Veterinary Microbiology 230 (2019) 62–71

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

First detection and characterization of *Psittaciform* bornaviruses in naturally infected and diseased birds in Thailand

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ARTICLE INFO

Keywords:
Parrot bornavirus
PaBV
Proventricular dilatation disease
PDD
PaBV-5
Cage mate
Southeastern Asia
Natural infection

ABSTRACT

In Thailand a proventricular dilation disease (PDD)-like syndrome commonly occurs in captive psittacine birds. The etiology, however, has been unknown to date and studies to detect parrot bornaviruses have never been performed in Southeastern Asia. Therefore, 111 psittacines (22 different species) including birds with suspected PDD based on clinical examination results (n = 65), cage mates of PDD suspected parrots without any clinical signs (n = 39) and dead birds with previous clinic suspicious for PDD (n = 7) were tested for bornaviruses using various reverse transcription polymerase chain reaction (RT-PCR) and realtime RT-PCR protocols, an enzyme-linked immunosorbent assay (ELISA), immunohistochemistry, and genome sequencing. Bornaviral infections, indicated by the presence of RNA or antibody positive reactions were detected in 60 birds (54.1%) belonging to 15 psittaciform species and originating from 41 owners. Occurrence of *Psittaciform 1 orthobornavirus* was confirmed by sequencing of PCR products in 24 of these birds. Parrot bornavirus (PaBV)-5, belonging to the species *Psittaciform 2 orthobornavirus* and found only in single birds in the United States of America, Japan and Hungary until now, was identified in a macaw. Full genome sequencing revealed features shared with other strains of this virus. PaBV-4 was the prevalent virus type and the viruses grouped in two of the five genetic PaBV-4 subclusters known so far while PaBV-2 was found in a single patient. Forty-five psittacines of the group of PDD-suspected birds (69.2%), 4 dead birds and 11 clinically healthy cage mates were positive in at least one test the latter suggesting inefficient horizontal transmission in natural infections. Lymphoplasmacytic infiltrations (non-purulent inflammation, ganglioneuritis) and bornavirus antigen were detected in diverse tissues confirming PDD as the disease involved. These results may have a major impact on conservation projects including the five near-threatened parrot species living in the wild in Thailand.

1. Introduction

In 2008, novel bornaviruses in parrots at that time designated as avian bornavirus (ABV) were identified as a possible etiological agent of proventricular dilatation disease (PDD) (Honkavuori et al., 2008; Kistler et al., 2008). Subsequently, several experimental studies confirmed the role of avian bornaviruses as the causative agent of PDD (Gancz et al., 2009; Piepenbring et al., 2012). To date, at least 20 different avian bornaviruses have been identified and have been divided into five species by a recent reclassification (Amarasinghe et al., 2018): *Passeriform 1 orthobornavirus*, *Passeriform 2 orthobornavirus*, *Psittaciform 1 orthobornavirus* (including the parrot bornaviruses PaBV-1, -2, -3, -4 and -7), *Psittaciform 2 orthobornavirus* (comprising PaBV-5) and *Waterbird 1 orthobornavirus*. Munia bornavirus 1 (MuBV-1) and the parrot bornaviruses PaBV-6, and -8 remained unclassified.

PDD is a devastating, often fatal disease that has been reported in more than 80 species of captive psittacine birds, and PDD-like symptoms have even been described in non-psittacine birds (Lubin et al., 2006; Rossi et al., 2017). PDD can affect both sexes equally and is generally found in adult birds but was also described in juveniles in

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https://doi.org/10.1016/j.vetmic.2019.01.013
Received 4 November 2018; Received in revised form 11 January 2019; Accepted 11 January 2019

both naturally (Kistler et al., 2010) and experimentally infected psittacines (Piepenbring et al., 2016, 2012). Typically, the disease spreads slowly; however, there have been reports of acute outbreaks with high mortality rates in psittacine aviaries (Kistler et al., 2008; Lubin et al., 2006).

The predominant clinico-pathological feature of the disease in psittacine birds is the dilation of the proventriculus and thus the term PDD is generally used for this disease. Clinical signs of PDD frequently include malfunction of the gastrointestinal tract with or without neurological signs such as tremor, ataxia, and seizures. Undigested seeds in the feces, regurgitation, impaction of the proventriculus, crop stasis, emaciation and ultimate death by starvation are commonly seen (Gregory et al., 1994; Staeheli et al., 2010). Subclinical bornavirus-infected carriers showing no signs of disease have been documented in several studies (Heffels-Redmann et al., 2011; Piepenbring et al., 2016, 2012).

The histopathological hallmark lesions of PDD are lymphoplasmacytic encephalomyelitis and ganglioneuritis. They are considered, by some authors, as pathognomonic and may, therefore, be used to confirm a tentative diagnosis of the disease (Rinder et al., 2009; Staeheli et al., 2010; Tizard et al., 2016; Weissenböck et al., 2009).

By now, cases of PDD have been diagnosed in many parts of the world (Heffels-Redmann et al., 2011; Kistler et al., 2008; Komorizono et al., 2016; Last et al., 2012; Philadelpho et al., 2014). Even though PaBVs are regarded circulating worldwide in captive psittacine birds, there have been, with regard to Asia, only few reports of PaBV infection in some countries where PaBV-1, -2, and −4 as well as, only once in Japan so far, PaBV-5, were detected in captive birds with or without clinical manifestation of PDD (Horie et al., 2012; Kistler et al., 2008; Komorizono et al., 2016; Sassa et al., 2013). Thailand is a country located in Southeastern Asia where the weather is optimal for various bird species. Due to this Thailand is an intensive trading center of a wide variety of pet birds and exotic animals from around the world. In addition, there are seven native psittacine species in Thailand and Southeastern Asia. Five of them have been categorized as Near Threatened (NT) by the International Union for Conservation of Nature including blue-rumped parrot (*Psittinus cyanurus*), red-breasted parakeet (*Psittacula alexandri*), blossom-headed parakeet (*Psittacula roseata*), alexandrine parakeet (*Psittacula eupatria*), and grey-headed parakeet (*Psittacula finschii*), whereas vernal hanging-parrot (*Loriculus vernalis*), and blue-crowned hanging-parrot (*Loriculus galgulus*) have been categorized as Least Concern (LC). Although there has been no report of PDD-like disease in the indigenous species described above so far, PDD-like signs have commonly been found in many other bird patients in Thailand. Nevertheless, it still remains unclear whether the underlying cause of the PDD-like disease in Thailand is actually related to PaBV. Measures appropriate to prevent spreading of PaBV and PDD in Thailand are not applied, and therapy is restricted to palliative treatment that may not address the real need of the patients.

To address this question, this study aimed to identify PaBV as the underlying cause of PDD-like disease in Thailand and to gain the first information on the occurrence of PaBV infection in Thailand as well as in Southeastern Asia. The genetic variants of the viruses occurring in Thailand were characterized and used for phylogenetic analysis. Finally, a purpose of the study was also to examine the clinico-pathological features of PDD-like disease in naturally infected birds in Thailand.

2. Materials and methods

2.1. Ethics statement

All of the sample collections and investigations were performed for reasons of medical indication and only by request and permission by bird owners. Ethical approval was thus not required. Nevertheless, sample collection was conducted in strict accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Animal Welfare act (AWA) on purpose to avoid suffering in sampled birds.

2.2. Sampling of captive Psittaciform birds in Thailand

From June 2016 until January 2017, samples of 111 psittaciform birds in Thailand were collected and examined for the presence of bornaviruses. The birds included in this study were classified into 3 groups as follows: birds with PDD suspected based on clinical examination ($n = 65$), cage mates of PDD-suspected parrots without any clinical sign ($n = 39$) and dead birds, all with natural deaths, and with clinical or postmortem suspicion of PDD ($n = 7$).

PDD suspicion was based on detection of 1) gastrointestinal signs: undigested seed in feces, regurgitation, crop stasis and proventricular enlargement from diagnostic imaging results, 2) neurological signs: ataxia, tremor, seizures, and inability to perch, with or without 3) other common signs: emaciation, lethargy, and sudden death. The range of diagnostic samples varied between birds as a consequence of varying sources of submission, which included veterinarians, private hobby bird owners, and also breeders. The collected samples consisted of crop and cloacal swabs, chest feathers, feather calami, and serum. To maximally avoid suffering of the birds, sample collection was performed according to the Institutional Animal Care and Use Committee (IACUC) sample collection guide. All birds were carefully restrained by skilled veterinary nurses and samples were taken by licensed and experienced veterinarians using standard techniques without anesthesia. Blood samples were collected from the right-side jugular vein or the brachial vein of the birds by experienced veterinarians to alleviate suffering and stress. Several birds included in this study had blood taken for health checks requested by the owners, and the same blood samples were used for multiple other diagnostic tests besides bornavirus serology. In case of post-mortem investigations, organs including brain, nerves, proventriculus, ventriculus, heart, and kidney were collected. In the group of cage mates of PDD-suspected parrots, samples were taken from the mates in the same manner as from the patients in order to detect subclinical PaBV infection. 103 out of 111 psittacines were examined and sampled by two of the authors (PS and BL) at the Exotic Pet Clinic, Kasetsart Veterinary Teaching Hospital and Diagnostic Center, the Faculty of Veterinary Medicine, Kasetsart University, Thailand, while the other 8 psittacines were examined and sampled by third party veterinarians in other clinics and these samples were then submitted by express mail.

Organ samples collected from fresh carcasses were fixed in 10% neutral buffered formalin for histopathological and immunohistological investigations. All other samples were stored at −80 °C until diagnostic tests for the detection of viral RNA or antibodies were performed at the end of every week. Diagnostic RT-PCR, qRT-PCR, and ELISA for virus identification as well as histopathology were performed at Kasetsart Veterinary Teaching Hospital and Diagnostic Center, the Faculty of Veterinary Medicine, Kasetsart University, Thailand. Immunohistochemistry techniques as well as PCR assays to obtain a full genome sequence of a single virus strain were performed at the Clinic for Birds, Small Mammals, Reptiles and Ornamental Fish, LMU Munich, Germany.

2.3. RNA extraction and reverse transcription

Viral RNA was extracted for individual birds from pooled samples of multiple sources. For crop and cloacal swabs, chest feathers, and feather calami, the QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) was used while RNA of tissue samples from post-mortem examination such as brain, proventriculus, and ventriculus was extracted by using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). Both RNA extraction kits were utilized according to the manufacturer's instruction.

The extracted RNA was then transcribed into first strand

complementary DNA (cDNA) with random hexamer primers (Roth, Karlsruhe, Germany) and the Invitrogen™ M-MLV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, USA) under the following conditions: 65 °C for 5 min, 37 °C for 2 min, 37 °C for 50 min, and 70 °C for 15 min.

2.4. Conventional polymerase chain reaction (PCR)

The cDNA served as a template for 3 PCR assays targeting conserved regions of the polymerase (L), matrix (M), and nucleoprotein (N) genes, each. The published primers (Kistler et al., 2008) were used as follows: ABV_LconsensusF (5′-CGCCTCGGAAGGTGGTCGG-3′) and ABV_LconsensusR (5′-GGCAYCKACTCTTRAYYGTRTCAGC-3′) for the L gene, ABV_MconsensusF (5′-GGRCAAGGTAATYGTYCCTGGATGGCC-3′) and ABV_MconsensusR (5′-CCAACACCAATGTTCCGAAGMCG-3′) for the M gene, and ABV_NconsensusF, (5′-CCHCATGAGGCTATWGATTGGATTAAC-3′) and ABV_NconsensusR, (5′-GCMCGGTAGCCNGCCATTGTDGG-3′) for the N gene. Amplification was put under process by the following temperature profile: 15 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 50 °C, and elongation for 30 s at 72 °C and a final incubation for 7 min at 72 °C. Per 25 µL of reaction mixture, 1 µL of cDNA, 2.5 µL of 10X buffer, 0.2 µL of each 50 µM primer, 0.25 µL of dNTP (25 mM each), 1.5 µL of 25 mM $MgCl_2$, 19.225 µL of H_2O and 0.125 µL of 5 units/µL HotstarTaq® polymerase (Qiagen, Hilden, Germany) were added. Control reactions included at least 1 negative control from each RNA extraction procedure, 1 positive reverse transcription control and a positive cDNA as a control for PCR or real-time PCR, both obtained from PaBV-4 strain 1440 (GenBank accession number FJ603671, FJ603677 and FJ603683), which originated from a naturally infected festive amazon (*Amazona festiva*) in Germany. PCR products were visualized using gel electrophoresis in 2% Tris-acetate-EDTA buffered agarose gel (Kistler et al., 2008; Weissenböck et al., 2009).

2.5. Quantitative real-time PCR (qRT-PCR)

A QuantiNova™ Probe PCR Kit (Qiagen, Hilden, Germany) was used to target the amino-terminal region of phosphoprotein (P) gene of PaBV-4; set 1032–1322 with the forward primer (5′-CAGACAGCACGT CGAGTGAGA-3′), the reverse primer (5′-AGTTAGGGCCTCCCTGGG TAT-3′) and the probe (6FAM-5′-AGGTCCCCGCGAAGGAAGCGA-/3′-6-TMR) (Honkavuori et al., 2008). The PCR kit was used according to the manufacturers' instruction.

The PCR reaction was performed using the real-time PCR device CFX96 Touch Real-Time PCR Detection System (Bio-Rad, California, USA) and the following temperature profile. An initial heat activation of the polymerase, performed at 95 °C for 2 min, was followed by a 2-step cycling protocol with denaturation at 95 °C for 5 s and a combined annealing/extension at 60 °C for 30 s, which was carried out in 50 cycles. Ct values greater than 36 were assessed as negative (Hogemann et al., 2017; Honkavuori et al., 2008).

2.6. Nucleotide sequence analysis

Extractions of PCR products from agarose gels ware performed by using FavorPrep™ GEL/PCR Purification Kit (Favorgen Biotech Corporation, Ping-Tung, Taiwan) as instructed by the manufacturer. Nucleotide sequences of bornavirus L, M and N gene fragments were determined by a commercial company (1st BASE, Selangor, Malaysia). The obtained sequences were analyzed using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with Psittaciform bornavirus species and other sequences stored in the GenBank database. Nucleotide sequence identities higher than 90% with a known bornavirus sequence were interpreted as indication for assignment to the same virus (Marton et al., 2015; Rubbenstroth et al., 2016).

Phylogenetic analyses of partial L gene (490 bp), M gene (306 bp) and N gene (342 bp) sequences were performed using the MEGA 7.0 software (www.megasoftware.net). After multiple sequence alignment was obtained by MUSCLE, the Neighbor-Joining method with Jukes-Cantor genetic distance model was applied. Bootstrap support was assessed by 1000 replicates (Rubbenstroth et al., 2016).

2.7. Whole-genome sequencing of a PaBV-5 strain from Thailand

The cDNA obtained from a blue and yellow macaw (case number: 16021), where PaBV-5 had been identified using diagnostic conventional PCR and sequence analysis, was used as a template for PCR assays on the same reaction conditions described for the conventional PCR above. Diverse primers (Supplemental Table S1) were designed based on the canonical sequence of complete and partial PaBV-5 genome sequences (AB519144, KR612223, KT378600, LC120625) and likewise various complete *Psittaciform 1 orthobornavirus* genome sequences retrieved from GenBank database. Gel extraction and PCR product purification were performed by using QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. The PCR products were directly sequenced using the Sanger technique by a commercial company (Eurofins GATC Biotech, Constance, Germany). The obtained genomic sequences were assembled by using the MEGA 7.0 software. BLASTn, BLASTx, and BLASTp were used to determine nucleotide and amino acid identities to sequences deposited in GenBank. Phylogenetic analyses including the PaBV-5 strains known so far and other viruses in the genus *Orthobornavirus* were performed by using MEGA 7.0 software based on the nucleotides coding for the N, X, P, M, G and L proteins and excluding the non-coding regions.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Serum samples that were obtained from 106 psittacines were tested for the presence of bornavirus-reactive antibodies using an ELISA as described before (Hogemann et al., 2017) with minor modifications. Sera were obtained from blood after centrifugation for 5 min at 3500 × g. In short, recombinant N protein (Reuter et al., 2010) derived from *Psittaciform 1 orthobornavirus* and diluted in coating buffer (pH 9.6) to a final concentration of 4 µg/mL was used for coating wells of a medium binding polystyrene ELISA plate and incubated overnight at 4 °C. After washing with phosphate-buffered saline containing 0.1% Tween (PBST), wells were blocked with 4% non-fat dry milk in PBST and incubated at 37 °C for 1 h. Wells were washed with PBST and incubated at 37 °C for 1 h with 1:2000 serum dilution in Tris-buffered saline containing 0.1% Tween (TBST). The wells were washed again with PBST and incubated at 37 °C for 1 h with 1:1000 dilution of a rabbit anti-pigeon-IgG polyclonal antibody (Daum et al., 2009) in TBST. Subsequently, the wells were again washed with PBST and incubated at 37 °C for 1 h with 1:400000 dilution of peroxidase-linked goat anti-rabbit-IgG (Jackson ImmunoResearch, Newmarket, UK) in TBST (Reuter et al., 2010; Rinder et al., 2010). After washing with PBST, 100 µL of Sureblue™ TMB Microwell Substrate (KPL, Gaithersburg, USA) was applied to the wells and incubated for 10 min in the darkness at room temperature. The reaction was stopped with 50 µL of 1 M H_2SO_4, and the HRP activity was measured at 450 nm in a BioTek™ ELx800™ Absorbance Microplate Readers (Fisher Scientific, Hampton, USA). Negative controls (blanks without patient sera and sera obtained from uninfected parrots) were included in every run to check for unspecific binding of reagents. Absorptions higher than the mean value of three negative parrot control sera plus threefold SD were considered positive (Hogemann et al., 2017).

2.9. Histopathology and immunohistochemistry

In order to confirm that presence of common histopathological and

immunohistochemical features of PDD in Thailand, histopathology and immunohistochemistry were performed as additional diagnostic methods. Organ samples were collected from 3 fresh carcasses (case number: 16006, 16037, and 16053) and fixed in 10% neutral buffered formalin. Tissues were then cut into small pieces, paraffin-embedded, cut into 2–4 µm sections and stained with hematoxylin and eosin (H&E) according to standard protocols.

Immunohistochemistry (IHC) was performed by using a previously published method (Löffler, 2011) with some modification. Polyclonal antiserum raised in rabbits against recombinant N protein of *Psittaciform 1 orthobornavirus* was used as a specific primary antibody. The paraffin-embedded tissue blocks were cut into 2–4 µm sections and mounted to Superfrost™ Plus Adhesion Slides (Thermo Fisher Scientific, Waltham, USA). After deparaffinization, the sections were rehydrated, blocked with 0.3% hydrogen peroxide for 13 min, and then incubated in 1% goat serum at a dilution of 1:100 in phosphate-buffered (PBS) for 45 min. The sections were then incubated with the primary antibody at a dilution of 1:500 in antibody solution (PBS containing 1% goat serum) for at least 60 min. As negative controls, the sections were incubated with antibody solution without the anti-ABV-N antibody. After washing with PBS, the sections were incubated with biotinylated goat anti-rabbit antibodies (Vector Laboratories, Biozol, Eching, Germany) at a dilution of 1:100 in antibody solution and stained using a Vectastain ABC Elite detection kit (Vector Laboratories, Biozol, Eching, Germany) for 30 min each. The sections were washed again with PBS, immersed in 3,3′-diaminobenzidine (DAB) solution for 10 min, rinsed with distilled water, counterstained with hematoxylin, dehydrated and mounted with Eukitt® (Orsatec, Bobingen, Germany) under a cover-slip.

3. Results

3.1. Detection of Psittaciform 1 and 2 orthobornaviruses in various psittacine species

A total of 60 out of the 111 psittacine birds (54.1%) reacted positive for bornavirus in at least one test. These included 45 PDD-suspected alive parrots corresponding to 69.2% of the suspected alive birds, 11 cage mates of PDD suspected parrots (not clinically ill, 28.2%), and 4 (57.1%) out of 7 dead birds with clinical suspicion of PDD (Tables 1and 2). Virus RNA or antibodies were found in birds of both sexes. Their age varied between 2 months and 6 years (Supplemental Table S2). The positive birds belonged to 15 of the 22 different psittacine species included in this investigation and originated from 41 different owners.

Table 2
Association between PDD-like disease and PaBV infection in captive psittacine birds in Thailand.

PDD-like disease	PaBV		Total number of birds	% PaBV-positive birds
	Positive[a]	Negative		
Positive[b]	49	23	72	68.06
Negative[c]	11	28	39	28.21
Total	60	51	111	54.05
Yates' corrected chi-square = 14.61[d]				

[a] PaBV-positive, bird that reacted positive for PaBV in at least one test (RT-PCR, qRT-PCR, ELISA).
[b] PDD-like disease-positive, tentative diagnosis of PDD, birds with clinical signs of PDD such as gastrointestinal signs (undigested seed in feces and proventricular enlargement from diagnostic imaging results) and/or central nervous system signs.
[c] PDD-like disease-negative, birds without clinical signs of PDD.
[d] Highly significant ($P < 0.001$).

Partial genome sequences of products of the conventional RT-PCR assays were obtained from 25 of 33 PCR-positive birds (Table 1). Eleven, 9, and 22 partial L, M, and N gene sequences, respectively, with satisfactory quality were recovered and submitted to GenBank under the accession numbers MH559279 and MH581096 to MH581119. Based on BLAST analyses, viruses from 23 birds were identified to be PaBV-4, while one virus (GenBank accession numbers; MH581096) was identified as PaBV-2, and another one (GenBank accession number; MH559279) as PaBV-5 (Table 1).

The phylogenetic analysis of partial L gene (490 bp; Fig. 1A), M gene (306 bp; Fig. 1B), and N gene (342 bp; Fig. 1C) sequences confirmed in Thailand the occurrence of both bornavirus species described

Table 1
Results of PaBV investigations in captive psittacine birds originating from Thailand.

Family	Genus/ Species	Number of investigated birds	PaBV-positive birds[a]	RT-PCR-positive birds	ELISA-positive birds	PaBV type identified by sequencing
Psittacidae	*Amazona ochrocephala*	1	0	0	0	N
	Anodorhynchus hyacinthinus	1	1	1	1	NA
	Ara ararauna	12	5	4	4	PaBV-4 (n = 1), PaBV-5 (n = 1)
	Ara chloropterus	3	3	2	2	PaBV-4 (n = 2)
	Ara macao	2	1	0	1	NA
	Aratinga nenday	2	2	1	2	PaBV-4 (n = 1)
	Aratinga solstitialis	52	31	17	30	PaBV-4 (n = 13)
	Diopsittaca nobilis	5	4	2	3	PaBV-4 (n = 1)
	Forpus sp.	3	0	0	0	N
	Myiopsitta monachus	1	1	1	1	PaBV-2 (n = 1)
	Pionites melanocephalus	1	0	0	0	N
	Psittacara wagleri	1	1	0	1	NA
	Psittacus alexandri	1	0	0	0	N
	Psittacus erithacus	6	3	1	2	PaBV-4 (n = 1)
	Psittacus roratus	9	1	1	1	PaBV-4 (n = 1)
	Pyrrhura molinae	1	0	0	0	N
	Pyrrhura perlata	1	0	0	0	N
Cacatuidae	*Cacatua ducorpsii*	1	0	0	0	N
	Cacatua galerita	1	1	1	1	PaBV-4 (n = 1)
	Cacatua galerita triton	1	1	0	1	N
	Cacatua moluccensis	5	4	1	3	PaBV-4 (n = 1)
	Cacatua ophthalmica	1	1	1	1	PaBV-4 (n = 1)
Total		111	60	33	54	25

NA, no PCR product available; N, negative diagnosis for parrot bornavirus.
[a] All of these 60 birds reacted positive for PaBV in at least one test. Note: not all birds were tested by both RT-PCR and ELISA.

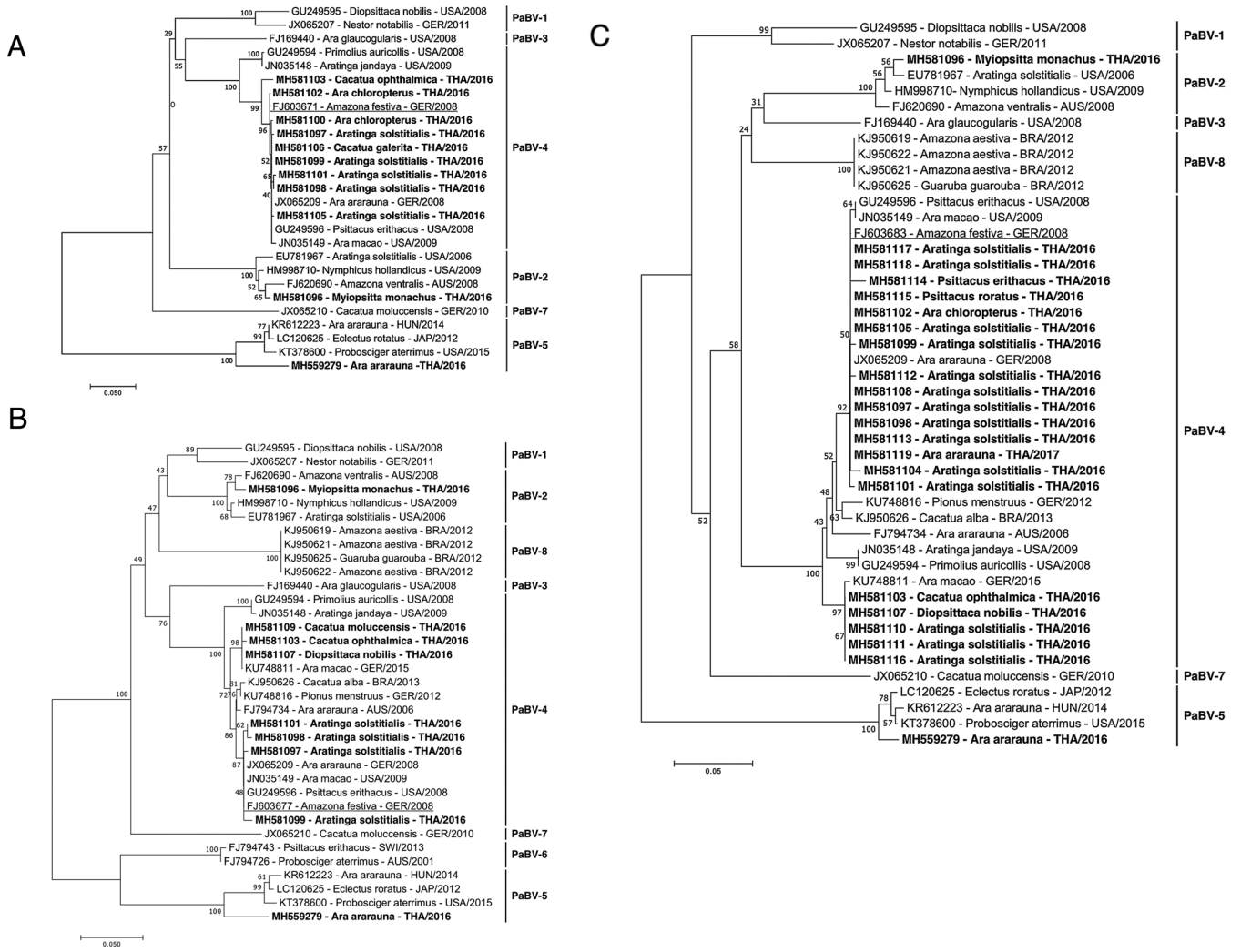

Fig. 1. A - 1C. Phylogenetic analysis based on partial PaBV L gene (490 bp; Fig. 1A), M gene (306 bp; Fig. 1B), N gene (342 bp; Fig. 1C) sequences from captive psittacines in Thailand. The tree was constructed including public PaBV reference sequences stored in the GenBank database. The GenBank accession number and the host species as well as the country and year of detection is given for the strains. Sequences emphasized in bold were generated during this study. Sequences from the PaBV-4 strain 1440, which was used as positive control in RT-PCR, were underlined.

so far in parrots, *Psittaciform 1 orthobornavirus* and *Psittaciform 2 orthobornavirus* and did not reveal any association of individual clusters to the geographic origin, time of sampling or host species. Several sequences of viruses from Thailand were nearly identical to each other and to virus sequences from Germany and the United States of America, indicating a close genetic relationship.

3.2. Whole-genome sequence of a PaBV-5 strain from Thailand

Using PCR and Sanger sequencing, 8892 nt of the genome of the PaBV-5 strain 16021 from this study was obtained. BLAST analysis of the N, X, P, M, G and L gene revealed a nucleotide (nt) sequence identity of 91% with the three full-genome sequences of PaBV-5 strains 2014-A, Cockg 5, and 2012/Japan (Genbank accession numbers KR612223, KT378600, and LC120625, respectively) reported so far (Guo and Tizard, 2015; Komorizono et al., 2016; Marton et al., 2015). A deletion of nucleotides in the L gene found only in strain Cockg 5 so far was not present in strain 16021. With regard to the deduced amino acid (aa) sequences, an overall identity of 95–96% among virus 16021 and the other three PaBV-5 strains was calculated (Table 4).

Regarding the N/X intergenic region, of PaBV-5 strain 16021 was regarded, a short upstream open reading frame (uORF) of the same length (18 nt) as in the other PaBV-5 strains was found (Supplemental Fig. S1).

As shown in Table 4, based on nt sequence identities, the analysis of individual genes showed that PaBV-5 was closely related to the single PaBV-6 strain described only by a partial M gene sequence up to now (FJ794743). 82% nt and 93% aa sequences identities were calculated.

Phylogenetic analysis of the PaBV-5 and other viruses in genus *Orthobornavirus* based on nucleotides coding for the N, X, P, M, G and L protein and excluding the non-coding regions revealed a separation of the phylogenetic tree into three major clades (Fig. 2), as demonstrated in previous studies (Guo and Tizard, 2015; Komorizono et al., 2016). The current PaBV-5 strain 16021 was grouped together with the other three PaBV-5 strains (*Psittaciform 2 orthobornavirus*), in clade 2 common with *Passeriform 1 orthobornavirus*, *Passeriform 2 orthobornavirus*, and *Waterbird 1 orthobornavirus*, whilst the first clade consisted of *Mammalian 1 orthobornavirus* and *Mammalian 2 orthobornavirus*. The PaBVs of *Psittaciform 1 orthobornavirus* were classified into clade 3.

3.3. Comparison of various tests for Psittaciform bornavirus diagnosis

3.3.1. Enzyme-linked immunosorbent assay (ELISA)

As shown in Table 3, specific antibodies against parrot antibodies reactive were detected in 54 of the 106 sera samples tested by ELISA. Forty-five parrots (68.2%) with clinical signs of PDD revealed

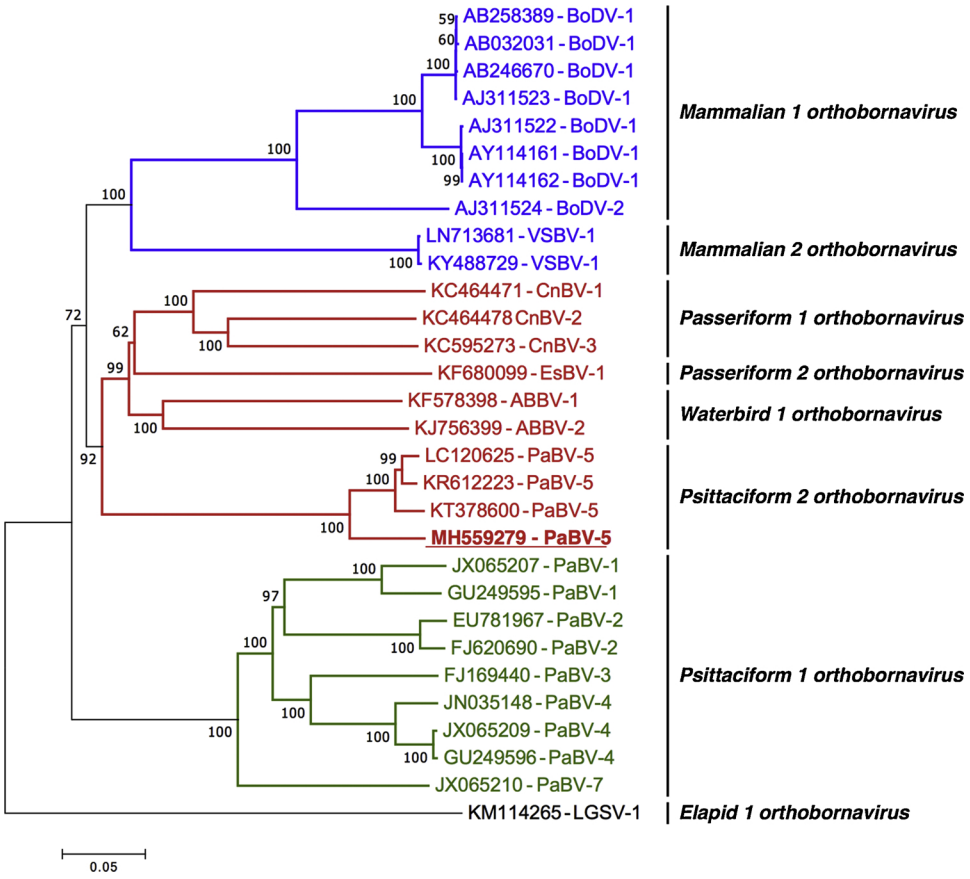

Fig. 2. Phylogenetic analysis of the PaBV-5 strain found in Thailand and other viruses of the genus *Orthobornavirus* based on nucleotides coding for the N, X, P, M, G and the partial L protein without the non-coding regions. This tree was constructed using the Neighbor-Joining method with Jukes-Cantor genetic distance model and 1000 bootstrap replicates by MEGA 7. The PaBV-5 strain 16021 of this study is emphasized in bold and underlined.

antibodies against parrot bornavirus nucleoprotein, which included 25 of the 45 birds that were also tested in PCR assays and found positive there. Nine birds (22.5%) of the group of PDD-suspected-parrots cage mates with no clinical sign of PDD were positive in the ELISA. (Supplemental Table S2 and Supplemental Fig. S2)

3.3.2. Conventional RT-PCR

The conventional RT-PCR was carried out by using assays for three different genes (L, M, and N gene). Thirty-three of 111 birds (29.7%) were positive in at least one of the assays (Table 3). These included 25 alive birds with suspected PDD, 4 clinically healthy cage mates of PDD suspected birds, and 4 dead birds with clinical suspicion of PDD. Twelve (36.4%) of these birds reacted positive for all three ABV genes (Supplemental Table S2). 20 (60.6%), 14 (42.4%), and 28 (84.8%) of these birds were positive for L, M, and N gene respectively, while only 14 out of 111 birds were positive for PaBV in the qRT-PCR which is regarded as specific for PaBV-4 (Table 3).

3.4. Confirmation of characteristic signs of PDD by histopathology and immunohistochemistry

The histologic examinations revealed lymphoplasmacytic inflammatory infiltrates within ganglia of the proventriculus and ventriculus of the three examined birds. Other signs such as perivascular lymphocytic inflammatory infiltrates (cuffing) as well as occasional gliosis in the brains was also noted. All three birds showed additional signs of nephrosis and two birds had an additional serositis. The histopathologic lesions in general were mild (Fig. 3).

Immunohistochemical stains revealed positive viral immunolabeling in myenteric ganglia and neurons of the brain and in cells of diverse tissues of other organs such as epithelial cells of the crop, glandular cells of the proventriculus, as well as epithelial cells and muscle cells of the ventriculus (Fig. 3).

Table 3
Comparison of the number of PaBV-positive birds detected by antibody assay (ELISA) and genomic RNA tests (conventional RT-PCR and qRT-PCR), in different parrot groups.

Groups	ELISA	Conventional RT-PCR				qRT-PCR	PCR total	ELISA and PCR[a]	PaBV-positive[b]
		L gene	M gene	N gene	Total				
Alive birds with suspected PDD based on CE	43/64	13/65	10/65	23/65	25/65	12/65	25/65	23/64	45/65
Cage mates of PDD suspected parrots (not clinically ill)	9/39	3/39	1/39	2/39	4/39	0/39	4/39	2/39	11/39
Dead birds with clinical or postmortem suspicion of PDD	2/3	4/7	3/7	3/7	4/7	2/7	4/7	2/3	4/7
Number of positive birds/ number of tested birds	54/106	20/111	14/111	28/111	33/111	14/111	33/111	27/106	60/111
Number of positive birds/ number of tested positive birds	54/58	20/60	14/60	28/60	33/60	14/60	33/60	27/58	60/60

CE, clinical examination.
[a] Number of birds with positive results both in ELISA and in at least one PCR assay related to the number of birds investigated with all tests.
[b] All of these 60 samples reacted positive for PaBV in at least one test.

Table 4
Comparison of nucleotide (NT) and amino acid (AA) sequence identities among the PaBV-5 strain 16021 characterized in the present study and representative psittaciform orthobornaviruses.

Species	Virus (Abbreviation)	Accession No.	Sequence identity of the genomic coding region (%)													
			N gene		X gene		P gene		M gene		G gene		L gene		Overall	
			NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA
Psittaciform 1 orthobornavirus	PaBV-1	GU249595	72	76	77	59	70	70	75	86	68	67	68	67	69	69
	PaBV-2	FJ620690	72	77	76	49	70	69	76	87	69	68	68	67	69	69
	PaBV-3[a]	FJ169440	73	75	76	59	70	68	75	84	68	68	69	74	70	72
	PaBV-4	JN035148	73	75	76	56	71	69	76	85	68	69	68	67	69	69
	PaBV-7	JX065210	72	76	74	55	71	69	77	87	69	70	68	68	70	70
Psittaciform 2 orthobornavirus	PaBV-5	LC120625	91	97	95	92	94	99	92	98	92	95	91	96	91	96
	PaBV-5	KT378600	91	98	95	91	93	98	93	99	92	95	91	95	91	95
	PaBV-5	KR612223	91	98	94	90	93	99	92	97	91	96	91	96	91	96
Unclassified viruses	PaBV-6[a]	FJ794743	NA	NA	NA	NA	NA	NA	82	93	NA	NA	NA	NA	NA	NA
	PaBV-8[a]	KJ950619	72	83	NA	NA	NA	NA	71	85	NA	NA	NA	NA	NA	NA

NA, data not available.
[a] Only partial sequences were available for PaBV-6 (M), PaBV-8 (N and M) and PaBV-3 (L and overall).

3.5. Association between PDD-like disease and PaBV infection in captive psittaciform birds

Forty-five of the 65 alive birds with PDD-like disease and 4 of 7 birds found dead (summing up to a total of 68.1% of the suspected birds, Table 2) reacted positive for PaBV in at least one test (RT-PCR, qRT-PCR and ELISA). When the relationship between PDD-like disease and the detection of parrot bornavirus in captive psittacine birds in Thailand was statistically analyzed by the chi-square test with Yates's continuity correction, the value of 14.6 indicated an association in the PDD-like disease and PaBV infection in captive psittacines in Thailand at a 0.001 level of significance.

When the 39 cage mates without clinical signs of PDD were regarded, 11 birds (28.2%) were found to be PaBV-positive as was confirmed by PCR and sequence analysis. For 28 cage mates of diseased and infected birds, however, we did not find any indication for PaBV infection, corresponding to 71.8% which were negative for PaBV.

3.6. Presentation of clinical signs in PaBV-affected birds

Among the 60 PaBV-positive birds, there were 49 birds with clinical signs. Forty-three birds (71.7%) presented only with gastrointestinal (GI) signs including undigested seed in feces, proventricular dilatation detected by radiography examination, crop stasis and regurgitation; 11 birds (18.3%) showed only central nervous system (CNS) signs including ataxia, tremor, inability to perch, and seizure while 5 birds (8.3%) revealed a combination of GI and CNS signs (Fig. 4).

PaBV-5 was found in a blue and yellow macaw (*Ara ararauna*) showing severe dilatation of proventriculus during necropsy. Clinical signs of this bird included undigested seed in feces, abdominal enlargement and sudden death. The single bird with PaBV-2 infection, a Monk parakeet (*Myiopsitta monachus*) presented severe CNS signs (ataxia) before dying suddenly (Supplemental Table S2).

4. Discussion

In this study we were able to describe for the first time the occurrence of PaBV in Thailand, and even in Southeastern Asia. Infection was found in a high proportion (54.1%) of the birds included in this investigation and was highly associated with PDD-like diseases of psittaciform birds as 49 of 72 birds showing respective signs (68.1%) were positive for PaBV in at least one test (ELISA, RT-PCR, and qRT-PCR). These percentages can, of course, not be interpreted as prevalences because of non-random sampling of holdings with birds suffering from PDD-like disease. However, since this disease complex is frequently seen by veterinarians in Thailand (PS, unpublished observation), our data might suggest a high frequency of PaBV infections in parrots kept as companion birds in Thailand. Relevance of this finding is high not only with regard to veterinary medicine, but also to wild species conservation. Investigations on the seven native free-living parrot species in Thailand and Southeastern Asia which include five near threatened species, do not yet exist but are urgently needed.

Standard diagnostic tests were used to detect PaBV infections and included PCR protocols described already in 2008 when avian bornaviruses had been detected (Honkavuori et al., 2008; Kistler et al., 2008; Staeheli et al., 2010; Weissenböck et al., 2009) and an ELISA (Hogemann et al., 2017; Reuter et al., 2010). Because of known genetic heterogeneity of bornaviruses as well as presumed persistent bornavirus infections with late and intermittent virus shedding, a combination of multiple tests was applied. In agreement with former studies (Philadelpho et al., 2014; Weissenböck et al., 2009), the assay for the N gene appeared to be of high sensitivity and was more consistent than that for the M gene, which, in contrast to other studies (Gancz et al., 2010; Raghav et al., 2010; Rubbenstroth et al., 2012), seemed to yield false negative results even more often than the L gene PCR (Table 3). The possibility that, in the investigation presented here, the L gene PCR results may have provided false positives cannot be excluded but is not supported by the results of the other assays. Assays targeting different highly conserved genomic regions used in parallel proved appropriate for sample testing in this study, especially for the detection of new bornaviruses not identified so far or of distantly related species (Philadelpho et al., 2014).

Partial L, M, and N gene sequences derived from RT-PCR products revealed a genetic variation of PaBV in Thailand as found in other investigations elsewhere. Both Psittaciform bornavirus species known so far were detected in Thailand. PaBV-5, a virus belonging to the species *Psittaciform 2 orthobornavirus*, which was detected only in three countries around the world up to now, in birds in Japan, Hungary and the United States of America (Guo and Tizard, 2015; Horie et al., 2012; Kistler et al., 2008; Komorizono et al., 2016; Marton et al., 2015), was found in a blue and yellow macaw (*Ara ararauna*) revealing severe dilatation of proventriculus during necropsy. As described in several studies from other countries (Rubbenstroth et al., 2012, 2016; Staeheli et al., 2010; Weissenböck et al., 2009), PaBV-4 was the most prevailing psittaciform bornavirus in the birds of this study (n = 23). We found only one bird with PaBV-2 infection, which presented severe neurological signs before dying suddenly. Phylogenetic analyses performed from multiple partial L, M, and N gene sequences (Fig. 1A–C) demonstrated a genetic heterogeneity of PaBV-4 viruses of this study which were distributed in two (1 and 2) of the five genetic clusters previously

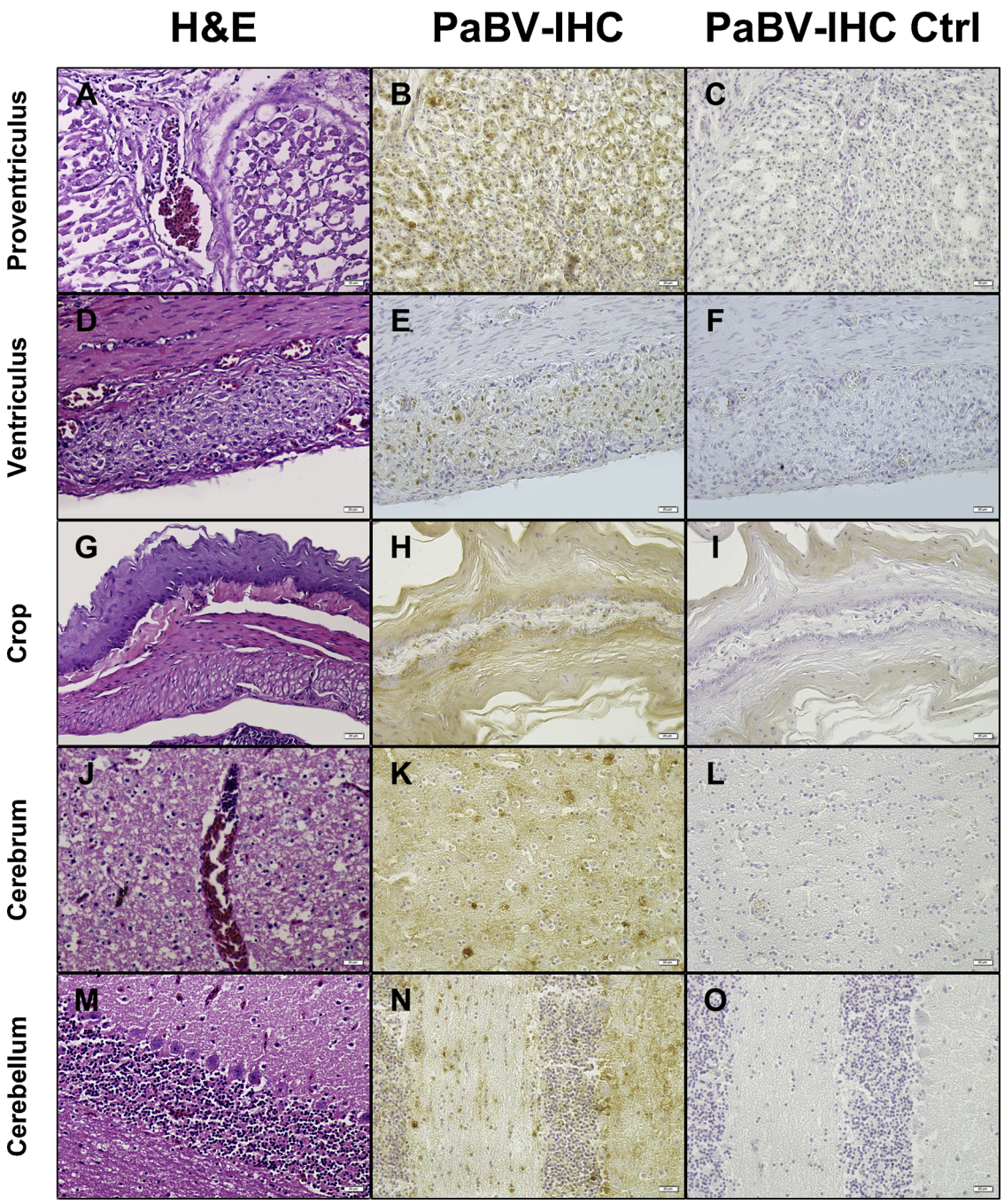

Fig. 3. Photomicrographs of histological findings (Hematoxylin and Eosin; H&E) and immunohistochemical (IHC) labeling of *Psittaciform 1 orthobornavirus* N protein from proventriculus (A–C), ventriculus (D–F), crop (G–I), cerebrum (J–L) and cerebellum (M–O) of PaBV-affected parrots in Thailand. Proventriculus with unspecific mild inflammatory reaction (A), lymphoplasmacytic infiltrates in myenteric ganglia of the ventriculus (regarded as pathognomonic; D), crop with no specific lesions (G), cerebrum with unspecific lymphocyte accumulation in the blood vessel (J), cerebellum with no specific lesions (M), PaBV-IHC with positive brown staining mainly of cell nuclei in various tissues due to specific anti-PaBV-N-antibody binding: glandular cells (B), within myenteric ganglion (E), epithelial cells (H), neurons and glia cells (K) neurons and Purkinje cells (N); PaBV-IHC Ctrl: negative controls without use of anti-PaBV-N antibody and therefore without specific staining (C, F, I, L, O).

identified for PaBV-4 (Rubbenstroth et al., 2016).

The apparently lacking association with geographic origin might reflect extensive trading of these birds, international exchange and a nearly global distribution of various genetic variants (Rubbenstroth et al., 2016).

The PaBV-5 mentioned above was suspected after finding that the partial L, M and N gene sequences clustered with those of PaBV-5 strains known so far (Fig. 1A–C). When the complete genomic sequence of this PaBV-5 strain 16021 was analyzed, a nt sequence identity of only 91% with other full-genome sequences of PaBV-5 (KR612223, KT378600, and LC120625) was found indicating considerable genetic differences. Results of BLAST and phylogenetic analysis nevertheless confirmed that this virus belonged to PaBV-5. Particularly, a short uORF (18 nt) in the N/X intergenic region, found only in PaBV-5 so far, was also detected in strain 16021 and was exactly identical to that of known PaBV-5 strains (Horie et al., 2012; Marton et al., 2015).

With regard to clinical aspects of the disease in Thailand, gastrointestinal signs were most common, they were presented by 71.7% of

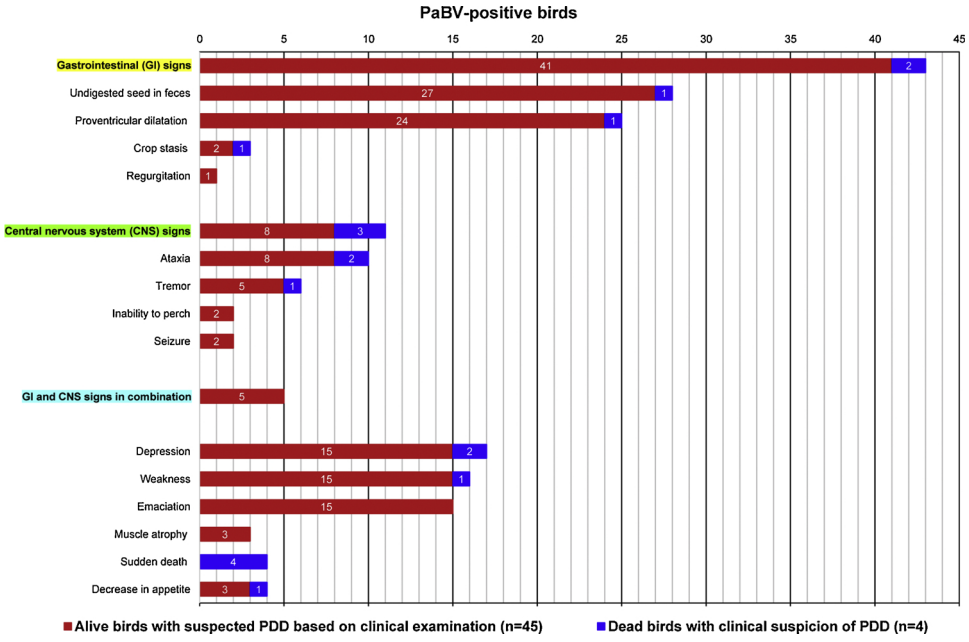

Fig. 4. Clinical signs found in PaBV-affected parrots in Thailand.

the PaBV-infected birds in this study, whereas neurological signs were detected in only 18.3% of the patients. These findings do not correspond with several other studies where neurological signs were dominating (Philadelpho et al., 2014; Piepenbring et al., 2016, 2012; Sassa et al., 2013). The causes for this are unknown to us. Besides clinical signs of PDD, characteristic pathologic and histopathologic lesions as well as bornavirus proteins were detected in psittacine birds in Thailand as shown by three representatives, two birds with clinical signs infected with PaBV-2 and -4 and one bird with a postmortem diagnosis of PaBV-4 infection. These findings confirm that birds in Thailand are infected with PaBV and can die due to PDD. Because of the low number of only one bird, each, found to be infected with PaBV-2 and PaBV-5 in this study, conclusions about possible differences among bornavirus types or species with regard to pattern of PDD lesions and clinical disease are, however, not possible.

Interestingly, 28.2% of PaBV-positive birds included in this study were clinically healthy, they were cage mates of diseased parrots. All these cage mate birds lived in very close contact (in the same cage) to their infected and diseased partners.

At the same time, our results emphasize and support the findings of former studies (Heffels-Redmann et al., 2011; Leal de Araujo et al., 2017; Piepenbring et al., 2016, 2012; Villanueva et al., 2010) that carrier birds with subclinical PaBV infections commonly occur. In general, PDD is a chronic disease and it is expected that many infected birds of this study were still in the incubation period. Nevertheless, although the incubation period has been described to vary and be very long (sometimes (more than 10 years), it can at present not be excluded that some of these birds might never develop clinical signs. The pathogenesis of this chronic disease is still unclear but immune-mediated reaction, likely involving T cells, may play an essential role in disease development (Hameed et al., 2018).

On the other hand, it is remarkable that most of the cage mates of diseased birds (71.8%) did not reveal any indication for PaBV infection. They showed negative results in all diagnostic tests used here. This confirms, in naturally infected birds, the conclusions deduced from experimental infections that horizontal bornavirus transmission from bird to bird is inefficient (Piepenbring et al., 2016, 2012; Rubbenstroth et al., 2014). In addition, these results show that removing of birds with diagnosed PaBV infection from aviaries is a reasonable measure for flock sanitation.

In conclusion, our investigation revealed, for the first time, the occurrence of two psittaciform bornavirus species and of PDD in psittacines kept by humans in Southeastern Asia. Future investigations of the infection status of wild parrots, especially of the five near threatened species, are urgently needed. Based on the results of our study, techniques for PaBV diagnostic are to be established in the routine diagnostics of veterinary clinics. These would make identification of aviaries with PaBV infections in Thailand possible in order to establish appropriate control and sanitation measures. These activities would also lead to an increased knowledge on occurrence and relevance of PaBV-5 as a pathogen for psittaciform birds.

Conflict of interest statement

The authors declare no financial or personal relationships with other people or organizations that could inappropriately influence their work.

Acknowledgements

P. Sa-ardta received a study grant from the German Academic Exchange Service (Deutscher Akademischer Austauschdienst, DAAD, Germany; grant number 57129429). This work was additionally supported by Prince of Songkla University (PSU) and Kasetsart University (KU), Thailand. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors would like to thank Bernd Kaspers, Department of Veterinary Science, Veterinary Medicine Faculty of LMU Munich, for sharing the rabbit anti-Psittaciform bornavirus N protein polyclonal antibody. We are immensely grateful to Sakuna Phatthanakunanan, Siriluk Jala, Pattrawut Sangnual, Patcharida Dittawong and Chotinun Angkachatchai for excellent technical assistance. We also thank Nichapa Jaraspongpisuth, and Chananya Chaiya for their comments that greatly improved the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.01.013.

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