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

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To my beloved King Rama IX

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TABLE OF CONTENTS

INDEX OF ABBREVIATIONS

aa	Amino acid
ABBV	Aquatic bird bornavirus
AG	Avian ganglioneuritis
ABV	Avian bornavirus
APMV	Avian paramyxovirus
AST	Aspartate transaminase
AWA	Animal Welfare act
BD	Borna disease
BL	Benchapol Lorsunyaluck
BLAST	Basic Local Alignment Search Tool
BoDV	Borna disease virus
CBC	Complete blood count
cDNA	Complementary DNA
CE	Clinical examination
CK	Creatine kinase
CnBV	Canary bornavirus
CNS	Central nervous system
ct	Computed tomography
Ctrl	Control
DAAD	Deutscher Akademischer Austauschdienst
DAB	3.3’ Diaminobenzidine tetrahydrochloride

DNA	deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EsBV	Estrildid finch bornavirus
G	Gravity
GI	Gastrointestinal
H&E	Hematoxylin and eosin
HRP	Horseradish peroxidase
IACUC	Institutional Animal Care and Use Committee
IAS	International Aviculturists Society
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IIFA	Indirect immunofluorescence assay
JCPV	Jungle carpet python virus
kb	Kilobase
KU	Kasetsart University
LC	Least Concern
LGSV	Loveridge's garter snake virus
LMU	Ludwig-Maximilians-University of Munich
min	Minute
mRNA	Messenger RNA
MuBV	Munia bornavirus

NA	Not Available
nm	Nanometer
NT	Near Threatened
nt	Nucleotide
OD	Optical Density
PaBV	Parrot bornavirus
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline withTween
PCV	Packed cell volume
PDD	Proventricular dilatation disease
pH	Negative log of hydrogen ion concentration in a water-based solution
PMV	Paramyxovirus
PS	Phirawich Sa-ardta
PSU	Prince of Songkla University
qRT-PCR	Quantitative real-time RT-PCR
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
s	Second
SD	Standard Deviation
ssRNA	Single-stranded RNA
SWCPV	Southwest carpet python virus
TBST	Tris Buffered Saline with Tween
TMB	3,3',5,5'-Tetramethylbenzidine

uORF	Upstream open reading frame
VSBV	Variegated squirrel bornavirus
WBC	White blood cell
µg	Microgram
µL	Microliter
µM	Micromolar
µm	Micrometer

I. INTRODUCTION

In 2008, avian bornavirus (ABV) has been identified as a possible etiological agent of proventricular dilatation disease (PDD) in parrots (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; GRAY et al., 2010; MIRHOSSEINI et al., 2011; PIEPENBRING et al., 2012; RUBBENSTROTH et al., 2014a; GENTRY et al., 2015; PIEPENBRING et al., 2016; HÖGEMANN et al., 2017). 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* (WEISSENBÖCK et al., 2009b; PHILADELPHO et al., 2014).

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 (GREGORY et al., 1994; BERHANE et al., 2001; SCHMIDT et al., 2003; LUBIN et al., 2006; GANCZ et al., 2010; HEFFELS-REDMANN et al., 2011; ENCINAS-NAGEL et al., 2014; SASSA et al., 2015; ROSSI et al., 2017). PDD can affect both sexes equally and is generally found in adult birds but was also described in juveniles in both naturally (KISTLER et al., 2010) and experimentally infected psittacines (PIEPENBRING et al., 2012; PIEPENBRING et al., 2016). Typically the disease spreads slowly; however, there have been reports of acute outbreaks with high mortality in psittacine aviaries (LUBIN et al., 2006; KISTLER et al., 2010)

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. The clinical signs of PDD frequently include malfunction of the gastrointestinal tract with or without neurological signs (GREGORY et al., 1994; BERHANE et al., 2001; STAEHELI et al., 2010). Subclinical ABV-infected carriers showing no signs of disease have been documented in several studies (DE KLOET & DORRESTEIN, 2009; LIERZ et al., 2009; OUYANG et al., 2009;

HERZOG et al., 2010; VILLANUEVA et al., 2010; HEFFELS-REDMANN et al., 2011; HEFFELS-REDMANN et al., 2012; PIEPENBRING et al., 2012; PIEPENBRING et al., 2016; LEAL DE ARAUJO et al., 2017b).

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 such as Israel, Japan, and Korea. Among these countries, 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 (KISTLER et al., 2008; OGAWA et al., 2011; HORIE et al., 2012; SASSA et al., 2013; KIM et al., 2014). Surprisingly, in Southeastern Asia, where the weather is optimal for various bird species, PaBV detection has not been reported so far. Thailand is a country located in Southeastern Asia. This condition allows Thailand to become 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. 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 to be 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.

II. LITERATURE REVIEW

1. Proventricular dilatation disease in avian species

Proventricular dilatation disease (PDD) is a viral progressive neurologic and often fatal disease affecting predominantly psittacine birds and has also been reported in many other bird orders (DAOUST et al., 1991; GREGORY et al., 1994; GREGORY et al., 2000; BERHANE et al., 2001; SCHMIDT et al., 2003; SHIVAPRASAD, 2005; LUBIN et al., 2006; PERPIÑÁN et al., 2007; RINDER et al., 2009; WEISSENBÖCK et al., 2009a; GANCZ et al., 2010; SMITH et al., 2010; HEFFELS-REDMANN et al., 2011; HOPPES et al., 2013; RUBBENSTROTH et al., 2013; ENCINAS-NAGEL et al., 2014; SASSA et al., 2015; PIEPENBRING et al., 2016; ROSSI et al., 2017). It was recognized by the International Aviculturists Society (IAS) as an extremely dangerous disease concerning the aviculture of psittacine birds, if not the most threatening to parrots (KISTLER et al., 2008; GANCZ et al., 2009; PHILADELPHO et al., 2014). The disease affects the central nervous system (CNS), especially the myenteric plexus of the gastrointestinal (GI) tract (BEAUFRÈRE & TAYLOR, 2013) and the brain (HONKAVUORI et al., 2008; GANCZ et al., 2009; OUYANG et al., 2009; WEISSENBÖCK et al., 2009b; LEAL DE ARAUJO et al., 2017b).

The term PDD is generally used in this disease because of the dilation of the proventriculus as the predominant clinico-pathological feature in psittacine birds. Other synonyms have also been derived from the underlying clinico-pathological features of the disease such as macaw wasting/fading disease/syndrome, proventricular dilatation syndrome, neuropathic gastric dilatation of Psittaciformes, psittacine encephalomyelitis, myenteric ganglioneuritis, lymphoplasmacytic encephalomyelitis, infiltrative splanchnic neuropathy (GANCZ et al., 2010; STAEHELI et al., 2010; DELNATTE, 2013). In a recent publication, the terms avian myenteric ganglioneuritis, nonsuppurative ganglioneuritis, avian autoimmune ganglioneuritis, or simply, avian ganglioneuritis (AG) were purposed to use as newer terms due to expressing the disease process better and shifting the focus from the proventriculus to the nervous system (ROSSI et al., 2017).

In 2008, the disease has been attributed to novel bornaviruses in parrots which were, at that time, designated as avian birnavirus (ABV) and were confirmed as the

causative agents of PDD by several experimental studies afterward (HONKAVUORI et al., 2008; KISTLER et al., 2008; GANCZ et al., 2009; GRAY et al., 2010; KORBEL et al., 2011; MIRHOSSEINI et al., 2011; PIEPENBRING et al., 2012; RUBBENSTROTH et al., 2014a; GENTRY et al., 2015; PIEPENBRING et al., 2016).

1.1. Descriptive epidemiology of proventricular dilatation disease

PDD was initially reported in North America in the late 1970s as a clinical syndrome in young macaws (*Ara* sp.) newly imported from the Santa Cruz area of Bolivia. (GREGORY et al., 1994; HOPPES et al., 2013). It has been described in more than 80 species of both captive and free-living psittacines (GREGORY et al., 1994; BERHANE et al., 2001; SCHMIDT et al., 2003; LUBIN et al., 2006; GANCZ et al., 2010; HEFFELS-REDMANN et al., 2011; ENCINAS-NAGEL et al., 2014; SASSA et al., 2015; ROSSI et al., 2017). The most prevailing psittaciform species affected are African grey parrots (*Psittacus erithacus*), blue and gold macaws (*Ara ararauna*), Amazon parrots (*Amazona* sp.), and cockatoos (*Cacatua* sp.) (SCHMIDT et al., 2003), while other species, such as Quaker parrots (*Myiopsitta monachus*) and lovebird species (*Agapornis*) appear to be minimally affected (ROSSI et al., 2017). Fascinatingly, budgerigars (*Melopsittacus undulatus*) seem to be resistant to the disease and the cause still remains unclear (GRAHAM, 1991; REAVILL & SCHMIDT, 2007; DELNATTE, 2013). Suggestive lesions of PDD have also been reported in numerous other avian orders, including the passeriform species canaries (*Serinus canaria*), a greenfinch (*Carduelis chloris*), Bengalese finches (*Lonchura striata f. dom.*), a yellow-winged pytilia (*Pytilia hypogrammica*), black-rumped waxbills (*Estrilda troglodytes*), honeycreepers (*Drepanidinae*), and a long-wattled umbrella bird (*Cephalopterus penduliger*), as well as bird from other zoological orders like a bearded barbet (*Lybius dubius*), Canada geese (*Branta canadensis*), trumpeter swans (*Cygnus buccinator*), toucans (*Rhamphastidae*), a roseate spoonbill (*Platalea ajaja*), a bald eagle (*Haliaeetus leucocephalus*) and a peregrine falcon (*Falco peregrines*) (DAOUST et al., 1991; GREGORY et al., 2000; SHIVAPRASAD, 2005; PERPIÑÁN et al., 2007; WEISSENBÖCK et al., 2009a; SMITH et al., 2010; RINDER et al., 2012; HOPPES et al., 2013; RUBBENSTROTH et al., 2013).

PDD can affect both sexes equally and may generally be found in adult birds, but has been detected in birds as young as 5 weeks of age as well (KISTLER et al.,

2010). Typically the disease spreads slowly; however, there have been reports of acute outbreaks with high mortality in psittacine aviaries (LUBIN et al., 2006; KISTLER et al., 2010).

Although the vast majority of cases have been described in Europe and North America, by now cases of PDD and PDD-like diseases have been diagnosed in many parts of the world including Australia, the Middle East, South America, South Africa, and Asia (LUBIN et al., 2006; KISTLER et al., 2008; RINDER et al., 2009; WEISSENBÖCK et al., 2009a; WYSS, 2009; HEFFELS-REDMANN et al., 2011; OGAWA et al., 2011; HORIE et al., 2012; LAST et al., 2012; SASSA et al., 2013; KIM et al., 2014; PHILADELPHO et al., 2014). It is assumed that extensive trading and bird smuggling are responsible for the spread of the disease (DONELEY et al., 2007; STAEHELI et al., 2010).

1.2. Clinical signs and symptoms of proventricular dilatation disease

The clinical signs of PDD frequently include malfunction of the gastrointestinal tract with or without neurological signs, which are unspecific and commonly seen in pet birds. Bird owners usually notice the disease only in advanced states, when the birds show reduced appetite or polyphagia associated with weight loss and abdominal enlargement with other non-specific signs including depression, lethargy, muscle atrophy, polyuria and polydipsia (BERHANE et al., 2001; DONELEY et al., 2007; GANCZ et al., 2010; DELNATTE, 2013). The digestive form of the disease commonly includes maldigestion, undigested seeds in the feces, regurgitation, impaction of the proventriculus, crop stasis, emaciation and ultimate death by starvation. Neurological signs, such as tremor, ataxia, behavioral changes and seizures, have also found alone or in a combination with gastrointestinal signs. Other signs concurrently with PDD include ophthalmological abnormalities, feather picking, and cardiac conduction abnormalities. Some studies have reported that ophthalmological abnormalities, especially lesions located at the fundus oculi including chronical multifocal chorioretinitis and chorioretinal coloboma formation, are an important part of the clinical feature of PDD and could be early signs of the disease (STEINMETZ et al., 2008; KORBEL & RINDER, 2011; BEAUFRÈRE & TAYLOR, 2013).

These clinical signs are variable and depend on host species and are – at least regarding to the ophthalmological findings – non specific. They are also varying

from patient to patient (ROSSI et al., 2017). Acute form of the disease was developed by some birds while others develop a chronic or subclinical form that may persist very long sometimes (more than 10 years) (LEAL DE ARAUJO et al., 2017a).

1.3. Differential diagnosis of proventricular dilatation disease

Differential diagnoses of PDD can be divided into gastrointestinal diseases of infectious or non-infectious origin and diseases with neurologic presentations.

The infectious causes can further be divided into 1) bacterial infections: such as *Chlamydia psittaci, Mycobacterium* spp., *Salmonella* spp. and other Enterobacteriaceae can cause proventriculitis, enteritis, and upper digestive tract (crop and esophagus) disease, 2) viral infections: paramyxoviruses, herpesviruses, adenoviruses and particularly polyomavirus in young birds can cause gastrointestinal disease, 3) fungal infection: *Aspergillus* spp. (aspergillosis), *Candida* spp. (candidiasis) and especially *Macrorhabdus ornithogaster* (macrorhabdosis or megabacteriosis), 4) parasitic infections: roundworms and tapeworms can cause enteritis, and upper digestive tract disease (WOERPEL et al., 1984; GRAHAM, 1991; RINDER et al., 2009; GANCZ et al., 2010; HÖGEMANN et al., 2017).

Non-infectious differential diagnoses include 1) physiologic dilatation of the proventriculus of young psittacines or fruit/nectar-eating species, some Eclectus parrots, 2) mechanical problems or obstruction in the transportation of ingesta through the gastrointestinal tract: caused by foreign body, neoplasia, stricture, parasitism (e.g., cestodes, nematodes), bezoar or by a coracoid fracture (WOERPEL et al., 1984). 3) congenital disorder: myoventricular dysgenesis, koilin dysgenesis (BEAUFRÈRE & TAYLOR, 2013), 4) intoxication: poisoning with plant toxins and mainly with heavy metals, for example, lead or zinc must be ruled out (RIDGWAY & G-ALLERSTEIN, 1983; WOERPEL et al., 1984; GRAHAM, 1991; GANCZ et al., 2010), 5) nutritional deficiencies: potential vitamin E / selenium deficiencies should also be considered (WOERPEL et al., 1984; BEAUFRÈRE & TAYLOR, 2013).

Additionally, diffuse neurologic conditions should be considered including 1) vascular: atherosclerosis, ischemic infarction, cerebrovascular accident, 2) infectious: viral (encephalitis, paramyxoviruses, polyomavirus, reovirus, avian

viral serositis), bacterial (*Chlamydia psittaci, Mycobacterium* spp., *Listeria monocytogenes, Salmonella* spp.), fungal (*Aspergillus* spp., *Candida* spp.) and parasites (*Baylisascaris procyonis*, *Toxoplasma*, *Sarcocystis*), 3) traumatic: head trauma, cranial hypertension, 4) toxic: heavy metals, primarily chronic lead poisoning, insecticides, botulism, 5) metabolic: hypocalcemia (e.g., African greys), hypoglycemia, hepatic encephalopathy, other electrolyte imbalances (e.g., salt toxicity, magnesium) 6) idiopathic epilepsy, idiopathic tremors, 7) neoplasms of the nervous system, 8) degenerative diseases, 9) nutritional deficiencies: vitamins E, B1, B6, selenium (BEAUFRÈRE & TAYLOR, 2013).

1.4. Pathological lesions of proventricular dilatation disease

The pathological and histopathological lesions of PDD have been widely studied (GRAHAM, 1991; SHIVAPRASAD et al., 1995; REAVILL & SCHMIDT, 2007; OUYANG et al., 2009; RAGHAV et al., 2010; DELNATTE et al., 2013). The hallmark lesions of PDD are lymphoplasmacytic encephalomyelitis and ganglioneuritis. These lesions have been considered, by some authors, as pathognomonic and may, therefore, be used to confirm a tentative diagnosis of PDD (RINDER et al., 2009; WEISSENBÖCK et al., 2009b; RINDER et al., 2010; TIZARD et al., 2016).

Macroscopic lesions in birds with PDD include dilatation of the esophagus, proventriculus, ventriculus, or small intestine distension, atrophy of the proventricular muscle and pectoral muscles and mild to severe emaciation (BERHANE et al., 2001; GANCZ et al., 2010). Some birds with neurological signs have been reported a transparent fluid accumulation in the subarachnoid space (BERHANE et al., 2001).

Whilst microscopic lesions of PDD were characterized by non-suppurative inflammations in many tissues especially in peripheral, central and autonomic nervous tissues (SHIVAPRASAD et al., 1995; BERHANE et al., 2001; SCHMIDT et al., 2003). Lymphoplasmacytic infiltrations particularly in myenteric ganglia and nerves of the proventriculus and ventriculus are frequently presented (SCHMIDT et al., 2003). Perivascular cuffing related the similar infiltrations may also present in various tissues, regularly in the brain, spinal cord, peripheral nerves and adrenal glands (SHIVAPRASAD et al., 1995; BERHANE et al., 2001), then less frequently in myocardium and conductive tissue of the heart (VICE, 1992). The thickness of

the perivascular cuffing and the number of affected blood vessels varies greatly (SHIVAPRASAD et al., 1995). Furthermore, non-suppurative inflammations in many tissues, multifocal chorioretinitis, chronic retinal degeneration and coloboma formation resulting in clinical partial or complete blindness have also been found (SCHMIDT et al., 2003; DONELEY et al., 2007; STEINMETZ et al., 2008; KORBEL & RINDER, 2011).

1.5. Diagnosis of proventricular dilatation disease

Since the discovery of the etiologic agent of PDD in 2008 (HONKAVUORI et al., 2008; KISTLER et al., 2008), a variety of diagnostic methods have been developed for the detection of avian bornavirus infection. These methods will be described further in chapter 2.4. Diagnosis of Psittaciform bornavirus infection. This chapter, in contrast, is focussing on the detection of clinical and histopathological signs which, in combination, are characteristic of PDD. This must be differentiated from the pure detection of a pathogen.

The physical examination, including ophthalmologic and cardiac examination (VICE, 1992; STEINMETZ et al., 2008; KORBEL & RINDER, 2011), should always be the initial step for diagnosing PDD; nevertheless, most of the clinical features of PDD mentioned previously are unspecific (GREGORY et al., 1994). Crop and fecal cytologic examination, as well as bacteriologic examination, may be further steps aimed to rule out a secondary infection from bacteria and fungus (BEAUFRÈRE & TAYLOR, 2013). As well as that, blood tests including CBC and plasma biochemistry panel are inconsistent but supportive to assess the patient's health status and provide information to rule out other diseases such as lead and zinc intoxication. A reduced PCV or mild non-regenerative anaemia has been described in several birds suffering from PDD, as a result of gastrointestinal malabsorption and starvation (RIDGWAY & G-ALLERSTEIN, 1983; KELLER et al., 2010; HÖGEMANN et al., 2017). Slight lymphocytosis and monocytosis have occasionally been reported in PDD affected birds. Elevated WBCs may also be seen in a combination with heterophilia in acute cases of PDD (GANCZ et al., 2010; KELLER et al., 2010; BEAUFRÈRE & TAYLOR, 2013). Mild to moderate elevation of muscle origin enzymes levels including AST, CK and lactate dehydrogenase have been reported in psittaciform birds infected with PaBV (GANCZ et al., 2010; KELLER et al., 2010). However, elevated CK levels might be due to the excitement of patients when handling or muscle trauma (FUDGE,

2000). Hypoproteinemia, respectively hypoalbuminemia, and hypoglycemia have frequently been observed in birds with advanced, severe disease or decompensating patients (RIDGWAY & G-ALLERSTEIN, 1983; KELLER et al., 2010).

The further advanced diagnosis is diagnostic imaging, including plain radiographs of a dilated proventriculus, which is commonly seen in advanced cases with predominantly GI signs, even though this feature is not pathognomonic for PDD and it must also be reminded that this is a physiological condition in young parrots, especially from hand rearing (BOND et al., 1993), proventriculus/keel ratio measurement (DENNISON et al., 2009), contrast radiography using barium sulfate may useful to determine increased transit times and underline the GI morphology (BEAUFRÈRE & TAYLOR, 2013). Furthermore, if the technical possibilities are given, gastrointestinal fluoroscopic study, is the most helpful method to indicate reduced GI motilities, contractions and structural abnormalities associated with the disease (GANCZ et al., 2010). Endoscopy may be used to investigate serosal inflammation and evaluate the outer surfaces of the proventriculus and the ventriculus (BEAUFRÈRE & TAYLOR, 2013).

Ante-mortem diagnostic tests mentioned earlier may provide the important information for diagnosing PDD; nevertheless, they are not considered confirmatory for the disease (BOND et al., 1993; DELNATTE, 2013; HOPPES et al., 2013). Crop biopsy and histopathology remain the safest ante-mortem test to confirmatory diagnose PDD so far even high false-negative rate of approximately 40 % and the variable sensitivity, which ranges from 22 % to 76 % have been reported (GRAHAM, 1991; SHIVAPRASAD et al., 1995; GREGORY et al., 1996; BERHANE et al., 2001).

Therefore, the gold standard for the diagnosis of PDD is still post-mortem examination and identification of the pathognomonic lymphoplasmacytic infiltration in digestive nerve plexus particularly the upper and middle digestive tract (SCHMIDT et al., 2003). Along with infiltrations in other peripheral nerves such as brachial, sciatic are characteristic of the disease (BEAUFRÈRE & TAYLOR, 2013).

1.6. Etiology of proventricular dilatation disease: historical overview

A plausible virus etiology for PDD has been suspected for a decade. Due to the histological changes in the ganglia and the finding of 80-100 nm virus-like particles

in nerve tissue, viruses with neurotropic properties were suspected (WOERPEL et al., 1984). Various viruses affecting nervous system including avian encephalitis virus (HUGHES, 1984), coronavirus (GOUGH et al., 2006), adenovirus (HELDSTAB et al., 1985), reovirus (HUGHES, 1984) or eastern equine encephalitis virus (GREGORY et al., 1997b), paramyxovirus (GRUND et al., 2005), avian herpesvirus, togavirus and polyomavirus (RITCHIE et al., 1994) have been discussed as candidate causative agents of PDD; nevertheless, neither were successfully confirmed as the underlying cause of the disease.

Etioloical agents of previously known diseases with similar symptoms like Newcastle Disease or avian encephalitis were quickly ruled out as the cause (HUGHES, 1984), Gough et al. (2006) suspected that coronaviruses might be involved because they cause a similar disease (vomiting and wasting disease) in pigs. This suspicion was reexamined years later when coronaviruses were isolated from a PDD-suspected Amazon (GOUGH et al., 2006). Adenoviruses were suggested because of the morphology of virus particles found in the nerve tissue of a bird dying from PDD, but this suspicion was refuted by the demonstration of intranuclear inclusions that are not found in adenoviruses (HELDSTAB et al., 1985; LÖFFLER, 2011). Reoviruses were suspected in the 1980s, as other juvenile diseases, such as malabsorption syndrome in chickens, showed similarities in clinical signs (HUGHES, 1984). With increasing knowledge of the PDD this suspicion was no longer tenable. The Eastern Equine Encephalitis virus is only common in North America, and only young birds show PDD-like signs of disease while infection in adult birds is usually asymptomatic. This virus has been recognized as the cause of Avian viral serositis and is no longer suspected to cause PDD (GREGORY et al., 1997b). The role of paramyxoviruses (PMV), in this disease, has not been fully clarified so far. The discovery of intranuclear and intracytoplasmic inclusion bodies, as well as the discovery of enveloped, virus particles of varying sizes drew attention to the paramyxoviruses (MANNL et al., 1987). Lastly, a lentogenic Avian paramyxovirus serotype-1 (APMV-1) was assumed to be the cause of PDD, as virus particles were detected in the feces of diseased animals (GRUND et al., 2005). With an ELISA, which was able to detect seroconversion in APMV-1 -infected African gray parrots as early as 14 days after infection, antibodies against APMV-1 were detected in many psittacines with PDD (GRUND et al., 1999). In addition, APMV-1 could be isolated from nerve tissue of

PDD-sick birds (GRUND et al., 2005). Using PCR, APMV-1 was detected in the blood of an individual case of an Amazon suffering from PDD symptoms, but without histological confirmation of PDD (OROSZ & DAHLHAUSEN, 2007). Moreover, the attempt to fulfill Koch's postulates also failed. PMV-like virus particles were not only secreted by diseased, but also by clinically healthy birds. Experiments with isolation of the microorganisms from a diseased animal, conversion into pure culture and production of the same disease after experimental infection of healthy animals with this isolate were unsuccessful. Triggering of a PDD after infection with PMV and the re-isolation of the microorganism were not achieved (GRUND et al., 2005).

Another hypothesis for the development of PDD was that an autoimmune process is triggered by a bacterial or viral infection, which itself does not cause a serious illness. Such post-infectious polyneuropathies are known in humans and animals and might also be the cause of PDD in birds. This would explain why no virus could be uniquely identified as the cause, since the virus would have been eliminated by the immune system even when the disease is outbreaking (GRAHAM, 1991). Arguing against this assumption is that autoimmune diseases of the nerves are usually associated with their demyelination (GREGORY et al., 1997a). However, a screening of parrot sera for anti-ganglioside antibodies showed very high titers in PDD-sick birds. In addition, PDD symptoms were induced by experimental application of purified but adjuvanted gangliosides (ROSSI et al., 2008). However, it must be noted critically that the purification was from peripheral nerves and no information was given on the degree of purification or added adjuvant. Furthermore, these autoantibodies are also detected in healthy animals or humans, so that they have been questioned as the sole cause of the disease (RAVINDRNATH et al., 2000). To date, however, an autoimmune hypothesis in PDD is not completely excluded and the recent studies show that immune-mediated reaction, likely involving T cells, may play an essential role in disease development (HAMEED et al., 2018).

In 2008, a research group identified a novel virus of bornaviruses as a probable etiological agent of PDD by using panviral DNA microarray analysis followed by ultra-high throughput sequencing combined with conventional PCR. The advanced molecular technologies revealed that Borna Disease virus related pathogens were present. These agents, at that moment designated as avian bornaviruses (ABV),

were found in 62.5 % of PDD cases and in none of the control animals (KISTLER et al., 2008). Similar results from another independent study were published almost simultaneously, half a year later. The avian bornaviruses were found by unbiased pyrosequencing analysis and real time PCR in PDD-affected birds (HONKAVUORI et al., 2008). Afterward, numerous experimental studies confirmed the role of ABV as the causative agent of PDD in birds (DE KLOET & DORRESTEIN, 2009; LIERZ et al., 2009; OUYANG et al., 2009; RINDER et al., 2009; WEISSENBÖCK et al., 2009b; GRAY et al., 2010; HERZOG et al., 2010; KISTLER et al., 2010; REUTER et al., 2010; VILLANUEVA et al., 2010; WEISSENBÖCK et al., 2010).

2. *Bornaviridae* viruses

Bornaviridae is a family of viruses in the order *Mononegavirales*. Bornaviruses are enveloped viruses with non-segmented, single-stranded negative-sense RNA genomes of approximately 8.9 nucleotides. To date, 18 diverse viruses have been identified and assigned to ten species by the recent reclassification (AMARASINGHE et al., 2018). The natural hosts of bornaviruses are horses, sheep, cattle, rodents, birds, reptiles, and also humans (HOPPES et al., 2013; HOFFMANN et al., 2015; HYNDMAN et al., 2018). Bornaviruses may cause a neurologic syndrome which were called Borna disease (BD), when they were first identified in horses or sheep in central Europe (TOMONAGA et al., 2002; GRAY et al., 2010). The name "Borna disease" was created by epidemic outbreaks between 1894 and 1896 in cavalry horses in the city of Borna in Saxony, Germnay (CARBONE, 2001). In 1924, virus etiology was confirmed for the first time by transmission and reinfection experiments and currently recognized as Borna disease virus (BoDV, originally BDV) (ZWICK & SEIFRIED, 1925). Even when the most commonly affected species are horses and sheep, there have been numerous individual reports of the occurrence of BD in various other mammals such as cats, dogs, rabbits, guinea pigs, macaques and other ungulates (NAKAMURA et al., 1999; DEGIORGIS et al., 2000; JACOBSEN et al., 2010; LUTZ et al., 2015).

Since the 1990s, a zoonotic potential of the BoDV has been suspected, also due to the fact that many different mammal species, including primates, can become infected. A causal relationship between the occurrence of antibodies to BoDV and chronic neurological or psychological disorders was assumed in human patients (BODE et al., 1993; BODE & LUDWIG, 2003), but was subject of controversial debates and was rejected by many other virologists and the Society for Virology (Gesellschaft für Virologie) (HORNIG et al., 2012; SCHLOTTAU et al., 2018).

In 2015, a zoonotic potential of a new bornavirus was suspected when a previously unknown bornavirus was found in three German breeders of variegated squirrels (*Sciurus variegatoides*), who previously had a disease with encephalitis and encephalomyelitis. The same virus could also be detected in a bunny that had direct contact with one of the breeders and was therefore tentatively referred to a novel bornavirus as variegated squirrel 1 bornavirus (VSBV-1) (HOFFMANN et al., 2015; PFAFF et al., 2017; TAPPE et al., 2019).

The *Bornaviridae* family has grown steadily over past several decades when multiple additional viruses of this family were discovered. Since the initial detection of ABV as the causative agent of PDD in psittacines in 2008 (HONKAVUORI et al., 2008; KISTLER et al., 2008), ABV has been studied extensively. Up to the present time, at least 12 different viruses have been discovered in several avian species for example in psittacines, passerines and anserines (WEISSENBÖCK et al., 2009a; PAYNE et al., 2011b; RUBBENSTROTH et al., 2012; RUBBENSTROTH et al., 2014b).

Correspondingly, at one time, "warm-blooded" animals were thought to be susceptible to bornavirus infection (HYNDMAN et al., 2018). Once the first bornavirus-like sequences in a Gaboon viper (*Bitis gabonica*) were detected in 2004 (HYNDMAN et al., 2018), reptiles have been suggested as other natural hosts of bornaviruses (FUJINO et al., 2012). However, association between a disease and host, prevalence, zoonotic potential as well as host specificity of these reptile bornaviruses is completely unclear so far (HYNDMAN et al., 2018).

2.1. Taxonomy of the family *Bornaviridae*

Recently, based on previously published phylogenetic analyzes and the detection of complete genome coding sequences and biological characteristics of bornaviruses, the taxonomy of the *Bornaviridae* family was revised (KUHN et al., 2015; AMARASINGHE et al., 2018). By the recent reclassification 18 distinctive viruses have been included in two genera and divided into ten species as illustrated in Appendix A.

Based on the recent reclassification, the genus *Carbovirus* contains two species isolated from reptiles: 1) *Queensland carbovirus* (jungle carpet python virus: JCPV) and 2) *Southwest carbovirus* (southwest carpet python virus: SWCPV), whilst the genus *Orthobornavirus* includes eight species isolated from reptiles, horses, sheep, cattle, rodents, birds and humans: 1) *Elapid 1 orthobornavirus* (Loveridge's garter snake virus: LGSV-1), 2) *Mammalian 1 orthobornavirus* (Borna disease virus: BoDV-1 and -2), 3) *Mammalian 2 orthobornavirus* (variegated squirrel bornavirus: VSBV-1), 4) *Passeriform 1 orthobornavirus* (canary bornavirus: CnBV-1, -2, and -3), 5) *Passeriform 2 orthobornavirus* (estrildid finch bornavirus: EsBV-1), 6) *Psittaciform 1 orthobornavirus* (parrot bornavirus: PaBV-1, -2, -3, -4 and -7), 7) *Psittaciform 2 orthobornavirus* (parrot

bornavirus: PaBV-5), 8) *Waterbird 1 orthobornavirus* (aquatic bird bornavirus: ABBV-1 and -2), and two tentative, so far unclassified viruses, Munia bornavirus 1 (MuBV-1) (RUBBENSTROTH et al., 2016) and parrot bornavirus 6 (PaBV-6) (WEISSENBÖCK et al., 2009b). In a recent publication a new virus, tentatively called parrot bornavirus 8 (PaBV-8), has been reported (PHILADELPHO et al., 2014).

2.2. Characteristics of bornaviruses

The genomic organization of bornaviruses is generally similar to other negative sense ssRNA viruses. However, in the *Mononegavirales* order, unique to the *Bornaviridae* is the virus replication and transcription taking place within the nuclei of infected cells and also a genome replication strategy concerning splicing of the viral mRNA and trimming of 5′-terminal nucleotides (SCHNEIDER et al., 2005). This feature allows the virus to escape recognition by the innate immune system in infected cells (SCHNEIDER et al., 2007; REUTER et al., 2010). The diameter of bornaviruses is around 70 to 130 nm and the size of the bornaviral genome is approximately 8.9 kb, which is smallest in the order *Mononegavirales*: *Filoviridae* (ca. 19 kb), *Paramyxoviridae* (ca. 15 kb) and *Rhabdoviridae* (ca. 11–15 kb) (DE LA TORRE, 2006).

The bornaviral genome encodes six essential proteins arranged in the order: 3′-N (nucleoprotein), P (phosphoprotein), X (accessory protein), M (matrix protein), G (glycoprotein), L (polymerase)-5′. The helical nucleoside protein (N) and the P protein are the main targets for the immune response (TOMONAGA et al., 2002), because they contribute to protect the genomic RNA and form the nucleocapsid (KOBAYASHI et al., 2001). Some studies therefore use the viral N or P proteins to detect the anti-viral antibodies e.g. an enzyme-linked immunosorbent assay (ELISA) or use full virus as antigens in indirect immunofluorescence assays (IIFA) (HERZOG et al., 2010; RINDER et al., 2010; DE KLOET et al., 2011; HORIE et al., 2016). G protein has been associated with the cell entry of BoDV by receptor-mediated endocytosis; however, the cellular receptor of BoDV is still unidentified (VILLANUEVA et al., 2010). In BoDV a regulatory upstream open reading frame (uORF) in the N/X intergenic-region plays a role in the regulation of X and P proteins expression which has been described in several studies (RINDER et al., 2009; HORIE et al., 2012; MIRHOSSEINI et al., 2012). Intriguingly, PaBV-1, -2,

-3, -4, and -7 lack the regulatory uORF and possess a 21-22 nt deletion in the N/X intergenic-region (KISTLER et al., 2008; RINDER et al., 2009; FUJINO et al., 2012; MIRHOSSEINI et al., 2012; RUBBENSTROTH et al., 2012). CnBV, in contrast, does not possess the entire regulatory uORF but lacks the nucleotide deletion at this region (RUBBENSTROTH et al., 2013). Whilst a slightly shorter uORF was found only in PaBV-5 and ABBV (PAYNE et al., 2011b; HORIE et al., 2012). This implies that there are different strategies for regulation of expression of X and P proteins in the different bornaviruses (DELNATTE, 2013).

2.3. Pathogenesis of Psittaciform bornavirus infection

Even a decade after the discovery of PaBV as the causative agents of PDD, the pathogenesis of PDD, for example, the route of transmission or the viral cell tropism along with others aspects still remains unclear. The most accepted pathogenesis' theory suggests that the nervous system including central and enteric with peripheral nerves are damaged by inflammatory and immunological changes as a result from direct triggering by PaBV (LEAL DE ARAUJO et al., 2017a). The significant pathologic conditions affect innervation; hence impaired peristalsis of the gastrointestinal tract leads to maldigestion and malabsorption (GANCZ et al., 2010; RINDER et al., 2010; DELNATTE, 2013; PIEPENBRING et al., 2016; TIZARD et al., 2016; HÖGEMANN et al., 2017; LEAL DE ARAUJO et al., 2017a).

Extensive studies were able to proof an underlying relationship between PDD and PaBV in psittacine birds and also fulfill Koch's postulates by experimental infection of psittaciform birds with isolates of known viruses: PaBV-2 and -4 (GRAY et al., 2010; MIRHOSSEINI et al., 2011; PAYNE et al., 2011a; PIEPENBRING et al., 2012; RUBBENSTROTH et al., 2014a; OLBERT et al., 2016; PIEPENBRING et al., 2016; RUNGE et al., 2017). In a recent study (LEAL DE ARAUJO et al., 2017b) the infection pathway was investigated by tracking the viral spread and lesion development using molecular, histological and immunohistochemical methods in cockatiels after experimental inoculation of PaBV-2. The study revealed that after inoculation, PaBV-2 initial targeted the CNS, then spread out to the ganglia in the peripheral tissues such as the GI system and then widely distributed to various tissues such as crop, proventriculus, ventriculus, intestine, kidneys, skin, and vessels at late points of infection (LEAL DE ARAUJO et al., 2017b).

Based on BoDV infections in some laboratory rodent models, the development of Borna disease in these species is immune-mediated and associated with cytopathic T cells (ROTT et al., 1988; STITZ et al., 1989; HOPPES et al., 2013). It is therefore hypothesized that suitable immunosuppressive protocols, especially selective removal of T cells or immunosuppressive drugs, such as cyclosporine A, may have therapeutic benefit. (STITZ et al., 1989; HOPPES et al., 2013). This hypothesis was also formulated with regard to birds infected with PaBV (HAMEED et al., 2018). Cyclosporine A treatment of cockatiels experimentally infected with PaBV-2 resulted in complete protection from disease, but not from infection. This result further supported the hypothesis that immune-mediated reactions, likely involving T cells, may play an essential role in the development of PDD in psittaciform birds (HAMEED et al., 2018).

2.4. Diagnosis of Psittaciform bornavirus infection

Presently, a variety of diagnostic methods have been developed for the detection of PaBV infections. Several molecular biological methods focusing on detection of viral RNA, and serological methods for identifying specific antiviral antibodies have been widely established.

2.4.1. Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR and quantitative real-time RT-PCR (qRT-PCR) are mainly used for determining the presence of bornavirus RNA (HONKAVUORI et al., 2008; KISTLER et al., 2008). Currently, there have been various PCR protocols which were developed to detect specific target genes, especially the N (nucleoprotein), M (matrix protein) and L (polymerase) genes (KISTLER et al., 2008; DE KLOET et al., 2011; DELNATTE et al., 2014; GUO et al., 2014; KIM et al., 2014; PHILADELPHO et al., 2014; RUBBENSTROTH et al., 2014b). Especially the M and N genes were shown to yield high sensitivity in numerous studies (GANCZ et al., 2010; RAGHAV et al., 2010; DELNATTE, 2013). RT-PCR can be employed on a diversity of samples (invasive or non-invasive sampling) and on necropsy materials. In ante-mortem detection, crop, choanal and cloacal swabs, feces and nonvascular contour (chest) feather are regularly suggested (DE KLOET et al., 2011; DELNATTE, 2013; HOPPES et al., 2013). Calami of plucked chest contour feathers have been recommended by De Kloet and colleagues as a minimally invasive and trustworthy method, which, to their experience, was even more

reliable than PCR examination of cloacal swabs (DE KLOET et al., 2011). However, PCR testing of the minimally or non-invasive samples mentioned above are not always adequately sensitive, as in some birds with a negative swab result, viral RNA was detected in the brain or in other post-mortem organ samples (LIERZ et al., 2009). Although the RT-PCR is highly sensitivity, a variety of genetic heterogeneity, mutation in the primer or probe region and the feature of PaBV to be shed intermittently in urofeces, may cause false negative results (HOPPES et al., 2013). Additionally, designing broad-range primers in order to amplify the whole variety of PaBVs have definitely been challenging because of their high genetic heterogeneity (RUBBENSTROTH et al., 2013; PHILADELPHO et al., 2014).

2.4.2. Sequence analysis

Genome sequencing is mainly significant in order to identify novel unknown bornaviruses or co-infections with multiple PaBV strains (WEISSENBÖCK et al., 2009a; WEISSENBÖCK et al., 2010; NEDOROST et al., 2012; RUBBENSTROTH et al., 2012), to study the epidemiology of the geographical circulations, along with to understand the ecology, evolution and pathogenesis of these variants (DELNATTE, 2013). Although, the complete genome of each PaBV should ideally be sequenced, nowadays, full-genome sequences of PaBV were only obtained in a small amount of strains. PaBV-2 and -4 have been the most reputable bornaviruses circulating worldwide and have correspondingly been the most frequent target of whole genome sequencing in various studies so far (KISTLER et al., 2008; WEISSENBÖCK et al., 2009b). The only full-genome sequence of PaBV-7 available so far was obtained from a salmon-crested cockatoo (*Cacatua moluccensis*) in Germany (RUBBENSTROTH et al., 2012). PaBV-5, which was found in single birds in the United States of America (GUO & TIZARD, 2015), Japan (KOMORIZONO et al., 2016) and Hungary (MARTON et al., 2015) so far, full-genome sequences were also achieved, with the exception of the strain Cockg 5, for which only a partial sequence is available. Common to these PaBV-5-strains is a deletion of nucleotides in the L gene (GUO & TIZARD, 2015). The genomes of PaBV-3, -6 and -8 were partially sequenced until now (HONKAVUORI et al., 2008; WEISSENBÖCK et al., 2009b; PHILADELPHO et al., 2014).

2.4.3. Immunohistochemistry

The presence of PaBV antigen in dead psittacine birds with suspected PDD is

examined in immunohistochemistry (IHC), this is particularly usefull when the histological lesions are mild or ambiguous. (GREGORY et al., 1996; OUYANG et al., 2009; RINDER et al., 2009; WEISSENBÖCK et al., 2009b; HERZOG et al., 2010; RAGHAV et al., 2010). Raghav et al. (2010) suggested IHC as the gold standard since 100 % sensitivity and 100 % specificity of IHC were found in their study (RAGHAV et al., 2010). In other studies, however, IHC was shown to be less sensitive than PCR in detecting PaBV infections (LÖFFLER, 2011). Various reagents were used for IHC; for example, polyclonal antiserum raised against the BoDV phosphoprotein (RINDER et al., 2009; WEISSENBÖCK et al., 2009b; WEISSENBÖCK et al., 2009a) and polyclonal antiserum directed against the N antigen (OUYANG et al., 2009; LÖFFLER, 2011).

2.4.4. Serology

Serological tests including enzyme-linked immunosorbent assays (ELISA) (RINDER et al., 2010; DE KLOET et al., 2011; VONDRÁČKOVÁ et al., 2014; HÖGEMANN et al., 2017), Western blot (DE KLOET & DORRESTEIN, 2009; LIERZ et al., 2009; VILLANUEVA et al., 2010) and indirect immunofluorescence assays (IIFA) (HERZOG et al., 2010; HORIE et al., 2016) are also commonly used at the present time to detect the presence of specific antibodies against PaBV.

2.4.4.1. Indirect immunofluorescence assays (IIFA)

Herzog et al. (2010) established an IIFA for the *intra vitam* detection of ABV-specific serum antibodies based on the cross-reactivity of psittacine antibodies with mammalian BoDV antigens. When PaBV antigen was used instead of BoDV antigen, a comparable sensitivity was achieved (HERZOG et al., 2010). However, in another study, Zimmermann et al. (2014) demonstrated sensitivity differences in IIFA using different antigens, and the highest sensitivity for the detection of anti-PaBV antibodies in parrots was achieved with PaBV as the antigen (ZIMMERMANN et al., 2014).

2.4.4.2. Western blot

The Western blot has also been tested as a serological method for the detection of anti-bornavirus antibodies in birds, particularly of antibodies to the N protein (DE KLOET & DORRESTEIN, 2009; LIERZ et al., 2009; RINDER et al., 2010; VILLANUEVA et al., 2010). In comparison to the ELISA, however, the Western blot has a lower sensitivity, is more expensive and also needs higher laboratory

skills so that this test is less suitable for routine diagnostics or larger numbers of samples (HÖGEMANN, 2018).

2.4.4.3. ELISA

The ELISA is a well-established serological diagnostic method (RINDER et al., 2010; DE KLOET et al., 2011). De Kloet et al. found ELISA testing for the presence of ABV nucleoprotein (p40) is more reliable than ELISA determining for the ABV phosphoprotein (p24) or the matrix protein (p16). This result also correlates well with the Western blot results (DE KLOET et al., 2011).

Nowadays, there is still no optimal screening protocol for PaBV infection in psittacine aviculture. Each of the diagnostic method described above has its advantages and its flaws, which depend on individual circumstances and aptness. Evaluation and comparison of specificity and sensitivity of these different serological tests are very difficult to be performed, due to the relatively poor correlation between the presence of antibodies and viral shedding (HOPPES et al., 2010; DELNATTE, 2013; HOPPES et al., 2013). Besides, antibodies do not seem to be associated with a protection from disease.

Consequencely, because of known genetic heterogeneity of bornaviruses as well as presumed persistent bornavirus infections with late and intermittent virus shedding, some authors recommended a combination of a serologic assay and RT-PCR (from crop, choanal or cloacal swabs) as the most reliable *intra vitam* diagnostic protocol to determine PaBV infections, (RINDER et al., 2009; HERZOG et al., 2010; RINDER et al., 2010; VILLANUEVA et al., 2010; HOPPES et al., 2013).

III. PUBLICATION

The information on materials and methods and the results are summarized in the following manuscript, which was accepted for publication. Published by Elsevier in Veterinary Microbiology in March 2019, available online at:

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Supplemental Figure S1-S2 mentioned in the publication are attached to the dissertation.

Supplemental Tables are available online at:

Supplemental Table S1. Primers used for whole-genome sequencing of PaBV-5 strain 16021 originating from Thailand. (XLSX)

https://ars.els-cdn.com/content/image/1-s2.0-S0378113518312884-mmc2.xlsx

Supplemental Table S2. Individual results of vital signs, clinical signs and diagnoses of the 111 psittacine birds. (XLSX)

https://ars.els-cdn.com/content/image/1-s2.0-S0378113518312884-mmc3.xlsx

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

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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.

Keywords: Parrot bornavirus; PaBV; Proventricular dilatation disease; PDD; PaBV-5; cage mate; Southeastern Asia; natural infection

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

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., 2012; PIEPENBRING et al., 2016).

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; WEISSENBÖCK et al., 2009b; STAEHELI et al., 2010; TIZARD et al., 2016).

By now, cases of PDD have been diagnosed in many parts of the world (KISTLER et al., 2008; HEFFELS-REDMANN et al., 2011; LAST et al., 2012; PHILADELPHO et al., 2014; KOMORIZONO et al., 2016). 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; SASSA et al., 2013; KOMORIZONO et al., 2016). 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 clinical 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., 2009b).

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′-CAGACAGCACGTCGAGTGAGA-3′), the reverse primer (5′-AGTTAGGGCCTCCCTGGGTAT-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 (HONKAVUORI et al., 2008; HÖGEMANN et al., 2017).

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 1,000 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 (HÖGEMANN 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 hour. Wells were washed with PBST and incubated at 37 °C for 1 hour 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 hour 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 hour 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 (HÖGEMANN 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 1-2). 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.

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).

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.

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 ($P < 0.001$).

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 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.

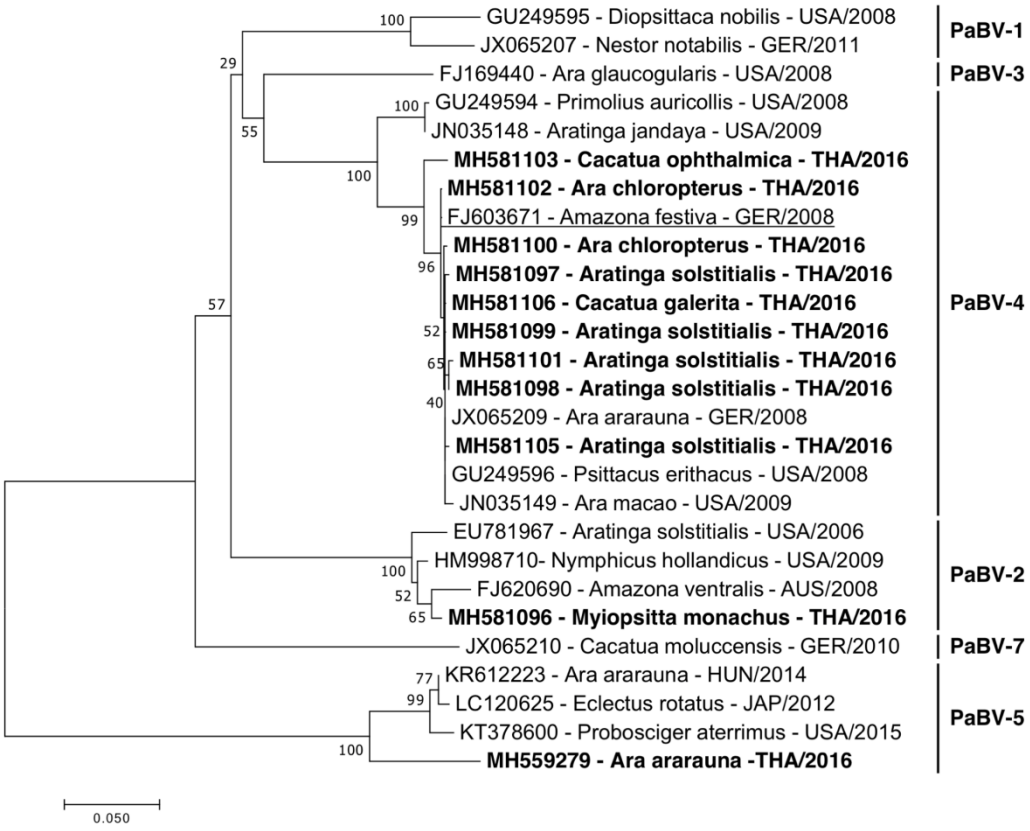


Fig. 1A. Phylogenetic analysis based on partial PaBV L gene (490 bp) sequences from captive psittacines in Thailand. The tree was constructed including public PaBV reference sequences stored in the GenBank database. The GenBank accession number and the host species as well as the country and year of 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.

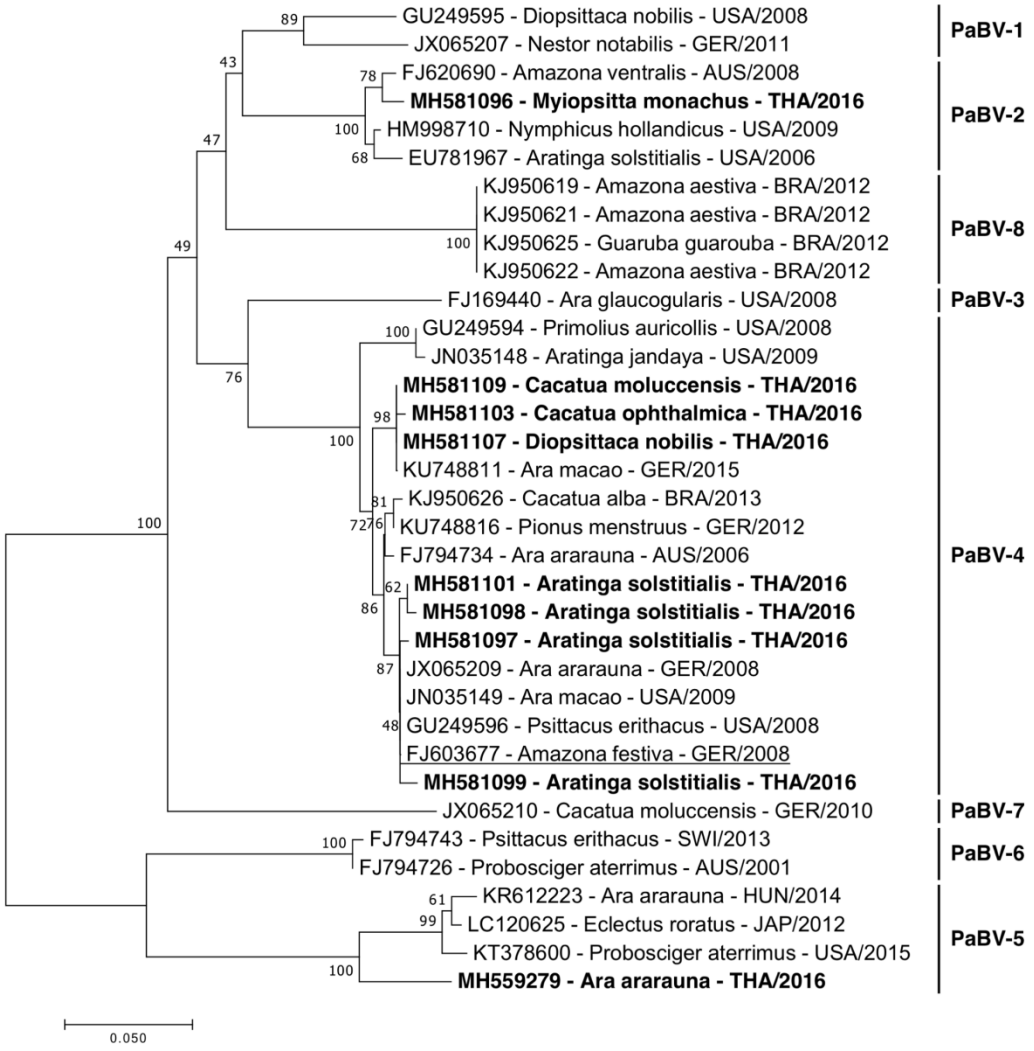


Fig. 1B. Phylogenetic analysis based on partial PaBV M gene (306 bp) sequences from captive psittacines in Thailand. The tree was constructed including public PaBV reference sequences stored in the GenBank database. The GenBank accession number and the host species as well as the country and year of 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.

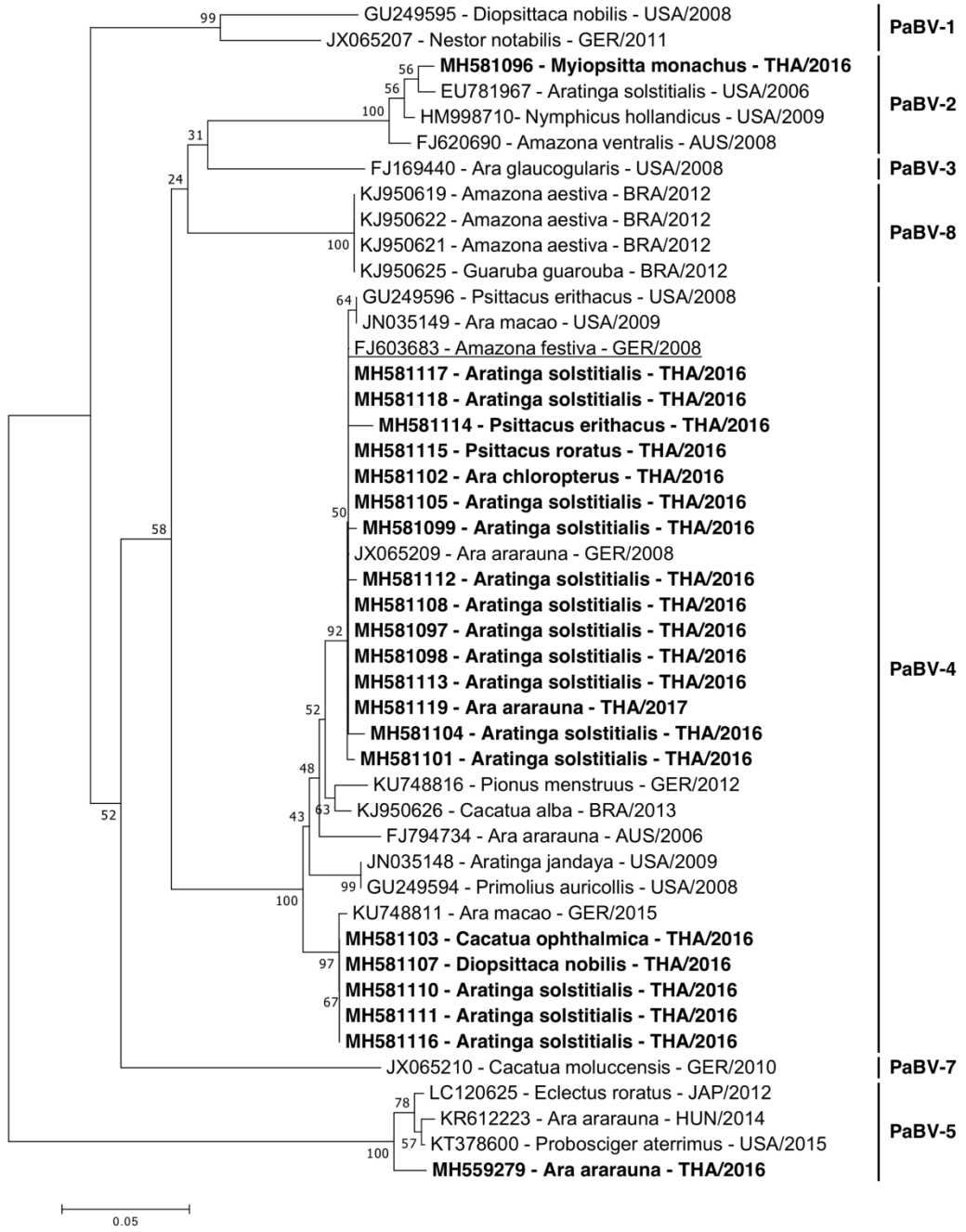


Fig. 1C. Phylogenetic analysis based on partial PaBV N gene (342 bp; Fig 1C) sequences from captive psittacines in Thailand. The tree was constructed including public PaBV reference sequences stored in the GenBank database. The GenBank accession number and the host species as well as the country and 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.

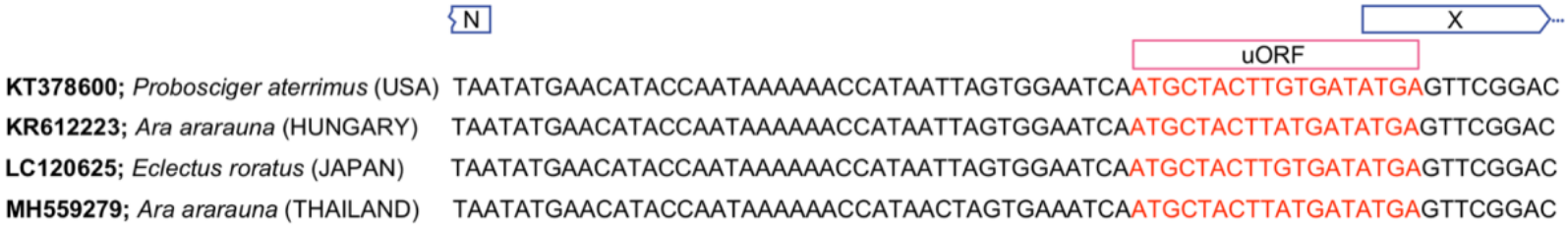
3.2. Whole-genome sequence of a PaBV-5 strain from Thailand

Using PCR and Sanger sequencing, 8,892 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 & TIZARD, 2015; MARTON et al., 2015; KOMORIZONO et al., 2016). 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 3).

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 Figure 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 & 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.



Supplemental Figure S1. Nucleotide alignment of the N/X intergenic region of PaBV-5 strain 16021 and the other PaBV-5 reported so far.

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

[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.

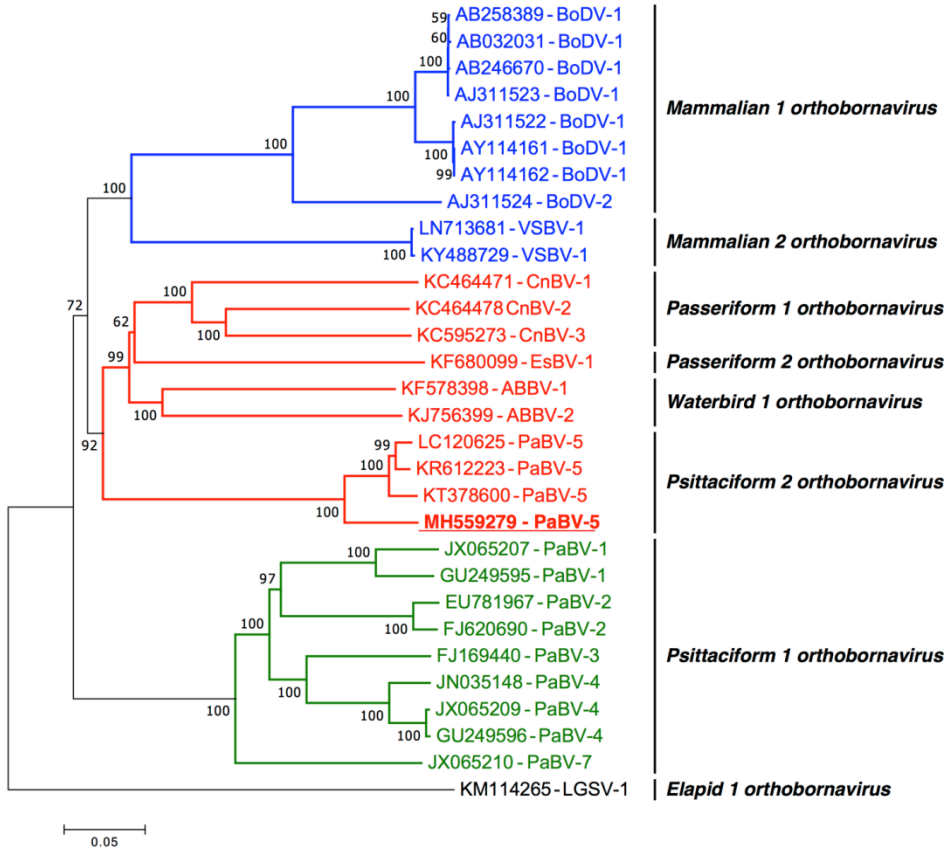


Fig 2. Phylogenetic analysis of the PaBV-5 strain found in Thailand and other viruses of the genus *Orthobornavirus* based on nucleotides coding for the N, X, P, M, G and the partial L protein without the non-coding regions. This tree was constructed using the Neighbor-Joining method with Jukes-Cantor genetic distance model and 1,000 bootstrap replicates by MEGA 7. The PaBV-5 strain 16021 of this study is emphasized in bold and underlined.

3.3. Comparison of various tests for Psittaciform bornavirus diagnosis

3.3.1. Enzyme-linked immunosorbent assay (ELISA)

As shown in Table 4, 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 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 Figure S2)

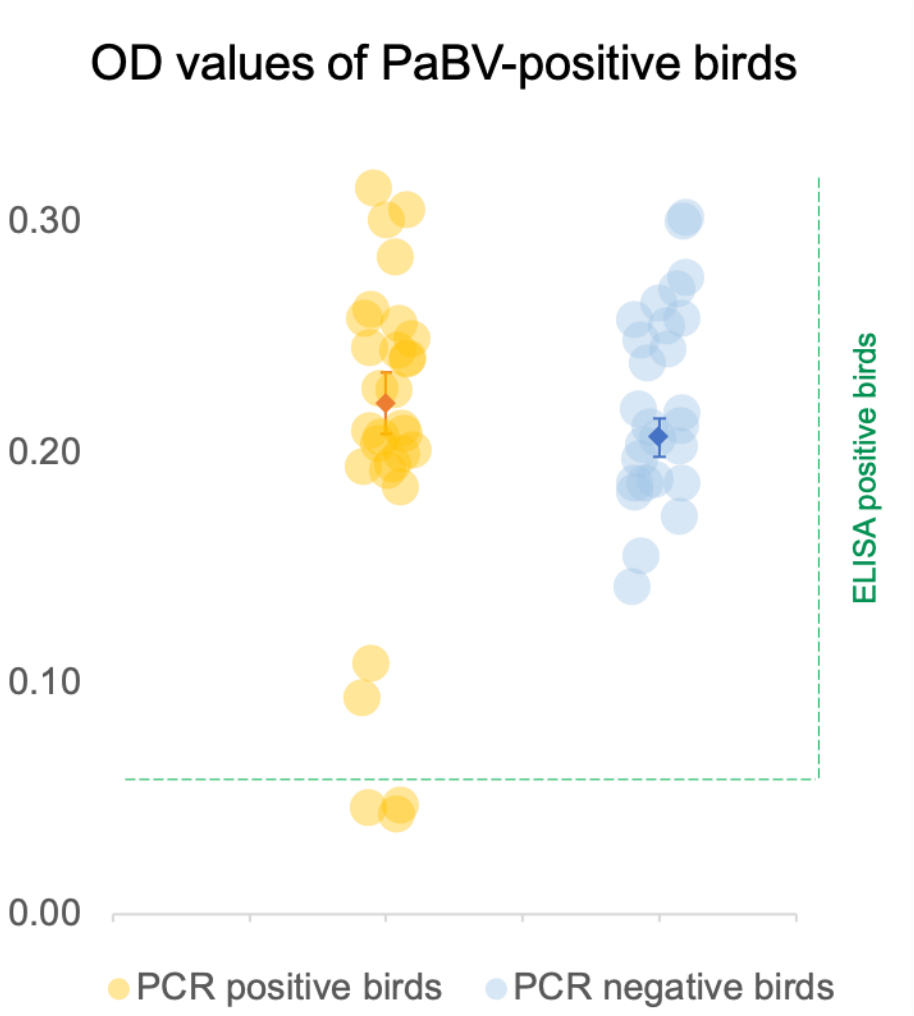
Table 4. Comparison of nucleotide (NT) and amino acid (AA) sequence identities among the PaBV-5 strain 16021 characterized in the present study and representative psittaciform orthobornaviruses.

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

[a]Only partial sequences were available for PaBV-6 (M), PaBV-8 (N and M) and PaBV-3 (L and overall).

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 4). 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 4).



Supplemental Figure S2. Scatter dot plot representation of ELISA optical density values (OD) of PaBV-positive birds (n = 57; 16021, 16054 and 16108 are not included as no serum was available for testing in ELISA). Error bars: 95 % confidence interval for mean.

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).

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.

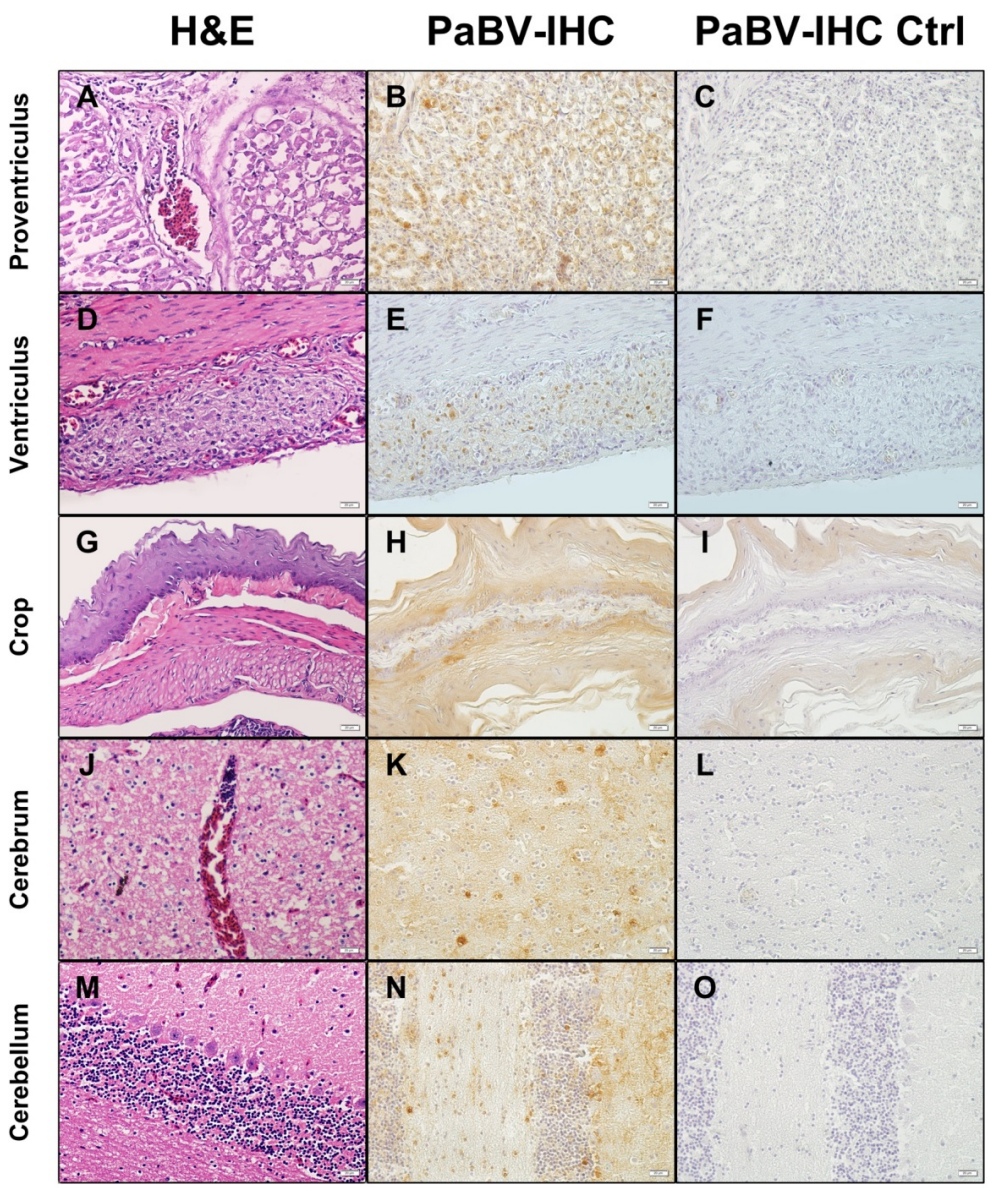


Fig 3. Photomicrographs of histological findings (Hematoxylin and Eosin; H&E) and immunohistochemical (IHC) labeling of *Psittaciform 1 orthobornavirus* N protein from proventriculus (A-C), ventriculus (D-F), crop (G-I), cerebrum (J-L) and cerebellum (M-O) of PaBV-affected parrots in Thailand. Proventriculus 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).

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).

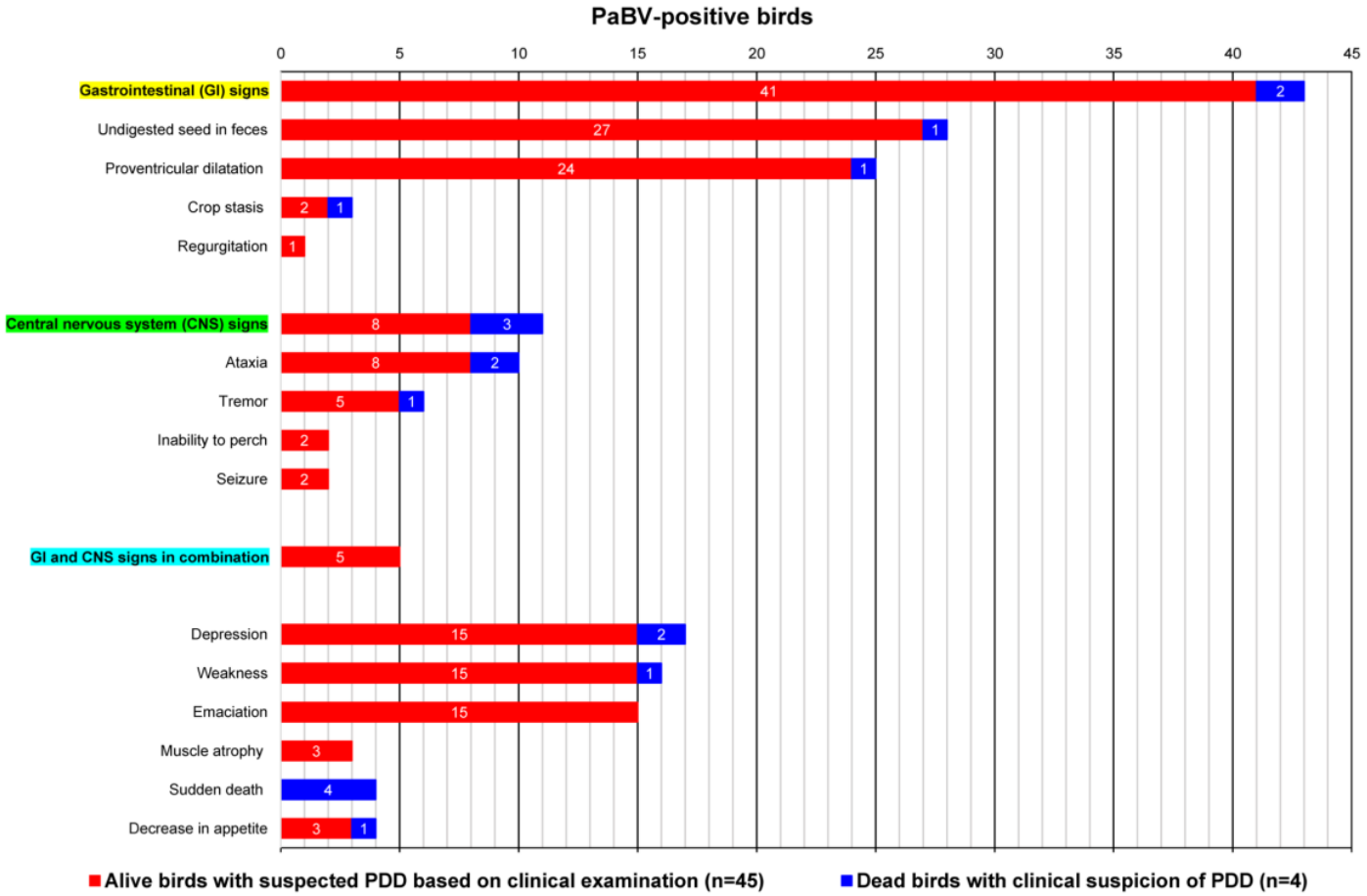


Fig 4. Clinical signs found in PaBV-affected parrots in Thailand.

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; WEISSENBÖCK et al., 2009b; STAEHELI et al., 2010) and an ELISA (REUTER et al., 2010; HÖGEMANN et al., 2017). 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 (WEISSENBÖCK et al., 2009b; PHILADELPHO et al., 2014), 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 4). 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 (KISTLER et al., 2008; HORIE et al., 2012; GUO & TIZARD, 2015; MARTON et al., 2015; KOMORIZONO et al., 2016), 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 (WEISSENBÖCK et al., 2009b; STAEHELI et al., 2010; RUBBENSTROTH et al., 2012; RUBBENSTROTH et al., 2016), 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, 1B, 1C) 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 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, 1B, 1C). 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 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 (PIEPENBRING et al., 2012; SASSA et al., 2013;

PHILADELPHO et al., 2014; PIEPENBRING et al., 2016). 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 (VILLANUEVA et al., 2010; HEFFELS-REDMANN et al., 2011; PIEPENBRING et al., 2012; PIEPENBRING et al., 2016; LEAL DE ARAUJO et al., 2017b) 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., 2012; RUBBENSTROTH et al., 2014a; PIEPENBRING et al., 2016). 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.

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IV. DISCUSSION

In this study we were able to describe for the first time the occurrence of PaBV not only in Thailand but 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 may, 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; WEISSENBÖCK et al., 2009b; STAEHELI et al., 2010) and an ELISA (REUTER et al., 2010; HÖGEMANN et al., 2017). 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. The sample collection has a pivotal role in overall accuracy of the test. Although plucked, contour feathers have been considered as an excellent source of ABV RNA detection of RT-PCR. The presence of RT-PCR inhibitors may also lead to false negative result anyway (DE KLOET et al., 2011). To prevent that situation, samples from crop and cloacal swabs in live birds were collected and pooled. In agreement with former studies (WEISSENBÖCK et al., 2009b; PHILADELPHO et al., 2014), 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 4). 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).

Serological analysis by ELISA showed the presence of bornavirus-reactive antibodies. Since this method can identify any psittacine birds with an infection, ELISA was the headmost diagnostic method in this present study. Intriguingly, there were three RT-PCR-positive birds that were negative for ELISA test. Two of them were clinically healthy cage mates of PaBV positive birds. These findings of positive RT-PCR but negative serology may occur in early stages of PaBV infection, as was shown in PaBV-4 experimental infection studies (PIEPENBRING et al., 2012; PIEPENBRING et al., 2016). False positive reactions caused by contaminations cannot completely be excluded but were not supported by extraction and PCR negative controls which always accompanied the tests. Notwithstanding, when the RT-PCR failed to detect PaBV infection, serological detection of anti-bornaviral protein antibodies also can be used to identify an exposure and *vice versa*.

With regard to qRT-PCR assay in this study, the primers were able to specifically detect PaBV-4 (HONKAVUORI et al., 2008). Although the sensitivity of qRT-PCR appeared to be greater than conventional RT-PCR, it was surprising that in this current study, only 14 birds reacted positive in the qRT-PCR. The false-negative results in this study might be induced by inhibition of the qRT-PCR assay, a low amount of target RNA and/or kind of sample used for extracting RNA. In addition, there might be some mismatches in the primer or probe region of the strains circulating in Thailand leading to a reduction in sensitivity of the real-time PCR protocol of Honkavuori et al (2008) used in this study.

Partial L, M, and N gene sequences derived from RT-PCR products revealed a genetic variation among PaBV circulating 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*, was found in a blue and yellow macaw (*Ara ararauna*) revealing severe dilatation of proventriculus during necropsy. This virus was detected only in three countries around the world up to now, in birds in Japan, Hungary and the

United States of America (KISTLER et al., 2008; HORIE et al., 2012; GUO & TIZARD, 2015; MARTON et al., 2015; KOMORIZONO et al., 2016). As described in several studies from other countries (WEISSENBÖCK et al., 2009b; STAEHELI et al., 2010; RUBBENSTROTH et al., 2012; RUBBENSTROTH et al., 2016), PaBV-4 belonging to the species Psittaciform 1 orthobornavirus was the most prevalent 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, 1B, 1C) demonstrated a genetic heterogeneity of PaBV-4 viruses which were distributed in two (1 and 2) of the five genetic clusters previously identified for PaBV-4 (RUBBENSTROTH et al., 2016).

The apparently lacking association with geographic origin which had been found in other studies as well (WEISSENBÖCK et al., 2009b; RAGHAV et al., 2010) 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, 1B, 1C). 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. When the results of BLASTn and BLASTp analysis are additionally considered (Table 4), a classification of strain 16021 into PaBV-5 is also supported by the orthobornavirus species demarcation criteria proposed by Marton et al. (2015) “i) greater than 75 % nt sequence identity can be observed between strains belonging to the same species, ii) 60-71 % nt identity values characterize strains belonging to different species but the same genus, iii) and less than 60 % identity is considered to be the cutoff value to demarcate viruses into different virus genera.” (MARTON et al., 2015). 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 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 (PIEPENBRING et al., 2012; SASSA et al., 2013; PHILADELPHO et al., 2014; PIEPENBRING et al., 2016). 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 showing PDD-like disease 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. Our results emphasize and support the findings of former studies (VILLANUEVA et al., 2010; HEFFELS-REDMANN et al., 2011; PIEPENBRING et al., 2012; PIEPENBRING et al., 2016; LEAL DE ARAUJO et al., 2017b) 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., 2012; RUBBENSTROTH et al., 2014b; PIEPENBRING et al., 2016). In addition, these results show that removing of birds with diagnosed PaBV infection from aviaries is

a reasonable measure for flock sanitation.

As aforementioned, there are seven indigenous psittacine species in Thailand. 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). These species are vulnerable to endangerment in the near future. It is not known whether PaBV can spread between captive parrots and wild parrots, and the diagnosis, prevention and control of PaBV infection are necessary as a matter of urgency. The results of this study provide a basis for further research, and they illustrate, that techniques for PaBV diagnostic may play a critical role to investigate the infection status of wild parrots and may be an initial step for further developments to maintain and improve the conservation status of these species.

In conclusion this 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 this 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 lead to an increased knowledge on occurrence and relevance of PaBV-5 as a pathogen for psittaciform birds. This study would also pave the way for veterinarians in Thailand and Southeastern Asia to further investigate on PaBV and PDD in captive and wild psittaciform birds.

V. SUMMARY

In Thailand, a PDD-like syndrome has commonly been found in captive psittacine birds. The etiology, however, still remained unclear, especially whether PaBV is actually the causal agent, and investigations to detect parrot bornaviruses have never been performed in Southeastern Asia so far. Importantly, the measures that are appropriate to prevent spreading of PaBV and PDD in Thailand, especially for the five near-threatened indigenous parrot species, have not been applied yet. Also, the therapy of diseased birds is restricted to palliative treatment which may not address the real need for the patients.

In order to identify PaBVs as the underlying cause of PDD-like disease in Thailand, the standard diagnostic protocol for identifying PaBV infections of the Clinic for Birds, Small Mammals, Reptiles and Ornamental Fish, LMU Munich, Germany, was applied to detect PaBVs in Thailand including RT-PCR targeting conserved regions of the polymerase (L), matrix (M), and nucleoprotein (N) genes, qRT-PCR and ELISA. From June 2016 to January 2017, diagnostic samples of 111 psittacine birds in Thailand comprising PDD-suspected birds based on clinical examination (n = 65), cage mates of PDD-suspected parrots without any clinical signs (n = 39) and dead birds, all by natural death, and with clinical or postmortem suspicion of PDD (n = 7) were collected and examined for the presence of bornaviruses at the Exotic Pet Clinic, Kasetsart Veterinary Teaching Hospital and Diagnostic Center, the Faculty of Veterinary Medicine, Kasetsart University, Thailand. The results of the investigations revealed, for the first time, the occurrence of PaBV in Thailand, and even in Southeastern Asia. Bornaviral infections were detected in 60 (54.1 %) of 111 psittacine birds, and were highly associated with PDD-like disease which was statistically analyzed by the chi-square test. Gastrointestinal (GI) signs, the most common signs found in this study, central nervous system (CNS) signs and combinations of GI and CNS signs were found in 49 (71.7 %) of 60 positive birds. However, interestingly, there were only 11 (28.2 %) clinically healthy cage mates that were positive in at least one diagnostic test thus suggesting inefficient horizontal virus transmission from naturally infected birds.

In order to further characterize PaBVs in Thailand, extracts of PCR products were sent to a commercial laboratory in order to determine nucleotide sequences of

bornavirus L, M and N gene fragments. The obtained sequences were analyzed by using BLAST and compared with Psittaciform bornavirus species and other sequences stored in the GenBank database. The results demonstrated that both bornavirus species described so far in parrots, *Psittaciform 1 orthobornavirus* (PaBV-2 and -4) and *Psittaciform 2 orthobornavirus* (PaBV-5), were detected in Thailand. Phylogenetic analyses were also performed and demonstrated a high genetic heterogeneity known from viruses in other parts of the world. In order to further characterize the suspected PaBV-5, whole genome sequencing was subsequently performed. cDNA of the suspected PaBV-5 was used as a template for RT-PCR assays using newly designed primers. The achieved genomic sequences were assembled and the full genome sequence was analyzed and compared with bornavirus sequences deposited in GenBank. The full genome sequence of the PaBV-5 strain from Thailand revealed a nucleotide identity of 91 % to three full-genome sequences of PaBV-5 strains reported so far. A short uORF in the N/X intergenic region found only in PaBV-5 viruses so far was also detected in the PaBV-5 strain from Thailand.

In order to confirm the presence of common histopathological and immunohistochemical features of PDD in psittacines in Thailand, histopathology and immunohistochemical investigations were performed as an additional diagnostic method by collecting various organ samples from three representatives, two birds with clinical signs infected with PaBV-2 and -4 and one bird with a postmortem diagnosis of PaBV-4 infection. The characteristic pathologic and histopathologic lesions, the lymphoplasmacytic infiltrations, and as well as the bornavirus antigen in diverse tissues altogether confirmed that birds in Thailand showing PDD-like disease are infected with PaBV and can possibly die due to PDD.

In summary, these results revealed the occurrence of two psittaciform bornavirus species and of PDD in captive psittacine birds in Thailand and Southeastern Asia. The application of different diagnostic tests was shown to be useful to avoid false negative results and to detect distantly related species. The diagnostic protocol to identify PaBV in psittaciform birds is to be established and used as a routine diagnostic protocol in veterinary clinics in Thailand. Furthermore, this study also provided information and a better understanding of PaBV, particularly of PaBV-5.

VI. ZUSAMMENFASSUNG

Erstnachweis und Charakterisierung von Bornaviren bei natürlich infizierten und klinisch erkrankten Papageienvögeln in Thailand

Phirawich Sa-Ardta

In Thailand wird ein Proventricular Dilatation Disease (PDD)-ähnliches Syndrom bei in Gefangenschaft gehaltenen Papageienvögeln häufig beobachtet. Die Ätiologie war bisher jedoch unklar und Untersuchungen zum Nachweis von Papageien-Bornaviren (PaBV) wurden bisher in Südostasien noch nicht durchgeführt.

Um PaBVs als Ätiologie der PDD-ähnlichen Erkrankung in Thailand zu identifizieren, wurde das Standard-Diagnostikprotokoll zum Nachweis von PaBV-Infektionen der Klinik für Vögel, Kleinsäuger, Reptilien und Zierfische der Tierärztlichen Fakultät der LMU München, Deutschland, in Thailand angewendt, welches eine RT-PCR, die auf konservierte Regionen der Polymerase (L), Matrix (M) und Nukleoprotein (N) Gene gerichtet ist, eine qRT-PCR und einen ELISA zum Nachweis von Antikörpern umfasst. Von Juni 2016 bis Januar 2017 wurden diagnostische Proben von 111 Papageienvögeln (22 Spezies aus 2 Familien) in Thailand gesammelt. Dabei wurden PDD-verdächtige Vögel (n = 65), Käfiggenossen der PDD-verdächtigen Papageien, die selbst keine klinischen Symptome zeigten (n = 39) und tote Vögel, die eines natürlichen Todes gestorben waren und bei denen anhand der klinischen Symptomatik oder aufgrund der postmortalen Untersuchung ein Verdacht auf PDD bestand (n = 7), in die Untersuchungen mit einbezogen. Diese Proben wurden in der Exotic Pet Clinic des Kasetsart Veterinary Teaching Hospital and Diagnostic Center der Tierärztlichen Fakultät der Kasetsart Universität, Thailand, auf Vorhandensein von Bornaviren untersucht. Anhand der vorliegenden Untersuchungsergebnisse konnte erstmals das Auftreten von PaBV in Thailand bzw. sogar erstmals das Auftreten in Südostasien aufgezeigt werden. Bornavirus-Infektionen wurden bei 60 (54,1 %) von 111 untersuchten Papageienvögeln nachgewiesen. Die statistischen Analysen (Chi-Quadrat-Test) belegten einen engen Zusammenhang eines PaBV-Nachweises mit einer PDD-ähnlichen Erkrankung. Gastrointestinale (GI-) Symptome, welche in der voliegenden Studie am häufigsten vorkamen, Symptome des zentralen

Nervensystems (ZNS) und Kombinationen von GI- und ZNS-Symptomen wurden bei 49 (71,7 %) der 60 positiven Vögeln gefunden. Interessanterweise wurde nur bei 11 (28,2 %) der klinisch gesunden Kontakttiere im Käfig durch mindestens einen positiven diagnostischen Test eine Infektion mit Bornaviren nachgewiesen. Dies lässt auf eine ineffiziente horizontale Übertragung des Virus durch natürlich infizierte Vögel schließen.

Analysen von Nukleotidsequenzen der in der konventionellen PCR erhaltenen Bornavirus-L-, M- und N-Genfragmente unter Verwendung von BLAST unter Einbeziehung der GenBank-Datenbank zeigten, dass beide bisher bei Papageien beschriebenen Bornavirus-Arten, *Psittaciform 1 orthobornavirus* (mit den Viren PaBV-2 and PaBV-4) und *Psittaciform 2 orthobornavirus* (mit dem Virus PaBV-5), in Thailand vorkamen. Die phylogenetischen Analysen zeigten eine hohe genetische Heterogenität, wie sie von Viren in anderen Teilen der Welt bekannt ist. Zur weiteren Charakterisierung eines PaBV-5-Virus, das bisher nur in drei Ländern der Welt nachgewiesen worden war, wurde eine Gesamtgenomsequenzierung durchgeführt. Die vollständige Genomsequenz des PaBV-5-Stammes aus Thailand zeigte eine Nukleotididentität von 91 % mit den drei bisher bekannten Vollgenomsequenzen von PaBV-5-Stämmen.

Zum Nachweis von für die PDD typischen Merkmalen bei Papageienvögeln in Thailand wurden histopathologische und immunhistochemische Untersuchungen durchgeführt. Dabei wurden verschiedene Organproben von drei Vögeln beispielshaft untersucht. Zwei Vögel mit klinischen Symptomen waren mit PaBV-2 und -4 infiziert und bei einem Vogel war postmortal die Diagnose einer PaBV-4-Infektion gestellt worden. Der Nachweis charakteristischer pathologischer und histopathologischer Läsionen, lymphoplasmatischer Infiltrationen sowie von Bornavirus-Antigen in verschiedenen Geweben bestätigten, dass Vögel mit einer PDD-ähnlichne Erkrankung in Thailand mit PaBV infiziert sind und durch PDD sterben können.

Diese Untersuchung zeigte damit zum ersten Mal das Vorkommen von zwei psittaziformen Bornavirus-Arten und von PDD bei von Menschen gehaltenen Papageienvögeln in Südostasien. Zukünftige Untersuchungen zum Infektionsstatus von wildlebenden Papageien, insbesondere der fünf in Thailand vorkommenden potentiell gefährdeten („near-threatened“) Arten, sind dringend erforderlich. Basierend auf den Ergebnissen dieser Studie sollten Techniken zur PaBV-

Diagnostik als Routinediagnostik in Tierkliniken etabliert werden, um auf diese Weise eine geeignete, valide Monitoringsituation zur Identifizierung von Volieren mit PaBV-Infektionen in Thailand sowie Voraussetzungen zur Etablierung geeigneter Kontroll- und Hygienemaßnahmen zu schaffen.

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VIII. APPENDIX

Appendix A. Taxonomy of Family *Bornaviridae*

Genus	**Species**	**Virus**
Carbovirus	*Queensland carbovirus*	jungle carpet python virus (JCPV)
	Southwest carbovirus	southwest carpet python virus (SWCPV)
Orthobornavirus	*Elapid 1 orthobornavirus*	Loveridge's garter snake virus 1 (LGSV-1)
	Mammalian 1 orthobornavirus	Borna disease virus 1 (BoDV-1)
		Borna disease virus 2 (BoDV-2)
	Mammalian 2 orthobornavirus	variegated squirrel bornavirus 1 (VSBV-1)
	Passeriform 1 orthobornavirus	canary bornavirus 1 (CnBV-1)
		canary bornavirus 2 (CnBV-2)
		canary bornavirus 3 (CnBV-3)
	Passeriform 2 orthobornavirus	estrildid finch bornavirus 1 (EsBV-1)
	Psittaciform 1 orthobornavirus	parrot bornavirus 1 (PaBV-1)
		parrot bornavirus 2 (PaBV-2)
		parrot bornavirus 3 (PaBV-3)
		parrot bornavirus 4 (PaBV-4)
		parrot bornavirus 7 (PaBV-7)
	Psittaciform 2 orthobornavirus	parrot bornavirus 5 (PaBV-5)
	Waterbird 1 orthobornavirus	aquatic bird bornavirus 1 (ABBV-1)
		aquatic bird bornavirus 2 (ABBV-2)

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