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Short Communication

Molecular Detection of *Chlamydiaceae* in Captive Birds from Five Animal Facilities in the Philippines

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Abstract

Background: Chlamydia psittaci is a zoonotic agent transmitted from birds to humans that causes psittacosis from asymptomatic infections to mild flu-like illness to severe pneumonia, even death in humans. Although some antibiotic treatments are effective, infection may become severe if treatment is delayed due to delayed diagnosis. Testing for *C. psittaci* is not routinely available in most hospitals. Therefore, the risk of contracting *C. psittaci* is an important public health concern. **Methods:** In this study, 303 fecal samples from 99 bird species in five different animal facilities in the Philippines were collected and subjected to PCR targeting envB of Chlamydiales DNA. Positive samples were retested by real-time PCR targeting 16S rRNA and confirmed Chlamydiales infection. **Results:** Two out of 303 samples (0.6%) were positive for Chlamydiales DNA. One positive sample was from facility 1 (7.14%; 1/14), and the other was from facility 3 (0.87%; 1/115). The 102 bp of envB nucleic acid sequences were successfully determined. Phylogenetic analysis revealed the samples were found to be with cluster of *C. psittaci*, *C. abortus, and C. buotenis*. **Conclusions**: Although species could not be determined, we molecularly detected *Chlamydiaceae* that are potentially pathogenic and zoonotic.

Keywords

Captive birds; *Chlamydia psittaci*; PCR; psittacine birds; psittacosis

1. Introduction

The Gram-negative, obligate intracellular bacterium *Chlamydia psittaci* is a zoonotic agent that can be transmitted from birds to humans that causes avian chlamydiosis in birds and psittacosis (ornithosis) in humans. In birds, the disease is characterized by fever, watery green diarrhea, anorexia, emaciation, respiratory distress, and conjunctivitis, and the severity of illness can range from subtle upper respiratory disease or mild conjunctivitis to death. Even while the birds have subclinical infection, *C. psittaci* may still be excreted in ocular and nasal discharges, and feces. Transmission of *C. psittaci* is usually by aerosols from secretions of infected birds. Infections in humans can cause systemic diseases, respiratory problems, and even death, making it a public health concern in people with psittacines as pets or in workers having a direct contact with potentially infected birds [1]. Recently, it has been reported that *C. psittaci* spilled over from wild parrots to horses is an important source of *C. psittaci* infection in humans [2, 3]. Human-to-human transmission has been reported but was considered rare [4, 5], with the most recent outbreak of human-to-human transmission from asymptomatic carriers and by healthcare workers to close contacts being reported in 2020 from China [6]. Although the antibiotics tetracyclines, macrolides, and new quinolones are effective treatments against *C. psittaci*, the infection may become severe if treatment is delayed due to a late diagnosis [7]. Diagnostic tests for *C. psittaci* are not routinely available in most

hospitals, especially in developing countries [8]. Therefore, the risk of contracting *C. psittaci* is an important public health concern in the Philippines.

In the Philippines, the seroprevalence of *C. psittaci* was first investigated among several species of birds in 1971 using the direct complement fixation test [9]. Subsequently, enzyme-linked immune sorbent assay (ELISA) tests were performed on 36 confiscated wild birds in 2007, with six psittacines and three raptors demonstrating antibodies reactive to *C. psittaci* [10]. It was also reported that 6/6 confiscated psittacines, 9/9 confiscated hornbills, 3/3 confiscated passerines had antibodies to *C. psittaci* in 2018 albeit low concentration [11-13]. The presence of antibodies to *C. psittaci* indicates that the birds were previously infected or exposed to *C. psittaci* but does not necessarily mean that birds are still infected and are excreting the organism that could be sources of infection. The use of PCR however, has a higher sensitivity, and positive results, especially from fecal samples will mean that the animal is currently shedding the bacteria, which is a zoonotic potential and also a threat to wildlife conservation [14].

In this study, we investigated the presence of *C. psittaci* using molecular methods in 303 fecal samples from different 99 bird species of 11 orders in three zoos and two wildlife facilities to evaluate the active infection with shedding of *C. psittaci* that may serve as an infectious source to other birds and to humans.

2. Materials and Methods

2.1 Sample collections

Three hundred three (303) fecal samples were collected from 99 bird species in three zoos and two wildlife facilities located in the Philippines from February to November 2019 (Table 1). Zoos 1 and 2 are government-owned and are situated in the urbans surrounded by residential and commercial establishments with man-made foliage. Zoo 3 is privately owned and situated in the suburban also surrounded by residential areas. Rescue Center 1 is situated in the urban area and is surrounded by commercial establishments and a man-made park while Rescue Center 2 is situated in the province and in the suburban bordered by a river and a land currently being developed. The birds were mainly *Psittaciformes* (247 samples from 66 species), although *Bucerotiformes*, *Accipitriformes*, *Passeriformes*, *Columbiformes*, *Galliformes*, *Strigiformes*, *Casuariiformes*, *Pelecaniformes*, *Falconiformes*, and *Anseriformes* were included. The population was not showing any clinical signs during the sample collection. The individual feces were collected from the cage floor using sterile cotton swabs, placed in individual 1.5ml tubes, and then transported to the laboratory on ice. The samples were stored at -80 °C until use. The protocols for animal use in this study (2019-0052) have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine, University of the Philippines Los Banos. A gratuitous permit was issued by the Department of Environment and Natural Resources for the use of the animals in this study.

Table 1. The animal facilities and bird species where fecal samples were collected from February to November 2019.

Bird Species	Facility Type					Total
	Zoo 1	Zoo 2	Zoo 3	RC* 1	RC* 2	
Psittaciformes	14	14	89	107	23	247
Bucerotiformes			16		6	22
Accipitriformes		1	5		4	10
Passeriformes			4		2	6
Columbiformes					4	4
Galliformes					4	4
Strigiformes		3			1	4
Casuariiformes					2	2
Pelecanformes					2	2
Falconiformes			1			1
Anseriformes					1	1
Total	14	18	115	107	49	303

*RC = Rescue Center

2.2 DNA extraction and PCR for detecting chlamydial DNA

DNAs were extracted from 100mg of fecal samples using SepaGene (Sekisui Medical, Tokyo, Japan) following the manufacturer's instruction. All samples were screened for chlamydial DNA using primers target for *envB* of *C. psittaci, C. abortus, C. felis, C. caviae* and *C. pecorum*: Env-F AACCTCGGATAGCAAATTAATCTGG and Env-R ATTTGGTATAAGAGCGAAGTTCTGG for generating 152 bp [15]. The reaction was performed with TaKaRa Ex Taq Hot Start Version (TaKaRa Bio, Kusatsu, Japan) on a ProFlex Thermal Cycler (Thermo Fisher Scientific, MA, USA) with initial degeneration at 98°C for 2 min, then 40 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec, then final extension for 5 min at 72°C and soaking at 4°C. Positive results were reconfirmed by real-time PCR targeting 16S rRNA of a broad range of *Chlamydiales* using primers of PanCh 16F2 CCGCCAACACTGGGACT, and PanCh 16R2 GGAGTTAGCCGGTGCTTCTTTAC, with a probe FAM-CTACGGGAGGCTGCAGTCGAGA ATC-BHQ1 [16]. The reaction was performed with the Premix Ex Taq (probe qPCR) (TaKaRa Bio) on a Thermal Cycler Dice TP800 (TaKaRa Bio) following the manufacturer's instructions.

2.3 Sequencing, genetic homology search, and phylogenetic analysis

PCR products targeting *envB* were subjected to agarose gel electrophoresis, and the bands were cut out, purified by silica monolith using MonoFas (Animos, Kawaguchi, Saitama), then submitted to outsourcing sequencing service (Takara Bio) for determining the nucleic acid sequence of DNA by fluorescent dideoxy terminator method. The determined 102 bp nucleic acid sequences were searched for gene homology using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). For phylogenetic analysis, nucleotide sequences were aligned using ClustalW and phylogenetically analyzed using Mega 11 using maximum likelihood method with 1000 bootstrap replicates [17].

2.4 Nucleotide sequence accession numbers

Nucleotide sequences obtained in this study have been deposited at the DNA Data Bank of Japan (DDBJ) under accession numbers LC741338 for PH1, and LC741339 for PH147.

3. Results

3.1 Detection of Chlamydiales DNA by PCR

Two out of 303 samples (0.6%) were positive for *Chlamydiales* DNA by conventional PCR targeting *envB* (Fig. 1). One positive sample named PH1 was from zoo 1 (7.14%; 1/14), and the other named PH147 was from zoo 3 (0.87%; 1/115). These positive samples were retested by real-time PCR targeting 16S rRNA and confirmed *Chlamydiales* infection. The PH1 was from African love bird (*Agapornis* spp.) of *Psittaciformes* and PH147 was from Brahminy kite (*Haliastur indus*) of *Accipitriformes*. Those positive birds were donated by private individuals.

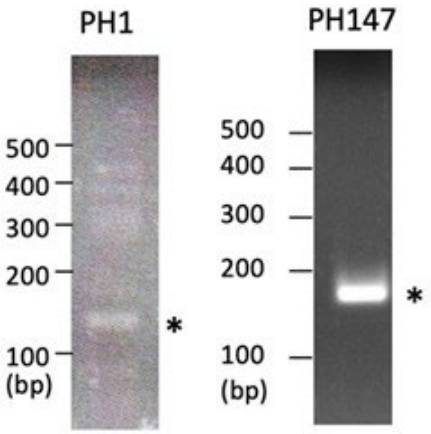

Fig. 1. PCR products of *Chlamydiales* DNA from PH1 and PH147. Agarose gel electrophoresis picture showing PCR bands between 100 to 200 bp. The targeted bands were 152 bp and indicated by asterisks. Numbers indicate base pairs (bp).

3.2 Phylogenetic analysis

The 102 bp of *envB* nucleic acid sequences were successfully determined. The nucleic acid sequences were different between PH1 and PH147 by two nucleic acids. By the genetic homology search, PH1 and PH147 were most adjacent to *C. psittaci* with identity of 100.00% and 98.04%, respectively. By the phylogenetic analysis, PH1 and PH147 were with cluster of *C. psittaci*, *C. abortus and C. buotenis* (Fig. 1). Although the short determined nucleic acid sequence made it difficult to determine the Chlamydial species, the detection of *Chlamydiaceae* by PCR was confirmed by phylogenetic analysis.

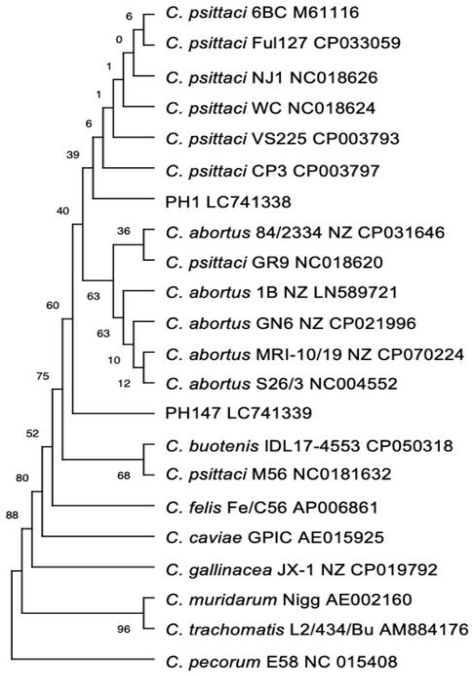

Fig. 2. Phylogenetic analysis of partial *envB* gene. Representative sequences of established *Chlamydiaceae* species were used. Numbers indicate percentages of the bootstrap replicates (n = 1,000). The species and strains are shown, and the accession number of each nucleotide in GenBank is provided. PH1 and PH147 were detected *Chlamydiaceae* in this study.

4. Discussion

In this study, we detected *Chlamydiaceae* in 2 out of 303 samples with cluster of *C. psittaci*, *C. abortus and C. buotenis*, although species could not be determined. The *Chlamydiales* order contains 7 different families, and *C. psittaci* is in *Chlamydiaceae* family that includes 13 species, namely *C. abortus, C. caviae, C. felis, C. muridarum, C. psittaci, C. pecorum, C. pneumonia, C. suis, C. trachomatis, C. avium, C. gallinacean, C. ibidis and C. buotenis* [18]. Of the 13 species, *C. psittaci* has been detected primarily in birds, and *C. avium, C. gallinacean, C. ibidis and C. buotenis* are newly found species in birds in the last decade [19-21]. *C. psittaci* and *C. abortus* are genetically close, and *C. abortus* was previously classified as serotype 1 of *C. psittaci*, but is now reclassified as separate species [22]. Some intermediate chlamydial species have been found, and *C. buotenis* is between *C. psittaci* and *C. abortus* [21]. *C. abortus* primarily infects ruminants especially sheep and goats that are associated with enzootic abortions [23], however, it can also infect birds and humans. In humans, *C. abortus* is a well-known zoonotic agent that most commonly affects pregnant women and causes abortion, stillbirth, gestational septicemia, pelvic inflammatory disease, and recently has been reported to cause atypical pneumonia [23, 24]. In birds, *C. abortus* has been detected in psittacine pet birds with and without symptoms [25, 26]. Although the role of birds in the transmission of *C. abortus* to humans is unclear, the potential risk of *C. abortus* infection from birds to humans is suggested. *C. buotenis* is a recently recognized species and only found in hawks and falcons so far and the pathogenesis and zoonotic potential has not been investigated yet [21, 27]. The *Chlamydiaceae* detected in this study, whether *C. psittaci, C. abortus*, or *C. buotenis*, are potentially pathogenic and zoonotic.

In this study, *Chlamydiaceae* are detected in 2 out of 303 healthy birds (0.6%), and those 2 positive birds were kept in different facilities, with positive rates of 7.14% (1/14) and 0.87% (1/115), respectively. This prevalence was similar to the prevalence in captive birds in Asian countries. In Japan, the molecular prevalence of *C. psittaci* has decreased from 5-8 % around 2006 to less than 1% in the last decade [25, 28], and 3.1% in Taiwan [29]. In the Philippines, the molecular prevalence found in this study was much lower than the seroprevalence of *C. psittaci* that were reported to be 25% (6/36) [10] and 100% [11-13] of confiscated wild birds. In general, seroprevalence is higher than molecular prevalence, which detects pathogenic nucleic acids. The presence of antibodies to *C. psittaci* indicates that the birds were previously infected or exposed to *C. psittaci* but does not necessarily mean that birds are still infected. And more, we used fecal samples for molecular detection of *Chlamydiacae* and all birds were apparently healthy during sample collection. In asymptomatic birds, even when infected, the shedding of *C. psittaci* is known to be intermittent and copy numbers of Chlamydial DNA in the samples might be limited [1]. By the meta-analysis of published papers, and prevalence of *C. psittaci* using non-PCR techniques was significantly higher than that using PCR techniques [14, 30]. In Colombia, the molecular prevalence of *C. psittaci* using feces was 29.9% [30] while seroprevalence has been reported to be 85% [31]. The antibody concentration against *C. psittaci* detected in previous studies in the Philippines were not high; of 27 birds serologically positive for *C. psittaci*, 26 had low positive with a concentration of <1:50 and 1:50, and one had positive with 1:200 [10-13]. These serologically positive birds had no clinical signs and assumed that active infection was

not seen by seroprevalence. The low molecular prevalence of *C. psittaci* in this study supports the low concentration of antibodies against *C. psittaci* in previous studies in the Philippines. The 2 birds positive for *Chlamydiaceae* were kept in zoos and the people frequenting the area as well as visitors might be at some risk of contracting the bacterium. Outbreaks of psittacosis had been reported in zoos and bird parks where infected animals were kept [33]. It is also important to be careful about *C. psittaci* infection in non-avian species because there are a variety of susceptible animals in zoos. Mammals may also be affected by the disease evidenced by an outbreak of psittacosis in a Siberian elk (*Alces alces cameloides*) in a zoo [33]. Although the active shedding of *C. psittaci* in bird feces was found to be low in the investigated population, attention must be paid to prevent transmission to other birds, mammalian animals, and humans. In order to estimate the risk accurately, isolation of *Chlamydiaceae* and determination of full-length sequence is desirable in the future.

5. Conclusions

In this study, we detected *Chlamydiaceae* in 2 out of 303 samples with cluster of *C. psittaci*, *C. abortus, and C. buotenis*, which are potentially pathogenic and zoonotic. Although the active shedding of *Chlamydiaceae* in bird feces was found to be low in the investigated population, attention must be paid to prevent transmission to other birds, mammalian animals, and humans.

Abbreviations

ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; IACUC, Institutional Animal Care and Use Committee

Author Contributions

Conceptualization, RBO and YSO; Methodology, YSO, RBO, and LLLL; Investigation, RBO, YSO, MSA and LLLL; Writing – Original Draft, MSA, RBO and YSO; Writing – Review & Editing, RBO and LLLL; Funding Acquisition, YSO; Resources, RBO and LLLL; Supervision, RBO, and YSO.

Ethics Approval and Consent to Participate

The protocols for animal use in this study have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine, University of the Philippines Los Banos (Protocol No. 2019-0052). A gratuitous permit was issued by the Department of Environment and Natural Resources for the use of the animals in this study (Wildlife Gratuitous Permit No. 289 and XI-2020-11).

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Conflict of Interest

The authors declare no conflict of interest.

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