not a new disease, but because of the lack of case reports or published research work in Malaysia, most bird owners still lack knowledge regarding the common species affected, the main signs and symptoms and the hazard of the shedding. Most respondents opted for "I don't know" in the knowledge criteria. However, the respondents showed positive attitudes towards and good practices to achieve control of the disease: most of the

practices enquired about were actually those needed to ensure the spread of this virus is reduced. This shows that pet owners have agreed and abided by the basic practices and believe that PDD is a disease that must be given high priority in order to avoid any risk of the birds contracting it. Most importantly, action to enhance ABV and PDD knowledge must come under consideration. Since most of the respondents learned from social media, it is recommended to create an official website on ABV and PDD, so that everyone can obtain accurate information. Increasing knowledge among the respondents will lead to better attitudes and practices being shaped in the future.

Among socio-demographic characteristics, age was the only significant factor associated with the KAP score. However, no corroboration of this association exists, because no KAP surveys have been conducted in other countries. The positive correlations between knowledge and attitude as well as attitude and practice in this study validate the relationship between knowledge-attitude and attitude-practice with respect to ABV and PDD as the P-value obtained <0.05 as shown in table 10. However, knowledge and practice were not significantly associated. It can be concluded that adequate knowledge is valuable and can lead to positive attitude shaping and result in better practices.

This study is the first attempt at molecular detection of ABV in Malaysia and its outcome is an overall low percentage of infection. The summarised prevalence result is that 4.5% (9/201) of pet birds, specifically Psittaciformes, were positive, while Passeriformes and Anseriformes were free from infection, indicating Psittaciformes species to have the highest incidence of infection. Of the birds which provided the nine positive samples, the two blue and gold macaws showed clinical signs of PDD. Potential risk factors were highlighted that included category, age and region, which are statistically associated. Detecting ABV among these groups of birds and listing possible risk factors may encourage the introduction of preventive measures; however, further research is still needed. This study also successfully surveyed the KAP of bird owners regarding ABV and PDD as the first such survey attempted in Malaysia on ABV infection. Although this study only evaluated a limited number of bird owners, it may serve as a good landmark for the orientation of efforts continuing to spread knowledge and educate pet bird owners on the importance of understanding ABV infections.

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