Prevalence of Psittacine Beak and Feather Disease and *Avian Polyomavirus* disease Infection in Captive Psittacines in the Central part of Thailand by Multiplex Polymerase Chain Reaction

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Abstract

Psittacine beak and feather disease and avian polyomavirus disease are the contagious viral diseases in psittacines that have similar clinical manifestations, characterized by feather disorder. To investigate the prevalence of Psittacine beak and feather disease virus (BFDV) and Avian polyomavirus (APV) in captive psittacines in the central part of Thailand, samples were collected from 124 psittacines (10 genera, 20 psittacine species) between May to October, 2006, interval. BFDV and APV were detected from dry blood spot and feather bulb samples using multiplex-polymerase chain reaction (Multiplex-PCR). The result showed that 64.5 percent (80/124) of the samples were positive to BFDV, 8.1 percent (10/124) were positive to APV, and 7.2 percent (9/124) were positive to BFDV and APV. Moreover, 81.2 percent (65/80) of BFDV infected birds showed asymptomatic infection ($P<0.001$). However, there was no significant difference between symptomatic and asymptomatic infection of APV. In addition, the feather bulb is more suitable than dry blood spot for BFDV detection with this method. However, both sample types are appropriated for APV detection.

Keywords: *Avian polyomavirus* (APV), Multiplex-polymerase chain reaction (Multiplex-PCR), Psittacines, Psittacine beak and feather disease virus (BFDV)

การสำรวจความชุกของเชื้อ Psittacine Beak and Feather Disease Virus และเชื้อ *Avian Polyomavirus* ในกลุ่มนกตระกูลปากขอในเขตพื้นที่ภาคกลางของประเทศไทย โดยวิธี Multiplex Polymerase Chain Reaction

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บทคัดย่อ

โรคขนและจงอยปากผิดปกติ และโรคโปลีโอมาเป็นโรคติดเชื้อไวรัสในวงค์นกแก้ว ซึ่งมีลักษณะทางคลินิกคล้ายกัน โดยเฉพาะความผิดปกติของขน เพื่อเป็นการสำรวจหาความชุกของเชื้อไวรัสก่อโรคขนและจงอยปากผิดปกติและไวรัสก่อโรคโปลีโอมาในวงค์นกแก้ว ในเขตพื้นที่ภาคกลางของประเทศไทย คณะผู้วิจัยได้ทำการเก็บตัวอย่างนกปากขอสายพันธุ์ต่างๆ ตั้งแต่เดือนพฤษภาคม ถึง ตุลาคม พ.ศ. 2549 รวม 10 วงค์ 20 สายพันธุ์ จำนวน 124 ตัว และทำการตรวจหาเชื้อไวรัสก่อโรคขนและจงอยปากผิดปกติและไวรัสก่อโรคโปลีโอมา จากเลือดและตุ่มขนของนกโดยวิธีปฏิกิริยาลูกโซ่โพลีเมอร์เรส จากผลการทดลองพบว่าจากตัวอย่างทั้งหมดที่ทำการสำรวจ พบนกที่ติดเชื้อ BFDV คิดเป็นร้อยละ 64.5 (80/124) นกติดเชื้อ APV คิดเป็นร้อยละ 8.1 (10/124) และมีการติดเชื้อร่วมกันร้อยละ 7.2 (9/124) โดยพบว่าร้อยละ 81.2 (65/80) ของนกที่ตรวจพบเชื้อ BFDV เป็นนกที่ไม่แสดงอาการ ($P<0.001$) ในขณะขณะที่นกที่พบการติดเชื้อ APV ไม่พบความแตกต่างอย่างมีนัยสำคัญระหว่างนกที่แสดงอาการและไม่แสดงอาการ นอกจากนี้ยังพบว่าตัวอย่างตุ่มขนมีความเหมาะที่จะใช้ตรวจหาเชื้อ BFDV อย่างไรก็ตามพบว่าทั้งตัวอย่างที่เป็นตุ่มขนและเลือดมีความเหมาะสมสำหรับการตรวจหาเชื้อ APV

คำสำคัญ : โรคโปลีโอมา ปฏิกิริยาลูกโซ่โพลีเมอร์เรส โรคขนและจงอยปากผิดปกติ

Introduction

Psittacine beak and feather disease is caused by psittacine beak and feather disease virus (BFDV). This virus is a non-enveloped icosahedral DNA virion with a diameter of 14-17 nm and belongs to a family *Circoviridae* (Ritchie et al. 1989). It is prosesed ambisense single-strand circular DNA with a genome size of approximately 2,000 nucleotides. BFDV genome consists of 7 Open reading frames (ORFs). However, only ORF1, ORF2 and ORF5 can be detected from all of BFDV isolates (Ritchie et al. 1989; Bassami et al. 1998; Bassami et al. 2001). BFDV cause feather dystrophy and beak deformities due to it can infect and replicate in the epithelial cell (Pass and Perry 1984) and also cause immunosuppression by infecting the thymus and the bursa, so the infected birds died from a secondary infection with bacteria and fungus (Ritchie et al. 1989; Adair 2000). Latently infected birds cause a severe environmental contamination because the transmission is occurred via feather dust and fecal particle (Ritchie et al. 1991). BFDV was first recognized in Australia (Pass and Perry 1984). Subsequently, the infection has been reported in Germany (Rahaus and wolff 2003), England (Baker 1996), Indonesia and Philippine (Ritchie 1995). Epidemiological study in Thailand showed

5% prevalence (Heng et al. 2003), while studies in other countries showed 8% prevalence in Italy (Bert et al., 2005), 5% prevalence in USA, 39.2% in Germany (Rahaus and Wolff, 2003), 3% in New Zealand (Dahlhausen and Radabaugh, 1997), and 25.3% in Poland (Piasecki and Wieliczko, 2010)

Avian Polyomavirus (APV) is classified into genus *Polyomavirus* subgenus *Avipolyomavirus* (Stoll et al. 1993). APV is a non-enveloped, icosahedral double-stranded circular DNA virion with a diameter of 45 nm and 4981 nucleotides in genome length (Dykstra and Bozeman 1982). APV genome composes of 2 parts, an early region that encode large tumor antigen (T) and small tumor antigen (t), and a late region which encode three structural proteins including, VP1, VP2 and VP3, respectively (Rott et al. 1988). Several reports indicated that APV infection causes vary symptoms, depended on the species infected as well as the age of bird at the time of infection. The previous study revealed 100% mortality in infected young bird and most of the birds died without premonitory signs of disease (Krautwald et al. 1989). The study in Italy showed 0.8% prevalence of APV infection (Bert et al. 2005), while 22.4% prevalence of APV and 6% prevalence of co-infection of APV and BFD were revealed in Poland (Piaski and Wieliczko 2010).

Due to similar feather disorders of Psittacine beak and Feather disease and Avian polyoma virus dusaese, clinical diagnosis cannot differentiate between these diseases. Therefore, laboratory diagnoses, such as immunohistochemistry (Sanada et al. 1999), *in situ* hybridization (Ramis et al. 1994) and electron microscopy (Sanada et al. 1999), are important tools for a definitive diagnosis. These methods are useful for the individual bird, but not suitable for routine numerous samples diagnostic screening. Haemagglutination and haemagglutination inhibition assays are simple procedures which routinely used in the diagnosis of psittacine beak and feather disease, however, these methods are limited due to suitable erythrocytes for different species of birds (Raidal et al. 1993). The detection of anti-BFDV antibody was also reported, however, this antibody cannot distinguish between infected birds and convalescent birds (Shearer et al., 2009a). Several problems can be solved by Polymerase chain reaction (PCR), which is suitable for large-scale diagnostic screening for BFDV and APV infection (Ypelaar et al. 1999; Phalen et al. 1991). Recently, the real-time PCR has been developed and showed high sensitivity (Shearer et al., 2009b). However, this method was required the special machine and spent high cost. Therefore, the aim of this study was to investigate the prevalence of PBFD and APVD in captive psittacines in the Central part of Thailand by Multiplex PCR and may help in establishing a control measurement of those diseases in Thailand.

Materials and Methods

Sample collection and DNA isolation

Between May to October, 2006, 124 of adult psittacine birds (10 genera and 20 species) were randomly selected from three zoos and three psittacine farms in Bangkok, Ratchaburi and Suphanburi province. Feather bulbs and 50 μl of blood samples were collected. Blood sample was collected as a dry blood spot by spotted and dried onto Whatman No. 1. The clinical status of both diseases was recorded. Clinical signs of these diseases included abnormal development of new feathers and abnormal beak elongation.

DNA isolation from dry blood spot was described by Sambrook and others (Sambrook at al. 1989). Briefly, a 2-3 pieces of dry blood spot were transferred into lysis buffer solution (0.1 M NaCl, 10 mM Tris-Cl (pH 8.0), 5% SDS) and 0.6 mg/ml Proteinase K then incubated at 56°C for 4 hours. Subsequently, phenol: chloroform: isoamylalcohol (25:24:1 v/v) extraction and DNA precipitation with absolute ethanol and 3 M sodium acetate. Pellet DNA was resuspended in TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0). For feather bulb samples, DNA was isolated according to a Rapid Alkaline lysis

method (Stoll et al. 1993). Briefly, feather bulbs were cut by the sterile blade into 2-3 mm and the blade must be disinfected between samples by framing. Pieces of feather bulbs were dropped into 20 µl of 0.2 N NaOH and incubated in water bath at 75°C for 20 minutes. After that, 0.04 M Tris-HCl (pH 7.5) 180 µl were added and the supernatant was transferred into a new eppendorf tube and used as a template for Multiplex-PCR.

Multiplex-PCR amplification

Multiplex-PCR in this study was modified from a single PCR system and the primers were listed in Table1. For amplification of the following conditions in a total volume of 50 µl reaction, 1x Multiplex PCR master mix (QIAGEN, Germany), BFDV and APV primers at a concentration of 0.2 µM, and 12s rDNA at a concentration of 0.1 µM were used. A MJ PCT200 thermocycler was used for initial denaturing at 95°C for 15 minutes, then 35 cycles with 45 seconds for denaturation at 94°C, 45 seconds annealing at 60°C and 1 minute extension at 72°C followed by a final extension step 3 minutes at 72oC. PCR products were separated on 2.0% agarose gel and stained with 0.5 µg/ml ethidium bromide. DNA bands were visualized by UV transluminator. The expected size for BFDV, APV, and internal control was 717, 550, and 436 bp, respectively. All of tested samples must be detected the internal control specific band.

Sensitivity and specificity of the Multiplex PCR

To determine the sensitivity of the test, DNA from BFDV and APV positive sample and 12S rDNA from bird were amplified by separated PCR. The BFDV (GenBank accession number FJ685978) was obtained from the Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animal (MOZWE), Faculty of Veterinary Science, Mahidol University, Thailand. APV was kindly provided from the Faculty of Veterinary Science, Chulalongkorn University. The PCR products were purified and calculated the copy number. Each of purified DNA products was mixed in an equal DNA copy and diluted in 10-fold serial dilutions from 105 to 100. These dilutions were used as a template for Multiplex-PCR to analyze the sensitivity. Amplification condition of the Multiplex PCR used as described above.

For specificity of test, the 10 negative BFDV and APV samples from PCR using other primer pairs (determined by MOZWE) were used as the negative controls for this Multiplex-PCR.

Statistical analysis

A Chi-square statistic and McNemar test were used for the statisyical analysis.

Results

Sensitivity of the Multiplex PCR

To determine the sensitivity of Multiplex-PCR, amounts of DNA from purified PCR product were diluted in serial 10-fold dilution and used in the Multiplex-PCR in a given conditions as given above. Although a non-specific band at 210 bp was found, however, it was not interfered the sensitivity of this test. An amplified product could be detected after stained with ethidium bromide when the original reaction mixture contained as few as 100 copies (0.713 fg) of BFDV and 10 copies (0.054 fg) of APV (Fig. 1). To control the false negative results, due to either the inhibition of PCR or the total absence of extracted DNA, a portion of the avian mitochondrial 12S rDNA gene was also amplified and generated PCR product 436 bps in size.

To determine the specificity of test, the negative BFDV and APV DNA sample from birds were detected using Multiplex-PCR. From figure 2, all of 10 negative control samples showed negative result when determined by Multiplex-PCR. The result indicated that the Multiplex-PCR developed in this study gave a high (100%) specificity.

Prevalence of BFDV and APV infection

To investigate the prevalence of BFDV and APV infection, 248 samples from 124 psittacines (10 genera, 20 species) were tested with Multiplex PCR and the results were showed in Table 1. All positive samples were tested twice and all of them showed clear results. A total of 80 of 124 birds samples were positive to BFDV, revealed 64.5% prevalence. Only 15 of 80 infected birds (18.8%) showed clinical signs, such as feather dystrophy and beak lesion, while most of them (81.2%) did not show any symptom (P<0.001) (Table 3). Most of the birds, which were susceptible to BFDV infection, belong to genus *Prosbosciger, Psittacula* and *Ara* (Table. 2).

Ten birds out of 124 (8.1% prevalence) were positive for APV infection and most of them belonged to genus *Ara, Psittacus* and *Psittacula* (Table. 2). However, APV infected birds did not show significantly difference among symptomatic and asymptomatic groups. Among BFDV and APV infected birds, 9 birds (7.2%) were co-infection (Table 2).

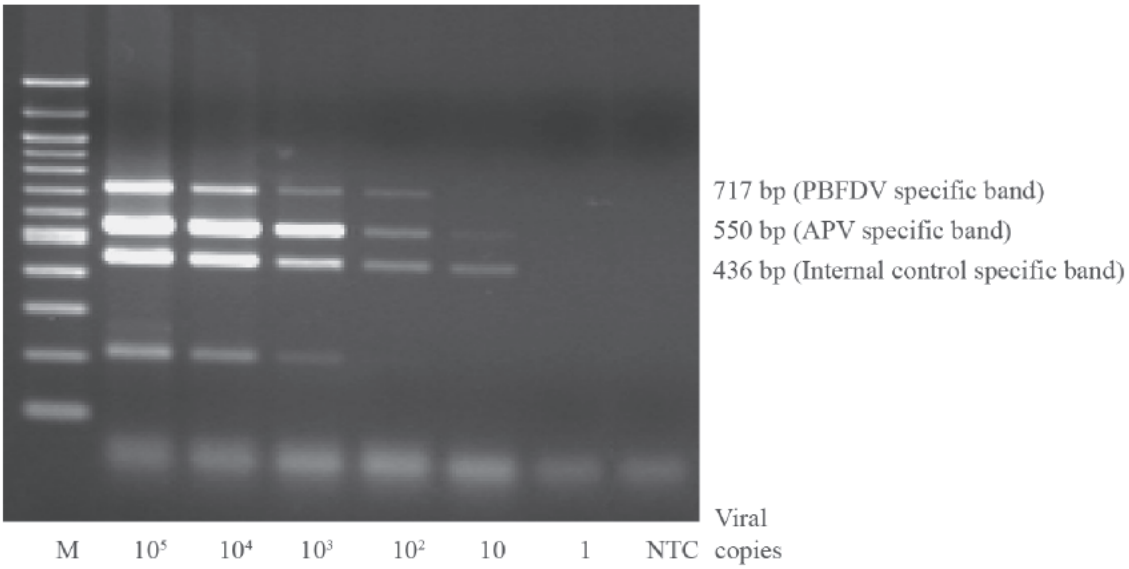

Figure 1 Determination of sensivity of Multiplex-PCR. Fifteen microliters of each reaction mixture was separated on 2.0% agarose gel and stained with ethidium bromide. The viral genome copies added to the initial reaction mixure are indicated as number as below figure. NTC; No template control, M; 100 bps DNA ladder.

Table1. Oligonucleotide primers used in multiplex PCR

Specific gene	Primers	Sequences (5' to 3')	Length (bases)	Size of products
BFDV *rep* genefl	pBFDV-F§	CTTATGTGGGAGGCTGCAGTGTT	23	717
	pBFDV-R§	TACTGAAATAGCGTGGTAGGCCTC	24	
APV VP1 gene	APV-mul-FΨ	AACCCTACAGACGGCGAG	18	550
	APV-mul-RΨ	GTCACAGTCCTCCTTGTACC	20	
12S rDNA gene	12S rDNA-Fφ	GGATTAGATACCCCACTATGC	21	436
	12S rDNA-Rφ	AGGGTGACGGGCGGTATGTACG	22	

§The primers described by Ypelaar and others (Ypelaar et al. 1999); Ψprimers described by Phalen and others (Phalen et al. 1991); φprimers described Miyaki and others (Miyaki et al. 1998).

Table 2. Genera of psittacine bird were detected for PBFDV and APV by using Multiplex PCR

Genus	Number of birds	BFDV		APV		Co-infection
		Positive	Negative	Positive	Negative	
Ara	12	11 (91.7%)	1 (8.3%)	3 (25%)	9 (75%)	3 (25%)
Psittacus	6	2 (33.3%)	4 (66.7%)	1 (16.7%)	5 (83.3%)	1 (16.7%)
Eclectus	8	7 (87.5%)	1 (12.5%)	0 (0%)	8 (100%)	0 (0%)
Cacatua	13	10 (76.9%)	3 (23.1%)	0 (0%)	13 (100%)	0 (0%)
Probosciger	3	3 (100%)	0 (0%)	0 (0%)	3 (100%)	0 (0%)
Psittacula	32	30 (93.7%)	2 (6.3%)	4 (12.5%)	28 (87.5%)	4 (12.5%)
Trichoglossus	2	1 (50%)	1 (50%)	0 (0%)	2 (100%)	0 (0%)
Agapornis	26	16 (61.5%)	10 (38.5%)	1 (3.8%)	25 (96.2%)	1 (3.8%)
Aratinga	17	0 (0%)	17 (100%)	0 (0%)	17 (100%)	0 (0%)
Lorius	5	0 (0%)	5 (100%)	1 (20%)	4 (80%)	0 (0%)
Total	**124**	**80 (64.5%)**	**44 (35.5%)**	**10 (8.1%)**	**114 (91.9%)**	**9 (7.2%)**

Table 3. Relationship of clinical sign and no clinical sign

Genus	PBFDV Positive (n = 80)		APV Positive (n = 10)	
	Clinical sign[a]	No clinical sign	Clinical sign	No clinical sign
Ara	3 (27.3%)	8 (78.7%)	1 (33.3%)	2 (66.7%)
Psittacus	1 (50%)	1 (50%)	0 (0%)	1 (100%)
Eclectus	5 (71.4%)	2 (28.6%)	0 (0%)	0 (0%)
Cacatua	2 (20%)	8 (80%)	0 (0%)	0 (0%)
Probosciger	0 (0%)	3 (100%)	0 (0%)	0 (0%)
Psittacula	4 (13.3%)	26 (86.7%)	0 (0%)	4 (100%)
Trichoglossus	0 (0%)	1 (100%)	0 (0%)	0 (0%)
Agapornis	0 (0%)	16 (100%)	0 (0%)	1 (100%)
Aratinga	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Lorius	0 (0%)	0 (0%)	1 (100%)	0 (0%)
Total	**15 (18.8%)**	**65 (81.2%)**	**2 (20%)**	**8 (80%)**

[a] clinical sign corresponding to feather dystrophy and beak lesion

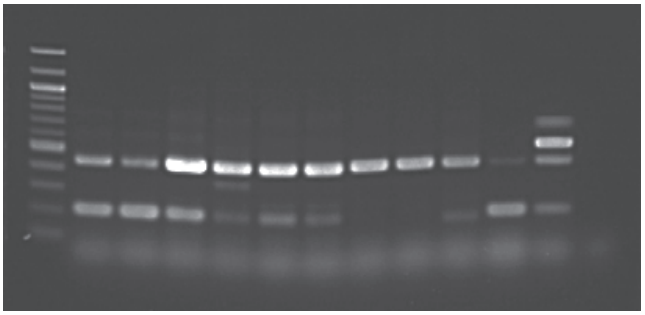

Figure 2 Determination of specificity of Multiplex-PCR. Lane1; 100 bps DNA ladder, lane2-11; Negative control, lane12; Positive control, lane13; No template control.

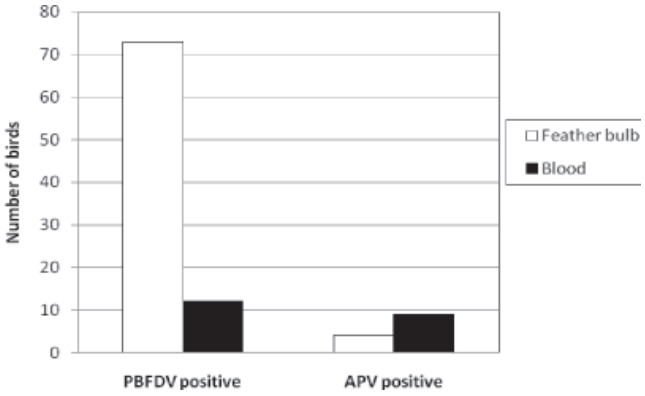

Figure 3 Comparison of suitable sample type between feather bulb and blood sample for PBFDV and APV detection. *Significant difference with $P<0.001$.

Comparison of sample type for BFDV and APV detection

To investigate the appropriate sample for BFDV and APV detection with Multiplex PCR, feather bulb and blood sample were collected and compared for the sensitivity of the viral detection. Seventy-three of 80 (91.2%) psittacine birds positive for BFDV were detected from feather bulb, whereas only 12 (15%) could be detected from blood sample ($P<0.01$). This result indicated that the more suitable sample for PBFDV detection is feather bulb. In contrast, the data did not show significant difference between feather bulb and blood sample for APV detection (Fig. 3).

Discussion

Psittacine beak and feather disease and Avian polyomavirus disease are the most common recognized viral disease of wild and captive psittacines. In this study, we investigated the prevalence of BFDV and APV in captive psittacines in the central part of Thailand by Multiplex PCR. The sensitivity of the test for BFDV was higher than APV which was 100 copies (0.713 fg) and 10 copies (0.054 fg), respectively. However, both viruses detection showed the same specificity. Our results revealed 64.5 % prevalence of BFDV, and 8.1% prevalence of APV infection. This study showed a higher prevalence of BFDV infection than previous study (Heng et al., 2003) Our explanation is that all of our samples were collected from adult birds, which have known to have well developed immune system and trend to be subclinical infection (81.2%). According to several reports, sub-clinical or asymptomatic infected birds can be a reservoir for the diseases, spreading virus via feather dust and fecal content. Subclinical infection, together with the open farming system and poor bio-security, result in a high chance of infection in the same area (Ritchie et al., 1991; Stoll et al., 1993). This study emphasize that detection of subclinical infection is a very important part for disease control. Our study showed a high prevalence of both diseases compared to studies in other countries, suggested that the monitoring program, especially in breeding is crucial as well as isolation of infection individuals. Moreover, a strict bio-security system should be established in order to control those diseases in Thailand.

Our results also indicated that 3 genuses of psittacines (Probosciger, Psittacula and Ara) showed more than 90 percent prevalence of PBFDV infection. Anyhow, the sample size of some genuses were too small. The appropriate sample for detection of BFDV in this study was feather bulb, which is related to the previous study (Hess et al., 2004), due to the prediction sites of BFDV are tissue in stratum germinativum at feather follicle and also epidermal tissue (Pass and Perry, 1984). However, both

of feather bulb and blood samples were not significantly differ in the detection of APV.

Conclusion

Multiplex PCR developed in this study gave a high sensitivity and specificity. BFDV genome detection in the feather bulb samples was more sensitive than in blood samples in this method. However, for the APV detection, there was no significantly difference between feather bulbs and blood samples. Our results revealed a 64.5 prevalence of BFDV, 8.1 prevalence APV, and 7.2 prevalence of co-infection with a multiplex PCR. In addition, most of BFDV infected birds (81.2%) were asymptomatic infection, while there was no significant difference between a symptomatic and a asymptomatic infection of APV.

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