Original Article

Genetic Detection and Identification of *Chlamydophila psittaci* in Captive Psittacine Birds in Thailand

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Abstract

Chlamydophilosis is an important, worldwide zoonosis caused by *Chlamydophila psittaci*. The aim of this study was to detect *C. psittaci* on choanal and cloacal swabs which were collected from 178 captive psittacine birds (23 different species) from eight provinces in Thailand. *ompA* gene detection using nested PCR showed that 7.87% (n=14) were positive for *C. psittaci*. The *C. psittaci* detection from two site collections (choanal and cloacal swabs) using nested PCR was more sensitive than from one site collection. Most positive samples (n=11) were from asymptomatic adult birds and three other positive birds showing clinical signs were juvenile birds. As a result, the adult birds which showed no clinical signs could be the explanation for the spread of the disease. Nucleotide sequences for all positive samples were identified as genotype A. Most positive samples had identical nucleotide sequences with *C. psittaci* isolated from humans in Japan (accession number AB468956). This study demonstrated that *C. psittaci* genotype A could be found in both symptomatic and asymptomatic infections from the captive psittacine birds in Thailand. Therefore, disease monitoring is necessary to prevent and control disease for bird owners and aviculture in general.

Keywords: *Chlamydophila psittaci*, Chlamydophilosis, nested PCR, *ompA*, psittacine birds

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Introduction

Chlamydophilosis is a common yet important zoonotic disease which is caused by *Chlamydophila psittaci*, an obligate, intracellular, Gram negative bacterium. The common name for the disease in avian is "parrot fever". Regarding its various clinical manifestations, the 'parrot fever' can affect the respiratory system (rhinitis, sinusitis, dyspnea), the gastrointestinal system causing diarrhea and yellowish-green urate or systemic diseases (depression, anorexia and ruffled feathers) (Harkinezhad et al., 2009[a]; West, 2011). Clinical signs can be divided into asymptomatic, mild, severe, or chronic form (Andersen and Vanrompay, 2000; Harkinezhad et al., 2009[a]; Smith et al., 2011; Sudler et al., 2004). Chlamydial transmission routes are mainly ingestion of contaminated materials from respiratory secretions or feces of infected animals and inhalation of contaminated airborne particles such as feather dander and fecal dust (Longbottom and Coulter, 2003; Smith et al., 2011; West, 2011).

C. psittaci is a member of the *Chamydiaceae* family, *Chlamydophila* genus, and classified into at least 9 genotypes including: A, B, C, D, E, F, E/B, WC and M56 (Beeckman and Vanrompay, 2009; Everett, 2000; Geens et al., 2005; Harkinezhad et al., 2009[a]). The genotyping is classified by molecular assay based on the outer membrane protein A (*ompA*) gene, which was reported to have strain specific sequences (Sudler et al., 2004). Most of the chlamydial genotypes are revealed in avian groups, except WC and M56 genotypes, which are in mammals (Andersen, 1991; Harkinezhad et al., 2009[a]; Longbottom and Coulter, 2003). In the psittacine group, A, B, F and E/B genotypes have been reported (Andersen and Vanrompay, 2000; Beeckman and Vanrompay, 2009; Harkinezhad et al., 2009[a]; Harkinezhad et al., 2007; Piasecki et al., 2012).

Psittacine birds, also known as parrots, are the most popular pet birds in several countries and classified into Order Psittaciformes, which is recognized as the main reservoir of *C. psittaci* worldwide (Chahota et al., 2006; Vanrompay et al., 2007). The *C. psittaci* prevalence in captive psittacine birds from pet shops and private aviaries was 3.4 to 56.1% depending on the country (Dovc et al., 2005; Eidson, 2002; McElnea and Cross, 1999; Piasecki et al., 2012; Raso Tde et al., 2002; Sheleby-Elias et al., 2013). In Thailand, human patients with psittacosis were described (Riantawan and Nunthapisud, 1996) and genotype B of *C. psittaci* was detected by nested PCR technique from feral pigeons in central Thailand (Sariya et al., 2015). However, infection rates and genotypes of *C. psittaci* in captive psittacine birds are still unknown. Therefore, the knowledge of prevalence in pet parrots is necessary for molecular epidemiology and disease management recommendations. Thus, the aim of this report was to evaluate the *C. psittaci* infection rates and genotypes from captive psittacine birds in Thailand.

Materials and Methods

Examined Sampling: Choanal and cloacal swabs were collected from 178 samples including twenty-three different parrot species (Table 1) between June, 2013 and May, 2014 in central (Bangkok, Pathum Thani, Nonthaburi, Nakhon Pathom, Ratchaburi and Suphan Buri provinces) and eastern (Chon Buri and Rayong provinces) Thailand. Fourteen captive psittacine birds showed some clinical signs of 'parrot fever' including dyspnea, green-yellowish droppings, or conjunctivitis and 164 samples were non-clinical, captive psittacine birds. Historical data were recorded and included avian species, age (adult or juvenile), clinical status and living area. Each choanal and cloacal swab was collected from all birds using sterile cotton buds and was spun into 500 µl of *sterile phosphate buffer* saline (PBS) in a microtube. The samples were kept on ice during transit to Kasetsart Veterinary Diagnostic Unit and were stored at -20°C until used.

Polymerase Chain Reaction: Total DNA was extracted from the swab sample using NucleoSpin® Tissue Kits (Macherey-Nagel, Germany) according to the manufacturer's buccal swab instructions. Each sample was eluted with 50 µl Nuclease free water for the nested PCR technique.

An amplified step was carried out by the nested PCR assay, which was developed from previous literature (Heddema et al., 2006). Two pairs of specific primers (outer and inner) were designed from a *ompA* gene region. A 1041 bp of PCR amplicon length was generated in a primary PCR round using outer primers (CPsittGenoFor: 5′-GCTACGGGTTCCGCTCT-3′ and CPsittGenoRev: 5′-TTTGTTGATYTGAATCGAAGC-3′). A 984 bp of PCR amplicon length was amplified in a nested PCR round using inner primers (CPsittFinner: 5′-CGCTCTCTCCTTACAAGCC-3′ and CPsittRinner: 5′-GATCTGAATCGAAGCAATTTG-3′).

Five µl of DNA sample and 1 µl of PCR product from the primary PCR were used as a DNA template for the primary PCR and the nested PCR round, respectively, which was added into the amplified mixture which contained 2.5 µl of 10X DreamTaq™ Buffer , 0.5 µl of 10 mM dNTPs mix, 0.25 µl of each primer (100 pmol/µl) and 0.125 µl of 5 U/µl Dream Taq™ DNA polymerase (Thermoscientific, Lithuania), and of which the volume was adjusted to 25 µl using RNase-free water. The primary PCR condition was performed at initial denaturation of 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, primer annealing at 55°C for 45 sec and extension of 72°C for 1 min. Then, the final extension was incubated at 72°C for 10 min. The condition of the nested PCR round was similar to the primary PCR except the primer annealing step was optimized at 62°C for 45 sec. Amplifications were performed in a T100™ Thermal Cycler (Bio-Rad, United Kingdom). Reaction products were stained with GelRed™ (Biotium Inc., United States) and after that were electrophoresed on 1% agarose gels and visualized under UV illumination.

DNA Sequence-Based Analysis: PCR amplicons of positive samples were purified using FavorPrep™ Gel/PCR Purification Kit (Favorgen Biotech Corporation, Taiwan) and sent to a private service laboratory (SolGent Co., Ltd., South Korea) for nucleotide sequencing. All nucleotide sequences were compared with nucleotide sequences that were

Table 1 Variety of tested captive psittacine species in Thailand

Species	Common name	Number of tested birds	Age		Clinical status		Living area							
			J	A	Yes	No	BK	PT	NT	NP	RB	SP	CB	RY
Agapornis spp.	Lovebird	7	0	7	0	7	2	0	0	5	0	0	0	0
Ara ararauna	Blue-and-gold macaw	14	3	11	0	14	5	0	1	3	2	0	3	0
Ara chloropterus	Green-winged macaw	1	0	1	1	0	0	0	0	0	1	0	0	0
Ara macao	Scarlet macaw	2	2	0	1	1	2	0	0	0	0	0	0	0
Aratinga nenday	Nanday conure	3	0	3	0	3	0	0	0	3	0	0	0	0
Aratinga solstitialis	Sun conure	69	3	66	0	69	36	1	0	17	0	0	14	1
Aratinga wagleri	Red-fronted conure	1	0	1	0	1	0	0	0	1	0	0	0	0
Cacatua alba	Umbrella cockatoo	6	1	5	1	5	3	0	1	2	0	0	0	0
Cacatua moluccensis	Salmon-crested cockatoo	6	0	6	0	6	2	0	0	4	0	0	0	0
Cacatua sulphurea	Yellow-crested cockatoo	1	0	1	0	1	1	0	0	0	0	0	0	0
Diopsittaca nobilis	Hahn's macaw	4	0	4	0	4	2	0	0	1	0	0	1	0
Eclectus roratus	Eclectus parrot	3	1	2	1	2	0	0	0	1	0	0	1	1
Forpus spp.	Forpus parrot	4	0	4	0	4	0	0	0	4	0	0	0	0
Nymphicus hollandicus	Cockatiel	6	1	5	3	3	5	0	0	1	0	0	0	0
Pionites melanocephalus	Black-headed parrot	1	1	0	1	0	0	0	0	1	0	0	0	0
Poicephalus gulielmi	Jardine's parrot	1	0	1	0	1	0	0	0	1	0	0	0	0
Poicephalus senegalus	Senegal parrot	3	0	3	0	3	2	0	0	1	0	0	0	0
Psittacula alexandri	Red-breasted parakeet	5	3	2	1	4	1	0	0	4	0	0	0	0
Psittacula krameri	Ringneck parakeet	1	0	1	0	1	1	0	0	0	0	0	0	0
Psittacus erithacus	African grey parrot	11	2	9	3	8	2	0	0	7	0	2	0	0
Pyrrhura molinae	Green-cheeked conure	25	0	25	0	25	6	0	0	18	0	0	1	0
Pyrrhura perlata	Crimson-bellied conure	1	0	1	0	1	1	0	0	0	0	0	0	0
Trichoglossus haematodus	Rainbow lorikeet	3	0	3	0	3	3	0	0	0	0	0	0	0
Total		**178**	**17**	**161**	**12**	**166**	**74**	**1**	**2**	**74**	**3**	**2**	**20**	**2**

A: Adult, J: Juvenile, BK: Bangkok, PT: Pathum Thani, NT: Nonthaburi, NP: Nakhon Pathom, RB: Ratchaburi, SP: Suphan Buri, CB: Chon Buri, RY: Rayong

Table 2 Results of *Chlamydophila psittaci* positive in captive psittacine birds by nested PCR

Marker	Species	Age	Symptoms	Swab type	
				choanal	cloacal
CP1	Sun conure	A	None	-	+
CP2	Sun conure	A	None	+	+
CP3	Sun conure	A	None	-	+
CP4	Sun conure	A	None	+	-
CP5	Umbrella cockatoo	A	None	+	+
CP6	Hahn's macaw	A	None	-	+
CP7	Eclectus parrot	A	None	+	-
CP8	Cockatiel	J	R	+	-
CP9	Red-breasted parakeet	J	C, R	+	+
CP10	Ringneck parakeet	A	None	+	-
CP11	African grey parrot	A	None	-	+
CP12	African grey parrot	A	None	-	+
CP13	African grey parrot	J	G	+	+
CP14	Green-cheeked conure	A	None	+	-
Total number (%)				**9 (5.06%)**	**9 (5.06%)**

A: Adult, J: Juvenile, C: Conjunctivitis, R: Respiratory symptoms, G: Gastrointestinal symptoms

published in GenBank using BLAST software (http://blast.ncbi.nlm.nih. gov/Blast.cgi).

To evaluate *ompA* gene variability among *C. psittaci* strains, positive nucleotide sequences were aligned and relationship to the genotypes of *C. psittaci* was analyzed using MEGA6 software (Tamura et al., 2013). Nucleotide position of the *ompA* gene was determined from the nucleotide position of strain 6BC (NC_015470). A phylogenetic tree was constructed using the Neighbor-Joining method from a distance matrix corrected for nucleotide substitutions by the Maximum Composite Likelihood mode. Data set was generated using 1,000 bootstrap resamplings.

Statistical Analysis: Historical information of captive psittacine birds compared with *C. psittaci* detected was analyzed by Pearson Chi-Square test using SPSS 18 for Windows (SPSS Inc., United States). Statistical significance was indicated at a p value of < 0.05.

Results

Choanal and cloacal swabs of 178 psittacine birds were used for this study. Fourteen of the 178 (7.87%) birds, which were collected from Bangkok (n=4), Nakhon Pathom (n=6), Suphan Buri (n=1) and Chon Buri (n=3), were positive for *C. psittaci* using the nested PCR based on the *ompA* gene. The positive samples were composed of nine species including sun conure, green-cheeked conure, red-breasted parakeet, eclectus parrot, African grey parrot, Hahn's macaw, umbrella cockatoo, ringneck parakeet and cockatiel (Table 2). In the positive samples, even though some positive psittacine birds could be detected by the pathogen in both choanal and cloacal swabs (2.25%), some positive psittacine birds were detected by the pathogen in either of the swabs (Table 2). Although the sensitivity rate was 5.06% for both choanal and cloacal swabs for detecting *C. psittaci*, different birds were found to be positive (Table 2).

Moreover, three of the fourteen positive cases presented clinical signs of the disease containing conjunctivitis, respiratory (dyspnea) or gastrointestinal (green-yellowish droppings) symptoms. The association between positive results of *C. psittaci* and clinical manifestations was analyzed. The positive results of *C. psittaci* detection was of a statistically significant difference (p = 0.022) compared with the clinical status (the presence and absence of clinical signs). Regarding the relationship between *C. psittaci* infection and age of the bird, the infection rate of the juvenile domesticated psittacine birds was 17.65% (3/17), of which all positive samples showed clinical signs of the disease, whereas the infection rate of the adult domesticated psittacine birds was 6.83% (11/161), of which all positive samples in the adult group were found to not have clinical signs.

The homology between the nucleotide sequences from the positive specimens and the published nucleotide sequences was analyzed by the BLAST program. The results displayed showed that the nucleotide sequences from this study were similar to genotype A of *C. psittaci* which was submitted to the GenBank database. The genetic variability of *C. psittaci* was explained from the nucleotide analysis with multiple nucleotide alignment and phylogenetic tree construction. The partial *ompA* sequences of all positive amplicons included CP1 to CP14 (KR010609 to KR010622, respectively), which were identified as genotype A of *C. psittaci* (Fig 1). In the results, most nucleotide sequences, except the sequences of CP5, CP8 and CP13, had identical sequences with the Kobe strain (AB468956), which was detected from *Homo sapiens* in Japan, whereas these sequences were different from the strains 84/55 (NC_018619), RD1 (NC_014796), C5/98 (EU682088) and Nose (AB284060) at position 283 of 984 bp of the *ompA* sequences and

were unlike at position 461 of 984 bp of the *ompA* sequences. The partial *ompA* sequences of CP5 and CP8 presented 100% sequence homology with the strains 84/55 (NC_018619), C5/98 (EU682088) and Nose (AB284060) isolated from a parakeet (*Amazona* sp.) in Germany, a calf in Germany and a budgerigar (*Melopsittacus undulates*) in Japan, respectively. Lastly, one sequence (CP13) presented 99.9% similarity with the strains 84/55, RD1 and Nose. The contradictory nucleotide position of these sequences was 940 of 984 and was the cause of the different amino acid sequences. The substitution of guanine for adenine resulted in threonine replacing alanine at position 314 of the amino acid sequence.

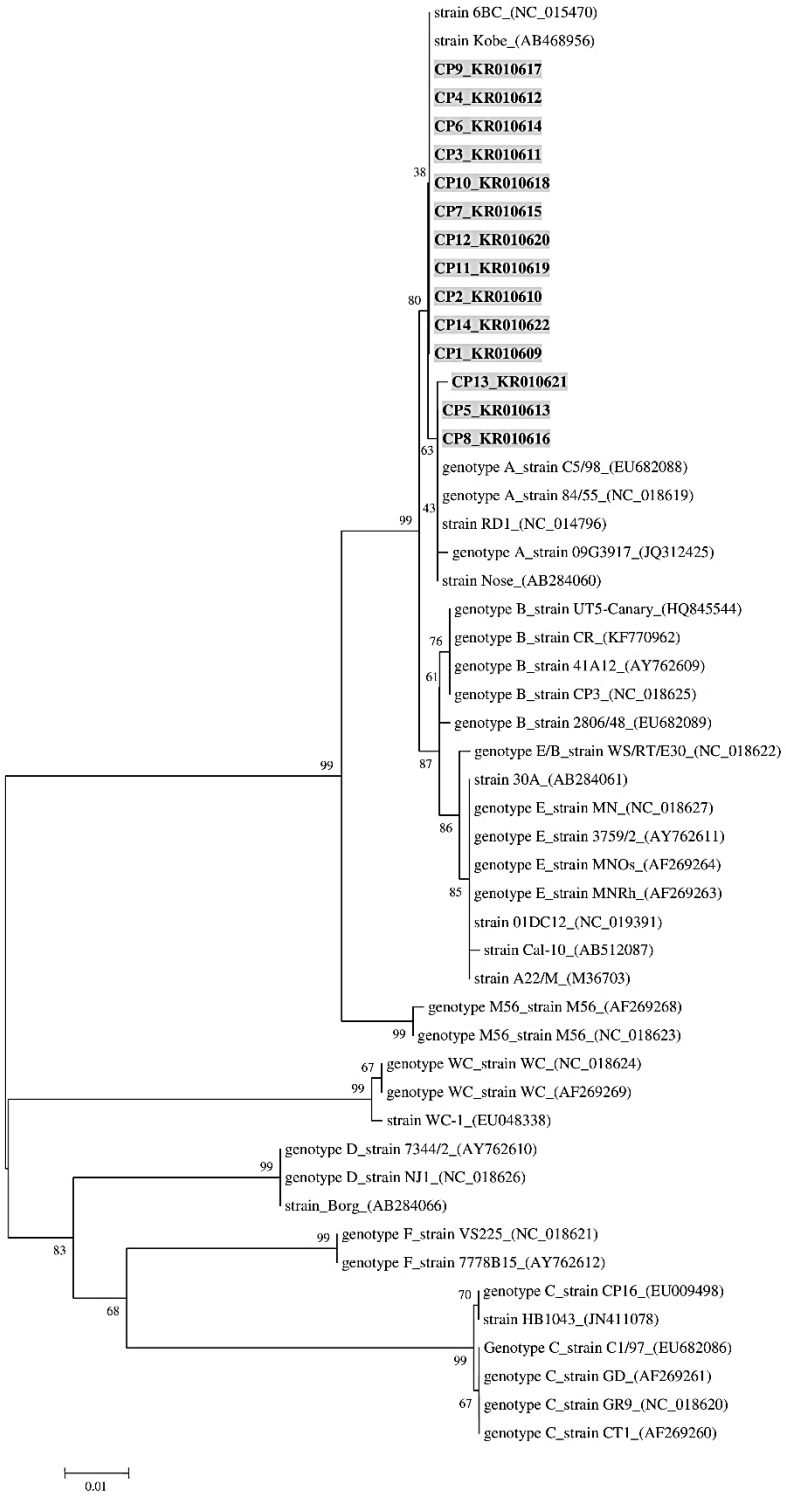

Figure 1 Phylogenetic analysis of *ompA* gene sequence of *Chlamydophila psittaci* in captive psittacine birds. The color gray with marker represents isolates from this study. Other sequences were obtained from GenBank (Accession numbers are indicated). Bootstrap values obtained from 1000 replications are shown at branch. The scale bar represents the number of substitutions for a unit branch length.

Discussion

The *C. psittaci* infectious disease or "parrot fever" is an important zoonosis disease in avian causing clinical and chronic diseases and producing subclinical carriers. Captive psittacine bird, which is the main reservoir of the pathogen that infects humans as well, is known as psittacosis (Frutos et al., 2012; Harkinezhad et al., 2009[b]; Harkinezhad et al., 2007; Kaibu et al., 2006; Vanrompay et al., 2007). The documented psittacosis case in Thailand was reported in 1996 (Riantawan and Nunthapisud, 1996).

The number of avian birds in Thailand increased from 2010 to 2012 as reported by the *Department of Livestock Development (DLD) (DLD, 2010; DLD, 2011; DLD, 2012)* but the *C. psittaci* infection data of psittacine birds is minimal as well as the health status and prevalence of the specific genotype of the organisms. In our specimens, the results of *C. psittaci* showed the infection rate of 7.87%. These results showed that positive *C. psittaci* was detected in birds from Thailand and also indicated the circulation of *C. psittaci* in birds or the environment of Thailand. This *C. psittaci* infection rate is similar to the *C. psittaci* infection rates in previous literatures (Chahota et al., 2006; de Freitas Raso et al., 2006; Madani and Peighambari, 2013; Marhold et al., 2012; McElnea and Cross, 1999; Piasecki et al., 2012; Sheleby-Elias et al., 2013). Furthermore, this prevalence study of *C. psittaci* in captive psittacine birds is similar to previous surveillance of *C. psittaci* in feral pigeons in central Thailand (10.8%) (Sariya et al., 2015). Conversely, the positive percentage is based on not only the species of birds but also the excretion periods of *C. psittaci*. The chlamydial excretion periods could be affected by various conditions such as stress, immune status, organism strains and infected dose (Harkinezhad et al., 2009[a]). Therefore, repeated diagnosis in suspected cases is suggested when negative samples are found (Vanrompay et al., 1995).

The nested PCR and nucleotide sequence based on the *ompA* gene are common methods to detect and identify the *C. psittaci* genotype (Andersen and Franson, 2007; Sachse et al., 2015). The genotype A was identified in all positive psittacine birds by the nucleotide sequence analysis based on the *ompA* gene. This finding corresponds with previous information that genotype A is predominantly isolated from psittacine birds (Andersen and Vanrompay, 2000; Beeckman and Vanrompay, 2009; Eidson, 2002; Harkinezhad et al., 2009[a]; Longbottom and Coulter, 2003; Zhang et al., 2015). Most sequences of this study had identical sequences with the chlamydial strain (Kobe strain accession number AB468956) isolated from humans in Japan. The captive psittacine birds may be carrying the virulent strain of *C. psittaci* found in humans. Recently, an article has reported the finding of genotype B of *C. psittaci* in feral pigeons in central Thailand, which is different from our study (Sariya et al., 2015). The genotypes of *C psittaci* infection are relatively host specific (Andersen and Vanrompay, 2000; Harkinezhad et al., 2009[a]).

In the experimental results, four out of the fourteen positive captive psittacine birds were inspected for this organism using a pair of swab types (choanal and cloacal) but ten samples detected DNA of *C. psittaci* from only one type of swabbing. Although the positive percentage of *C. psittaci* detection by nested PCR assay from choanal or cloacal swabs was alike (5.06%), the positive samples were different. One reason for the different result is the stage of infection which affects the profile of shedding routes. Moreover, the choanal swabs had higher levels of identified *C. psittaci* than the cloacal swabs or feces during the early stage of infection. The respiratory system is suspected to be the primary transmission route of *C. psittaci* infection in avian (Andersen, 1996; Harkinezhad et al., 2009[a]). Therefore, the result confirms that both cloacal and choanal swabs are required for the diagnosis of *C. psittaci* infection, which correlates with previous suggestion (Andersen, 1996).

Interestingly, this study found that all infected juvenile birds appeared to have clinical signs of the 'parrot fever' but all infected adult birds did not have clinical signs of this disease. The disease severity depends on the age of the hosts (Harkinezhad et al., 2009[a]; Smith et al., 2011; West, 2011). Additionally, an asymptomatic infection can usually be found in infected birds when they are a risk factor to shed this agent to humans and other animals (Harkinezhad et al., 2009[a]).

This study is the first to reveal the presence of *C. psittaci* infection and demonstrate the prevalence of positive findings in captive psittacine birds in Thailand. Surveillance of *C. psittaci* in various animals, including people with direct contact with psittacine birds, should be done. The information may raise the awareness of possible troubles and create the planning for prevention of this zoonotic pathogen.

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บทคัดย่อ

การตรวจสอบทางพันธุศาสตร์และการระบุชนิดของเชื้อคลามัยโดฟิล่าซิสตาซิ ในนกปากขอเลี้ยงในประเทศไทย

ปรุศก์ สุกใส[1,2] เบญจพล หล่อสัญญาลักษณ์[3] พัชริดา ดิษฐวงศ์[3] พรชัย สัญฐิติเสรี[4] ปรีดา เลิศวัชระสาระกุล[2,5*]

โรคคลามัยโดฟิโลซีสเป็นโรคสัตว์สู่คนที่สำคัญและพบการกระจายทั่วโลก โรคนี้มีสาเหตุจากเชื้อคลามัยโดฟิล่าซิสตาซิ วัตถุประสงค์ของการศึกษานี้คือการตรวจหาเชื้อคลามัยโดฟิล่าซิสตาซิจากตัวอย่างป้ายช่องโพรงจมูกและช่องทวารรวมจากนกปากขอเลี้ยง 178 ตัว (23 สายพันธุ์) จากพื้นที่ 8 จังหวัดของประเทศไทย โดยอาศัยการตรวจหายีน *ompA* ด้วยวิธีเนสเต็ดพีซีอาร์ พบผลบวกจำนวน 14 ตัว คิดเป็นร้อยละ 7.87 ความไวของการตรวจหาเชื้อคลามัยโดฟิล่าซิสตาซิด้วยวิธีเนสเต็ดพีซีอาร์จากการตรวจพบเชื้อจากตัวอย่างทั้ง 2 ตำแหน่งรวมกันสูงกว่าการตรวจจาก 1 ตำแหน่ง ตัวอย่างบวกส่วนมาก (จำนวน 11 ตัว) เป็นนกที่โตเต็มวัยและไม่แสดงอาการทางคลินิก มีเพียง 3 ตัวที่ให้ผลบวกและแสดงอาการทางคลินิก ซึ่งทั้งหมดเป็นนกอายุน้อย นกที่โตเต็มวัยที่ไม่พบอาการทางคลินิกอาจเป็นสาเหตุของการกระจายของโรค ลำดับนิวคลีโอไทด์ของตัวอย่างบวกทุกตัวอย่างถูกระบุเป็นจีโนไทป์ A โดยส่วนใหญ่มีลำดับนิวคลีโอไทด์เหมือนกับเชื้อคลามัยโดฟิล่าซิสตาซิที่ถูกแยกได้จากมนุษย์ในประเทศญี่ปุ่น (เลขทะเบียน AB468956) จากผลสามารถสรุปได้ว่าเชื้อคลามัยโดฟิล่าซิสตาซิ จีโนไทป์ A พบได้ในนกปากขอเลี้ยงในประเทศไทย ทั้งแสดงอาการและไม่แสดงอาการของโรค การเฝ้าระวังโรคจึงเป็นสิ่งจำเป็นเพื่อป้องกันและควบคุมโรคสำหรับเจ้าของนกและการเพาะเลี้ยงสัตว์ปีก

คำสำคัญ: คลามัยโดฟิล่าซิสตาซิ คลามัยโดฟิโลซีส เนสเต็ดพีซีอาร์ *ompA* นกปากขอ

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