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Original Study

Prevalence and Risk Factors of Avian Chlamydiosis Detected by Polymerase Chain Reaction in Psittacine Birds in Thailand

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Abstract: This study surveyed avian chlamydiosis, with the aim to estimate the prevalence and potential risk factors associated with *Chlamydia psittaci* infection in psittacine birds kept as domestic pets in Thailand. Oropharyngeal swabs were collected from 120 psittacine birds that were randomly selected from hospitals in the central (Bangkok) and northeastern regions (Khon Kaen) of Thailand between 2019 and 2021. The oropharyngeal swabs were subject to polymerase chain reaction testing to detect the *C psittaci ompA* gene. The prevalence of *C psittaci* was 2.5% (3/120, 95% confidence interval = 0.3–5.3). Of the 3 positive birds, 1 was a *Forpus* parrot (*Forpus* species)(CP43TH) and 1 was an African grey parrot (*Psittacus erithacus*)(CP49TH) from Bangkok; both were juvenile birds with clinical signs of disease. The third positive bird (CP12TH) was a subclinical adult sun conure (*Aratinga solstitialis*) from Khon Kaen. Two sequences of samples that were previously identified in human psittacosis cases (accession numbers MK032053.1 and HM450409.1) were also examined. Since there was a low number of infected birds, potential associations between *C psittaci* infection and various environmental variables (eg, cage cleaning, synanthropic birds, quarantine of new birds, and overcrowding) were assessed by Fisher exact tests. This study provides estimates of the prevalence and potential risk factors associated with *C psittaci* infection in psittacine birds from central (Bangkok) and the northeastern regions (Khon Kaen) of Thailand. The detection of *C psittaci* in captive psittacine birds demonstrates that there is a possibility for bird-to-bird transmission as well as some zoonotic potential for the human caretakers of these birds. Furthermore, larger-scale studies should be conducted to confirm these findings.

Key words: avian chlamydiosis, *Chlamydia psittaci*, psittacine birds, polymerase chain reaction, Thailand

INTRODUCTION

Avian chlamydiosis, or psittacosis (also known as ornithosis) in humans, is caused by *Chlamydia psittaci,* an obligate intracellular Gram-negative bacterium. According to the most recent classification, 9 different genotypes (A–F, E/B, M56, and WC) have been distinguished.[1] The classification is based on the *ompA* gene, which codes for the major outer membrane protein. Among the genotypes, 7 occur in the class Aves.[2] These *C psittaci* genotypes are considered the main agents of avian chlamydiosis in susceptible birds. *Chlamydia psittaci* has been detected in at least 467 species of birds belonging to 30 bird orders. The orders Psittaciformes and Columbiformes are reported to have the highest infection rates.[3]

In parrots, the prevalence ranges between 16 and 81%.[4] Many infected birds are subclinical carriers of the pathogen and expose other birds. Psittacine birds infected with *C psittaci* shed the pathogen regularly or intermittently in feces, lacrimal fluid, nasal discharge, and oropharyngeal mucus.[5] The incubation period of *C psittaci* infection is typically 3 days to several weeks prior to the appearance of the first clinical signs of disease. Clinical signs of avian chlamydiosis range from mild to severe systemic illness, especially in young birds, and

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may include anorexia, dehydration, depression, conjunctivitis, nasal and ocular discharge, dyspnea, and greenish diarrhea.[5–8] Extreme environments are considered to be one of the factors that increases the onset of clinical signs of disease.[1] Transmission to humans typically occurs by direct contact with birds or contaminated materials. The prevalence of *C psittaci* infection in humans having contact with domestic birds is 12.7% in Egypt and 13% in Belgium.[9,10] Human psittacosis normally causes influenza-like symptoms and conjunctivitis, with severe pneumonia in rare cases.[11]

There have been reports of avian chlamydiosis in various parts of the world, including Arizona, USA,[12] Australia,[13] Brazil,[14] China,[15] Costa Rica,[1] Egypt,[16] Iran,[3,17] Japan,[18] Mexico,[19] New Zealand,[20] the Philippines,[21] Taiwan,[22] and the Netherlands.[23] The first incident of *C psittaci* infection in psittacine birds in Thailand was reported in 1996.[24] The prevalence of *C psittaci* in pigeons (*Columba livia domestica*) with subclinical infection in central Thailand was 10.8%.[25] Another study of *C psittaci* in captive psittacine birds in Thailand reported a prevalence of 7.9% with subclinical infection.[7] Recently, the occurrence of *Chlamydia* species in wild birds in Thailand was reported at 0.64%.[26] Historically, the study of *C psittaci* infection in pet birds in Thailand has been limited. Therefore, data from animal hospitals in Bangkok and the northeastern region of Thailand were collected to determine current strains of *C psittaci* in these areas and the potential risk factors associated with the pathogen. Knowledge from these findings could be used to help form preventive strategies to reduce exposure for both birds and humans. The hypotheses for this cross-sectional study are that *C psittaci* will be identified in psittacine birds from Thailand and that some management schemes and individual characteristics will be identified as potential risk factors associated with this pathogen in psittacine birds from Thailand.

MATERIALS AND METHODS

Sampling and data collection

This cross-sectional study was performed according to the guidelines for the use and care of animals in science by the ethical principles of the Institutional Animal Care and Use Committee at Khon Kaen University (protocol no. 21/2021). Pet birds, regardless of their clinical disease signs, were selected from birds that were presented to animal hospitals for routine diagnosis in central (Kwuncum Animal Hospital Co, Ltd, Bangkok, Thailand) and northeastern (Kwuncum Animal Hospital Co, Ltd, Khon Kaen, Thailand) Thailand between June 2019 and April 2021. The birds included in this study were living in different provinces near Bangkok or Khon Kaen and were presented to the animal hospitals listed in Bangkok or Khon Kaen, respectively. Only 1 bird from each household was included in the study; therefore, when multiple birds were available in a household, a random number generator was used to select the study participant. Oropharyngeal swabs (n = 120) were collected from 18 species of psittacine birds to screen them for the presence of *C psittaci* (Table 1). Samples were collected from all birds using sterile cotton swabs and were placed into 500 μL of sterile phosphate-buffered saline, pH 7.4, in a microtube (1.5 mL microcentrifuge tubes, SBIO, Pathum Thani, Thailand).[17] After the birds were sampled, the swabs were placed on ice for transport to the Faculty of Veterinary Medicine, Khon Kaen University and were then stored at –20°C (–4°F) until DNA extraction was performed. A questionnaire for risk factors related to *C psittaci* infection was given to the bird owners. The questionnaire collected the following data about the birds: species; age; habitat; ventilation; housing; synanthropic birds; bird density; quarantine protocol for new birds; antibiotic use in sampled birds; cage cleaning routine; and presence of clinical signs including conjunctivitis, lethargy, ocular or nasal discharge, sneezing, dyspnea, and diarrhea with green-yellowish droppings. All bird owners who agreed to join the study signed a consent form for their bird to be included in this research investigation.

DNA extraction and polymerase chain reaction

A total of 120 swabs were examined for the presence of the *C psittaci ompA* gene using polymerase chain reaction (PCR) technology. For DNA extraction, a GF-1 Bacterial DNA Extraction Kit (Vivantis, Shah Alam, Malaysia) was used according to the manufacturer's instructions. Extracted DNA was eluted with 50 μL of Vivantis elution buffer and stored at –20°C (–4°F) until use. Positive samples were identified if nucleotide sequences with *C psittaci* strain PANH0624 (accession number MK030256) were detected. The negative DNA control included 200 μL of nuclease-free water. The method amplified the *ompA* gene using a conventional PCR technique with the primers CPsitt-F (5′-GCTACGGGTTCC GCTCT-3′; nucleotides 400 to 416) and CPsitt-R (5′-TTTGTTGATYTGAATCGAAGC-3′; nucle-

Table 1. Distribution of samples collected between June 2019 and April 2021 to measure *Chlamydia psittaci* in 18 different species of psittacine birds according to their ages, clinical status, location, and species.

Species	Common name	Age		Clinical status		Location		Total samples
		≤1 y	>1 y	Present	Absent	KKN	BKK	
Ara ararauna	Blue-and-yellow macaw	2	4	2	4	6	0	6
Aratinga solstitialis	Sun conure	23	11	17	17	20	14	34
Pionites melanocephalus	Black-headed parrot	1	0	0	1	1	0	1
Agapornis species	Lovebird	5	2	5	2	5	2	7
Psittacus erithacus	African grey parrot	8	11	14	5	12	7	19
Pyrrhura molinae	Green-cheeked conure	9	2	7	4	10	1	11
Anodorhynchus hyacinthinus	Hyacinth macaw	2	1	2	1	1	2	3
Nymphicus hollandicus	Cockatiel	4	6	3	7	6	4	10
Bolborhynchus lineola	Barred parakeet	1	0	1	0	1	0	1
Ara chloropterus	Green-winged macaw	0	2	2	0	2	0	2
Forpus species	*Forpus* parrot	2	0	2	0	1	1	2
Cacatua moluccensis	Moluccan cockatoo	2	5	6	1	4	3	7
Cacatua sulphurea	Yellow-crested cockatoo	1	1	2	0	1	1	2
Amazona ochrocephala	Yellow-crowned Amazon	1	1	2	0	1	1	2
Melopsittacus undulatus	Budgerigar	0	1	1	0	1	0	1
Eclectus roratus	Eclectus parrot	0	9	8	1	9	0	9
Psittacula krameri	Rose-ringed parakeet	1	1	1	1	1	1	2
Psittacula eupatria	Alexandrine parakeet	1	0	0	1	1	0	1
Total		63	57	75	45	83	37	120

Abbreviations: KKN, birds submitted to Khon Kaen animal hospitals; BKK, Bangkok animal hospitals.

otides 1420 to 1441). Reactions with 10 μL Taq PCR Master Mix 2X (Vivantis), 0.4 μL of 3.5 mM of MgCl, 0.4 μL of each primer (100 pmol/μL), and 8.8 μL of the DNA sample were prepared to a final volume of 20 μL. Amplification protocols were performed with an initial denaturation at 95°C (203°F) for 2 minutes; 35 cycles of denaturation at 94°C (201.2°F) for 30 seconds and alignment for 1 minute at 56°C (132.8°F); extension for 30 seconds at 72°C (161.6°F); and a final extension for 7 minutes at 72°C (161.6°F). The PCR results were visualized by 1.5% agarose gel electrophoresis in TBE (Tris Base, boric acid, EDTA, pH 8, 0.5 M) and stained with SYBR™ Green (Invitrogen, Ltd., ThermoFisher Scientific, Eugene, OR, USA). Sizer 1000 plus DNA (iNtRON Biotechnology, Seongnam, Korea) was used as a marker. The single target band of the *ompA* gene showing a product size of 1041 bp was an indication for a positive sample, and the positive PCR products were submitted for sequencing.

DNA sequencing and phylogenetic tree construction

Two randomly selected positive samples were sequenced at the U2Bio service laboratory (Seoul, South Korea). Nucleotide sequences were aligned with BioEdit Sequence Alignment Editor.[27] The *Chlamydia* species and strains were compared using BLAST software with the National Center for Biotechnology Information database (National Center for Biotechnology Information, Bethesda, MD, USA). The phylogenetic tree was constructed with MEGA7 for bigger datasets using the maximum likelihood method.[28] Bootstrap values (1000 replicates) were calculated to evaluate the support for branching of the tree.

Statistical analysis

The prevalence of avian chlamydiosis in psittacine birds from animal hospitals in 2 regions of Thailand was measured from the ratio of positive samples to the total number of sample swabs using an exact binomial confidence interval of 95% (95% CI). Univariate analyses of the association between *C psittaci* infection in individual birds and management factors were performed using Fisher exact tests in Epi-Info version 7. 2. 2. 6 (Centers for Disease Control and Prevention, Atlanta, GA, USA). The type I error rate was set at 5%.

RESULTS

A total of 120 live birds were screened for the presence of the *C psittaci ompA* gene. Three out of the 120 samples (2.5%; 95% CI: 0.3–5.3) were positive for *C psittaci*. The positive birds were from 3 different species. *Chlamydia psittaci* was identified in 50% (1/2) of *Forpus* parrots (*Forpus* species),

Table 2. Signalment and historical findings for the 3 *Chlamydia psittaci*–positive birds from Thailand.

	CP12TH Sun conure	CP49TH African grey parrot	CP43TH *Forpus* parrot
Species	*Aratinga solstitialis*	*Psittacus erithacus*	*Forpus* species
Age	>1 y	≤1 y	≤1 y
Clinical signs	None	D, L, S	C, D, N, O, S
Positive swab	Oropharyngeal	Oropharyngeal	Oropharyngeal
Habitat location	Khon Kaen	Bangkok	Bangkok

Abbreviations: C, conjunctivitis; D, dyspnea; L, lethargy; N, nasal discharge; O, ocular discharge; S, sneezing.

5.3% (1/19) of African grey parrots (*Psittacus erithacus*), and 2.9% (1/34) of the sun conures (*Aratinga solstitialis*). The *Forpus* parrot and African grey parrot were from Bangkok and were juveniles, while the sun conure was an adult from Khon Kaen (Table 2).

Two (66%) of the 3 positive samples presented with clinical disease signs, including conjunctivitis, dyspnea, lethargy, nasal discharge, ocular discharge, and sneezing. According to the univariate analysis, age ($P = 1.0$), clinical status ($P = 0.224$), location ($P = 0.224$), housing ($P = 1.0$), ventilation ($P = 0.093$), and antibiotic use ($P = 1.0$) were not associated with *C psittaci* infection. *Forpus* species had 58 times higher odds of being PCR positive for *C psittaci* than the other species (odds ratio [OR] = 58, 95% CI = 2.6–1293.1, $P = 0.049$). There were no significant associations found between the disease and bird species for sun conure and African grey parrot ($P > 0.05$). Four categories of cage cleaning included removing feces only, washing only, washing and disinfection, and never cleaning. Birds living in cages that had never been cleaned had 76 times (OR = 76, 95% CI = 5.3–1086.7, $P = 0.004$) greater chance of being PCR positive for *C psittaci* than any type of cleaned cage. All of the positive birds had contact with synanthropic birds. There was a significant risk of infection when exposure to synanthropic birds was present ($P = 0.006$). Furthermore, all 3 positive birds were in households that did not use quarantine to prevent the introduction of infectious disease into the household; thus, the lack of quarantine of new birds was a risk factor ($P = 0.003$). The density of birds categorized as overcrowding referred to a cage containing several birds such that the area for wingspan was limited according to McDonald and Noterman.[29] All of the positive cases were living in cages with limited space ($P = 0.001$). In summary, 4 factors, including cage cleaning, synanthropic birds, quarantine of new birds, and overcrowding, were significant risk factors associated with *C psittaci* infection in psittacine birds (Table 3).

The nucleotide sequences from 2 positive samples (CP12TH and CP49TH) were investigated for the *ompA* gene. The results showed a high homology of strains characterized as *C psittaci* genotype A (Fig 1). Two sequencing analyses identified 100% of the nucleotide sequences belonging to genotype A strains PAAC0728 (accession no. MK032053.1) and KMZ07 (HM450409.1). Other genotype A strains had 99% of the nucleotide sequences identical to the MN_Zhang (AF269281.1) strain and 08DC60 (CP002807.1) strain.

DISCUSSION

This study showed that the prevalence of *C psittaci* infection was 2.5% (3/120) in psittacine birds kept in captivity in 2 regions of Thailand. The prevalence was consistent with previous surveillance reports of *C psittaci* infection in other countries.[1,13,22,30,31] A previous report noted that the prevalence of *C psittaci* infection in Thailand was 7.9% among 178 captive psittacine birds in the central and eastern regions of Thailand, but there are no reports of the prevalence and risk factors from other provinces.[7] This study found *C psittaci* infection in the central (5.4%) and northeastern regions (1.5%) of Thailand. The slightly lower positive rate found in this study may have been associated with the conventional PCR methods, which are generally less sensitive than a nested PCR assay,[7] or a lower amount of pathogen in the sample, which could be a problem for amplification.[2,32,33] Furthermore, this study used only oropharyngeal swab, whereas other studies may have sampled more than 1 site or used more than 1 type of swab. Vanrompay[34] suggested that nasal swabs or oropharyngeal swabs are appropriate for detecting *C psittaci* at the early stage of disease; therefore, the authors only used oropharyngeal swabs for collecting samples. The 2 sequencing tests in this study showed identical sequences of *C psittaci* genotype A with the MN_Zhang strain (accession no. AF269281.1) and the 08DC60 strain (CP002807.1). All strains found in this study had

Table 3. Risk factor analysis of *Chlamydia psittaci* infection in psittacine birds from Thailand.

Variables	Positive	Negative	Prevalence (%)	OR (95% CI)	*P* value[a]
Species					
Sun conure	1	33	2.9	1.3 (0.1–14.5)	1.0
Grey parrot	1	18	5.3	2.7 (0.2–31.9)	0.41
Forpus parrot	1	1	50	58 (2.6–1293.1)	0.049
Other species	0	65	0	ref.	—
Age					
Juvenile	2	61	3.2	1.8 (0.2–20.8)	1
Adult	1	56	1.7	ref.	—
Clinical sign					
Absent	2	35	5.4	4.69 (0.4–53.4)	0.22
Present	1	82	1.2	ref.	—
Location					
Central Thailand	2	35	5.4	4.7 (0.4–53.4)	0.22
Northeastern Thailand	1	82	1.2	ref.	—
Antibiotic use in sampled birds					
>2 wk	3	20	3.1	Undefined	1.0
≤2 wk	0	97	0	ref.	—
Housing					
Cage	3	103	2.8	Undefined	1.0
Aviary	0	14	0	ref.	—
Cage cleaning					
Never	2	3	40	76 (5.3–1086.7)	0.004
Yes	1	114	0.9	ref.	—
Feces removal only	0	114	0	—	—
Washing only	1	114	0.9	—	—
Washing and disinfection	0	114	0	—	—
Overcrowding					
Yes	3	10	23.1	Undefined	0.001
No	0	107	0	ref.	—
Quarantine of new birds					
No	3	15	16.7	Undefined	0.003
Yes	0	102	0	ref.	—
Synanthropic birds					
Yes	3	20	13	Undefined	0.006
No	0	97	0	ref.	—
Ventilation					
Closed	3	52	5.4	Undefined	0.09
Open	0	65	0	ref.	—

Abbreviations: OR, odds ratio; CI, confidence interval; ref, reference.
[a] Using Fisher exact tests.

previously been identified in human psittacosis cases from the United States and Germany.[15,22,35] The results showed that pet birds may be reservoirs of psittacosis agents in humans who are at risk of direct contact or occupational exposure with these birds. The potential risk factors associated with *C psittaci* infection in psittacine birds were species and management factors. The *Forpus* species had significant odds of being positive for *C psittaci* infection (OR = 58) compared to African grey parrots and sun conures, but there were no significant differences (OR = 2.75 and 1.27, respectively). However, only 1 *Forpus* parrot was diagnosed with *C psittaci*. These results suggest that a larger sample size is needed to further assess these species differences.

One study reported that the prevalence of *C psittaci* infection in cockatiels (*Nymphicus hollandicus*) (60%) was higher than that in other species,[16] whereas another study reported that the highest prevalence (12.5%) was found in budgerigars (*Melopsittacus undulatus*).[22] Our study found that the highest prevalence was in birds living in cages that had never been cleaned (40%), whereas 0.87% of birds living in washed cages were positive. This finding was similar to previously reported

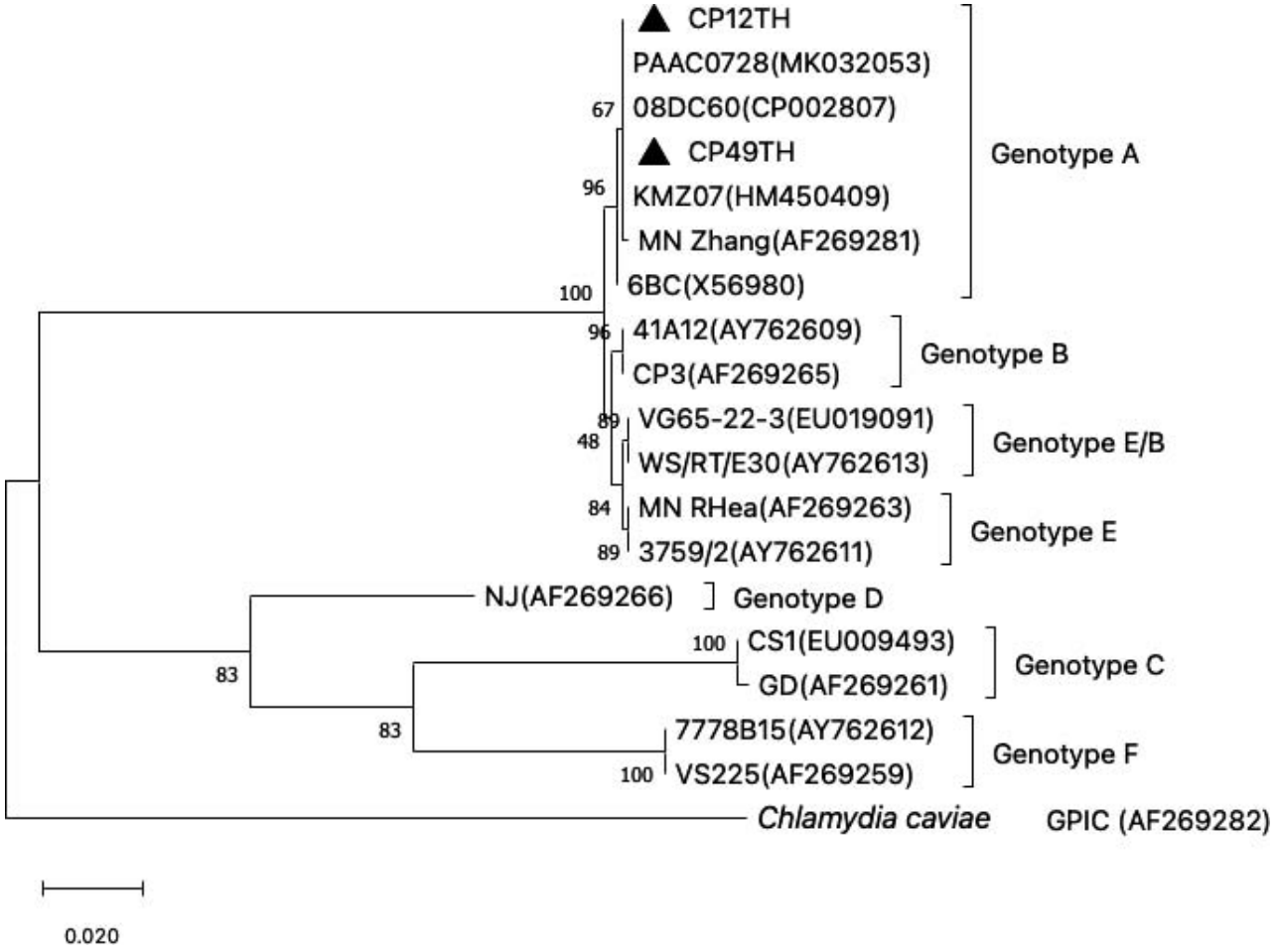

Figure 1. Phylogenetic tree based on *ompA* gene sequences from the samples of psittacine birds in Thailand that were positive for *Chlamydia psittaci*. Samples at nodes are bootstrap values. Sequencing of positive samples (CP12TH and CP49TH) from Khon Kaen and Bangkok, respectively.

studies that found washing a cage containing a significant amount of bird feces can reduce the likelihood of *C psittaci* infections.[36] Similar results were found in the current study, as never cleaning cages increased the likelihood of positive cases.

In this investigation, the prevalence of *C psittaci* infections in birds that had contact with synanthropic birds was 13%. A similar finding was recently reported in central Thailand and found subclinical feral pigeons that were positive for *C psittaci* genotype B, which could also be determined by *omp*A genotyping.[25] The current study found that the prevalence of *C psittaci* infection in households that did not quarantine newly acquired birds was 16.7%, compared to 0% in households that used a quarantine program. Therefore, a quarantine program may help reduce exposure of birds and humans to *C psittaci* from newly acquired birds.[36] In this study, all positive cases were living in overcrowded conditions (23.1%). This finding was consistent with the work of Smith et al,[37] who reported a higher risk of *C psittaci* infection in birds maintained in overcrowded conditions.

Due to the extremely low number of *C psittaci*–infected birds identified in this study, our findings require further investigation to determine whether these risk factors are independent of each other in multivariable models. This study represents the first attempt at estimating the prevalence and risk factors associated with *C psittaci* infections in psittacine birds in the central (Bangkok) and northeastern regions (Khon Kaen) of Thailand. Different real-time PCR diagnostic tests are recommended for future studies, as they have higher sensitivity for detection than the conventional PCR used in this study.[32,38] Long-term circulation of *C psittaci* in the birds and environments in Thailand is expected given that *C psittaci* infection is now an endemic infectious pathogen.

These results should be used to raise the general public's awareness of this zoonotic disease, and veterinarians should educate their clients about how they can mitigate the risk factors associated with *C psittaci*–positive cases.

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