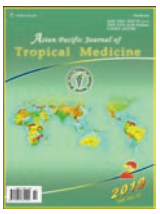

IF: 1.634

Asian Pacific Journal of Tropical Medicine

journal homepage: www.apjtm.org

doi: 10.4103/1995-7645.250839

Occurrence of *Chlamydia* spp. in wild birds in Thailand

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ARTICLE INFO

Article history:
Received 31 October 2018
Received in revised form 28 December 2018
Accepted 15 January 2019
Available online 1 February 2019

Keywords:
Asian openbill stork
Chlamydia spp.
Wild bird

ABSTRACT

Objective: To determine the occurrence of *Chlamydia* spp. in wild birds in Thailand. **Methods:** Cloacal and tracheal swabs of 313 wild birds from 11 orders, 27 families, and 51 species were tested to determine the occurrence of *Chlamydia* infection. The outer membrane protein A (*ompA*) gene was amplified from positive samples to construct a phylogenetic tree. **Results:** At the time of sample collection, none of the birds showed clinical signs of any disease. Of 313 wild birds, two Asian openbill stork (*Anastomus oscitans*) were positive for *Chlamydia* spp., representing 0.64% (2/313) and 4.9% (2/41) occurrence for birds overall and for the Asian openbill stork, respectively. Phylogram analysis based on deduced amino acid of the *ompA* gene showed that *Chlamydia* spp. in Asian openbill storks was closely related to that in wildfowl (*Pica pica* and *Cygnus olor*) from Poland in a different branch with a 95% bootstrap value and had a shorter evolutionary distance to *Chlamydia abortus*. **Conclusions:** Asymptomatic Asian openbill storks could be a potential source of *Chlamydia* infection in domestic animals, poultry, and humans who share their habitat.

1. Introduction

Chlamydosis is an infectious disease of several animal species, including wild birds and humans. The disease is caused by an obligate intracellular gram-negative bacteria in the family Chlamydiaceae. To date, Chlamydiaceae comprises 11 species, namely *Chlamydia psittaci* (*C. psittaci*), *Chlamydia felis(C. felis)*, *Chlamydia abortus* (*C. abortus*), *Chlamydia avium*, *Chlamydia caviae*, *Chlamydia gallinacea*, *Chlamydia muridarum*, *Chlamydia pecorum*, *Chlamydia suis*, *Chlamydia pneumoniae*, and *Chlamydia trachomatis*, and three candidate chlamydial species, namely *Chlamydia ibidis*, *Chlamydia sanzinia*, and *Chlamydia corallus*[1-6]. Within the chlamydial species, *C. psittaci*, *C. felis*, and *C. abortus* have zoonotic potential[7]. Chlamydiosis in birds can range from asymptomatic infection to severe disease with life-threatening illness, depending on the host species affected and the chlamydial species involved. Wild birds are important to public health because they can be infected with *Chlamydia* species that are transmissible to humans and domestic animals[8]. Several reports have shown the prevalence of *Chlamydia* in wild birds. In 2015, the positive rate for chlamydial DNA in wild birds in Poland was 7.3% (27/369)[9]. Two years later, a large number of wild birds in Poland were tested, and the results revealed Chlamydiaceae prevalence of 14.8% (132/894) [10]. Moreover, 10.3% (125/1 214) of wild birds in Austria and the Czech Republic have been found to be *Chlamydia* spp. positive[11].

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Foundation project: This work was financially supported by the Faculty of Veterinary Science, Mahidol University. The sample used in this study were collected by the project of Establishment of zoonotic viral networking system: developmental phase; subproject of Influenza A virus surveys in migratory and residence birds of Thailand granting from Cluster and Program Management Office (P-15-50535), the National Science and Technology Development Agency, Thailand.

How to cite this article: Parut S, Rattanaporn O, Witthawat W, Nareerat S, Paisin L, Ladawan S. Occurrence of *Chlamydia* spp. in wild birds in Thailand. Asian Pac J Trop Med 2019; 12(2): 67-71.

The lowest prevalence was reported in Korea[12]. Only 2.7% (6/225) of wild birds in Korea were found to be positive to *Chlamydia* DNA; four (1.8%, 4/225) and two (0.9%, 2/225) were positive for *C. psittaci* and *C. gallinacea*, respectively[12]. In Thailand, a few studies have sought to detect *Chlamydia* in wild birds. Of 407 feral pigeons, 44 (10.8%) were positive for Chlamydiaceae, with most of the positive samples *C. psittaci*[13]. One report examined *C. psittaci* in captive psittacine birds and found 7.9% (14/178) prevalence[14]. Thus, the aim of this study is to determine the occurrence of *Chlamydia* spp. in various species of wild birds in Thailand.

2. Materials and methods

2.1. Sample collection and genomic DNA extraction

During 2017-2018, tracheal and cloacal swabs of 313 wild birds from 11 orders and 51 species from seven provinces in Thailand were collected and examined (Table 1). The samples were kept in VB lysis buffer (Geneaid, Taiwan) and transferred to the laboratory in a cool chain within 48 hours. At the laboratory, genomic DNA was extracted from the samples using the viral nucleic acid extraction kit][(Geneaid, Taiwan). The animal handling protocol used during sample collection and the samples used in this study were approved by the Animal Care and Use Committee of the Faculty of Veterinary Science, Mahidol University (Protocol No. MUVS-2017-02-04 and MUVS 2018-01-02).

2.2. Housekeeping gene detection

Before detection of Chlamydiaceae, the samples were examined the quality of the DNA by detection of the *12S ribosomal (r) DNA* housekeeping gene using primers *12S rDNA*-F (5' GGATTAGATACCCCACTATGC 3') and *12S rDNA*-R (5' AGGGTGACGGGCGGTATGTAC G 3') and obtained a PCR product with a size of 436 bp[15]. In total a 25 μL, the PCR mixture contained 1× PCR buffer with 0.2 mM $MgCl_2$, 2.5 units of i-*Taq* DNA polymerase (iNtRON, South Korea), 1 mM of dNTPs, 0.5 μM of each primer, and 3 μL of template DNA. The PCR reaction was worked under the conditions of 2 min at 94 ℃ for initial denaturing, followed by 30 cycles of 15 s at 94 ℃, 15 s at 60 ℃, and 30 s at 72 ℃, and was terminated at 72 ℃ for 7 min.

2.3. Chlamydiaceae detection

Chlamydiaceae was detected by primers CHY-F (5' GCCTACCGGCTTACCAAC 3') and CHY-R (5' GGCGCAATGATTCTCGAT 3') targeting the *16S rRNA* gene of the Chlamydiaceae family[16]. The PCR mixture contained 1 mM of dNTPs, 1× PCR buffer with 0.2 mM $MgCl_2$, 2.5 units of i-*Taq* DNA polymerase (iNtRON, South Korea), 0.5 μM of each primer, and 3 μL of template DNA. Sterile DNase/RNase-free distilled water was added to increase the mixture to 25 μL. The PCR reaction was performed under the conditions of 2 min at 94 ℃ for initial denaturing, followed by 35 cycles of 15 s at 94 ℃, 30 s at 56 ℃, and 30 s at 72 ℃, and was terminated at 72 ℃ for 7 min. The primers generated a PCR product with a size of 230 bps.

2.4. ompA gene amplification and phylogenetic tree construction

The positive samples from the Chlamydiaceae detection protocol were used for amplification of the *ompA* gene. The *ompA* gene was amplified with primers CTU (5'-ATGAAAAAACTCTTGAAATCGG-3') and CTL (5' CAAGATTTTCTA GAYTTCATYTTGTT 3'). The primers generated a PCR product with a size of 1 070 bps[17]. The PCR mixture contained 3 μL of template DNA, 1× PCR buffer with 0.2 mM $MgCl_2$, 1 mM of dNTPs, 2.5 units of i-*Taq* DNA polymerase (iNtRON, South Korea), and 0.5 μM each of forward and reverse primer. The PCR reaction was worked under the conditions of 2 min at 94 ℃ for initial denaturing, followed by 35 cycles of 30 s at 94 ℃, 30 s at 58 ℃, and 30 s at 72 ℃, and was terminated at 72 ℃ for 7 min. After that, DNA fragment of each sample was ligated to the pGEM-T easy vector (Promega, USA) and transformed to competent *Escherichia coli* TOP10 (Invitrogen™, USA) using the calcium chloride method. Transformants were selected by blue-white screening method. Plasmid was extracted by the QIAprep spin miniprep kit (QIAGEN, Germany) and submitted to Macrogen Inc. (South Korea) for DNA sequencing. A phylogram of deduced amino acid sequences of the *ompA* gene was generated by the maximum likelihood method and the JTT matrix-based model with a bootstrap value based on 1 000 replicates[18]. Evolutionary analyses were conducted with MEGA7 version 7.0 software[19].

3. Results

For all bird samples, the housekeeping gene (*12S rDNA*) was detected to examine the quality of the DNA. All samples were found to be positive for the *12S rDNA* gene, indicating the good quality of the DNA. For Chlamydiaceae detection, of 313 wild birds, two (0.64%) were positive for Chlamydiaceae with asymptomatic infection. These birds were Asian openbill storks (*Anastomus oscitans*), which belong to the order Ciconiiformes. The positive rate for Asian openbill storks was 4.9% (2/41). The *ompA* gene of the positive samples was amplified and sequenced. Nucleotide sequencing of the *ompA* gene (Accession No. MK007613 and MK007614) in our study showed only 94.1% genetic similarity to *Chlamydia* spp. of Eurasian magpies (*Pica pica*) and mute swans (*Cygnus olor*) in Poland (Accession No. KX870484.1, KX424658.1, KX062052.1, KX062055.1). The *ompA* phylogenetic tree analysis

Table 1

Details and number of wild birds tested in the study.

Order	Family	Species	Common name	No. of tested bird
Anseriformes	Anatidae	*Anas platyrhynchos domesticus*	Domestic duck	4
Galliformes	Phasianidae	*Pavo cristatus*	Indian peafowl	4
Ciconiiformes	Ciconiidae	*Anastomus oscitans*	Asian openbill	41
Pelecaniformes	Ardeidae	*Egretta garzetta*	Little egret	1
		Ixobrychus sinensis	Yellow bittern	3
	Phalacrocoracidae	*Phalacrocorax fuscicollis*	Indian cormorant	2
		Microcarbo niger	Little cormorant	2
Gruiformes	Rallidae	*Amaurornis phoenicurus*	White-breasted waterhen	1
		Porphyrio poliocphalus	Grey-headed swamphen	3
Charadriiformes	Laridae	*Chronicocephalus genei*	Slender-billed gull	2
		Sternula spp.	Unidentified tern	5
		Chroicocephalus brunnicephalus	Brown-headed gull	113
Columbiformes	Columbidae	*Spilopelia chinensis*	Spotted dove	1
		Geopelia striata	Zebra dove	8
Strigiformes	Tytonidae	*Tyto javanica*	Eastern barn owl	3
Coraciiformes	Alcedinidae	*Halcyon smyrnensis*	White-throated kingfisher	2
		Alcedo atthis	Common kingfisher	4
Piciformes	Picidae	*Jynx torquilla*	Eurasian wryneck	1
Passeriformes	Laniidae	*Lanius cristatus*	Brown shrike	1
	Dicruridae	*Dicrurus macrocercus*	Black drongo	1
	Rhipiduridae	*Rhipidura javanica*	Malaysian pied fantail	3
	Monarchidae	*Hypothymis azurea*	Black-naped monarch	2
	Pycnonotidae	*Pycnonotus atriceps*	Black-headed bulbul	1
		Pycnonotus aurigaster	Sooty-headed bulbul	3
		Pycnonotus conradi	Streak-eared bulbul	15
	Hirundinidae	*Hirundo rustica*	Barn swallow	2
	Phylloscopidae	*Phylloscopus fuscatus*	Dusky warbler	19
	Acrocephalidae	*Acrocephalus bistrigiceps*	Black-browed reed warbler	12
		Acrocephalus orientalis	Oriental reed warbler	13
	Locustellidae	*Helopsaltes certhiola*	Pallas's grasshopper warbler	2
	Cisticolidae	*Prinia flaviventris*	Yellow-bellied prinia	1
		Prinia inornata	Plain prinia	3
	Pellorneidae	*Pellorneum ruficeps*	Puff-throated babbler	1
	Sturnidae	*Acridotheres grandis*	Great myna	1
		Sturnia malabarica	Chestnut-tailed starling	1
		Gracupica nigricollis	Black-collared starling	1
		Acridotheres tristis	Common myna	2
	Muscicapidae	*Muscicapa* spp.	Unidentified flycatcher	1
		Ficedula albicilla	Taiga flycatcher	3
		Calliope calliope	Siberian rubythroat	1
		Copsychus saularis	Oriental magpie-robin	2
		Saxicola stejnegeri	Stejneger's stonechat	2
	Passeridae	*Passer montanus*	Eurasian tree sparrow	1
		Passer domesticus	House sparrow	2
		Passer flaveolus	Plain-backed sparrow	2
	Ploceidae	*Ploceus hypoxanthus*	Asian golden weaver	1
		Ploceus philippinus	Baya weaver	3
		Ploceus manyar	Streaked weaver	8
	Estrildidae	*Lonchura punctulata*	Scaly-breasted munia	1
		Lonchura striata	White-rumped munia	1
	Motacillidae	*Anthus rufulus*	Paddyfield pipit	1

showed that the *Chlamydia* spp. detected in Asian openbill storks can be grouped together with 99% bootstrap support and was closely related to *Chlamydia* spp. detected in Eurasian magpies and mute swans in Poland but had a different cluster creation with a 95% bootstrap value (Figure 1). Additionally, the *Chlamydia* spp. found in this study had a closer relationship to *C. abortus* than any other known *Chlamydia*.

4. Discussion

Wild birds may play a role as a potential source of Chlamydiaceae that can be transmitted to humans, domestic animals, and poultry[8,20,21]. In the present study, we demonstrated the overall occurrence of *Chlamydia* spp. in several species of wild birds was 0.64%, suggesting low occurrence in wild birds in Thailand. The primers used in this study can detect Chlamydiaceae DNA as low

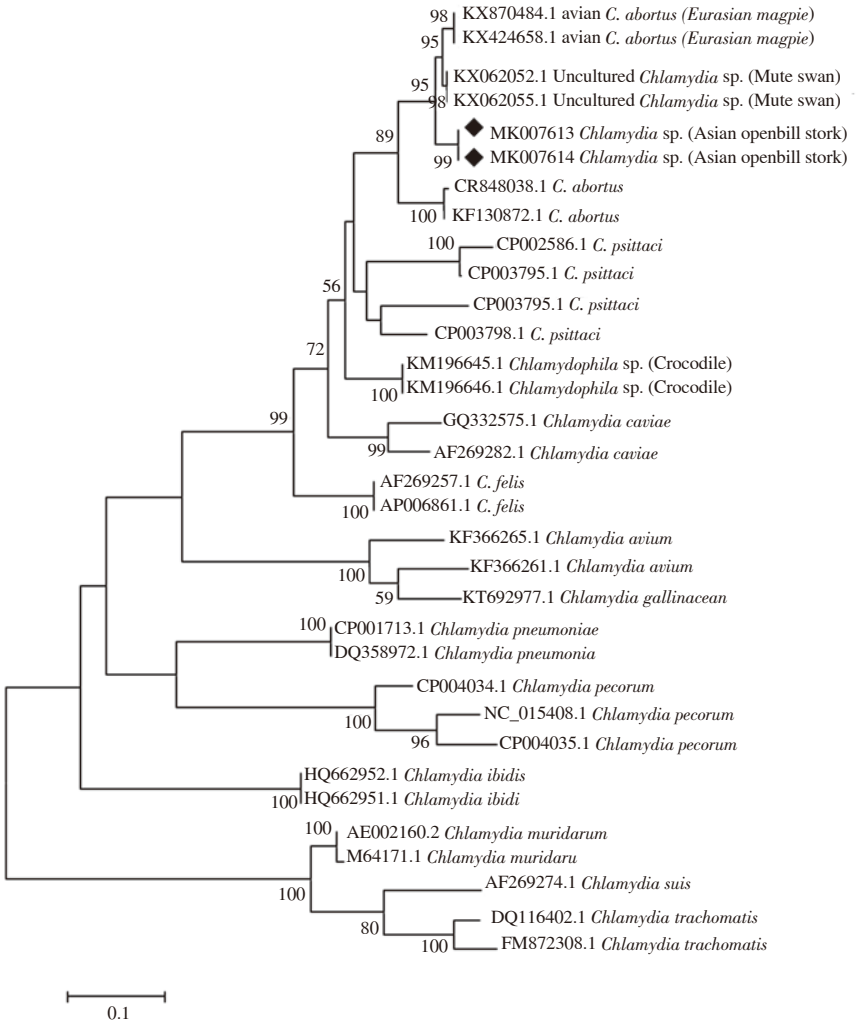

Figure 1. Phylogenetic tree resulting from analysis of deduced amino acid sequences of the Chlamydiaceae *ompA* gene.
The percentage of trees in which the associated taxa are clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The sequences analyzed in this study are indicated by a black rhombus.

as 1 fg, indicating high sensitivity of the test[16]. The occurrence found in the study was slightly lower than the rate in other countries, which ranges from 2.7% to 14.8%, depending on the bird species and detection method[9,10,12]. Phylogram-based *ompA* gene analysis of the positive samples found that the *Chlamydia* detected is closely related to *Chlamydia* detected/isolated in wildfowl in Poland and to *C. abortus*, which causes abortion and fetal death in ewes and goats, and abortion in women in close contact with aborting animals[7]. The wildfowl *Chlamydia* strains can presumably be classified as avian *C. abortus* based on MLST analysis. However, the pathogenicity of the avian *C. abortus* strains from wildfowl remains unknown[10]. The positive samples detected in our study were from Asian openbill storks in the order Ciconiiformes. A 4.9% prevalence level was found for these birds. Other species in Ciconiiformes were previously reported as having a Chlamydiaceae positive rate of 5.3% (2/38) for white storks[10] and 11.5% (13/113) for herons and allies[20], respectively. The variation of prevalence in Ciconiiformes may depend on the sample size of birds. However, to the best of the authors' knowledge, Chlamydiaceae has not been previously reported in Asian openbill storks. The Asian openbill stork is a migratory bird, and the migration of Asian openbill stork populations along various migration pathways may be a potential means of spreading of Chlamydiaceae. Asymptomatic birds can transmit the bacterium to domestic birds and humans that share their environment or habitat or by handling *via* fecal shedding and direct contact. In conclusion, this study demonstrates the occurrence of *Chlamydia* spp. in wild birds in Thailand is 0.64%. *Chlamydia* spp. in Asian openbill stork could be a potential source of infection in domestic animals, poultry, and humans who share their habitat.

Conflict of interest statement

The author declared that they have no conflict of interest.

Foundation project

This work was financially supported by the Faculty of Veterinary Science, Mahidol University. The sample used in this study were collected by the project of Establishment of zoonotic viral networking system: developmental phase; subproject of Influenza A virus surveys in migratory and residence birds of Thailand granting from Cluster and Program Management Office (P-15-50535), the National Science and Technology Development Agency, Thailand.

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