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Prevalence and genotype of *Chlamydia psittaci* in faecal samples of birds from zoos and pet markets in Kunming, Yunnan, China

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Abstract: *Chlamydia psittaci* is an important zoonotic pathogen in birds and may be transmitted to humans and result in severe respiratory disease. To assess the prevalence and genotype of *C. psittaci* in birds in Kunming, Yunnan, China, a total of 136 specimens of psittacine birds involving 8 species were collected from the city's zoos (*n*=60) and pet markets (*n*=76). The frequency of *C. psittaci* infection was 19.9% (27/136) in the psittacine birds. The prevalence of *C. psittaci* was higher in pet birds (26.3%; 20/76) than in zoo birds (11.7%; 7/60) (*P*=0.034). In particular, among *Agapornis fischeri*, the *C. psittaci* infection (50%; 10/20) was significantly more frequent in the pet markets than in the zoos (*P*=0.006). In addition, the highest prevalence of 41.2% (7/17) was found in *Ara ararauna*. To determine the genotype of *C. psittaci*, 23 *OmpA* gene fragments (about 1.4 kb) in 27 positive samples were successfully amplified and sequenced. Phylogenetic analysis showed that all the 23 strains belonged to genotype A. Our results demonstrate the high prevalence of *C. psittaci* genotype A infection in psittacine birds in Yunnan Province, suggesting a potential threat to human health in this area. Therefore, it is necessary to take effective measures to prevent the spread of *C. psittaci* among psittacine birds, as well as among employees and customers.

Key words: *Chlamydia psittaci*, Genotype, *OmpA*, Prevalence, Yunnan
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1 Introduction

Chlamydia psittaci is a major causative agent of psittacosis. It most frequently infects Psittaciformes but can also infect many other avian species, as well as a wide range of mammalian hosts (Kaleta and Taday, 2003; Zhu *et al.*, 2013). The highest infection rates are identified in psittacine birds and pigeons (Donham and Zejda, 1992; Pilny *et al.*, 2012). It can infect humans by inhalation or close contact (Beeckman and Vanrompay, 2009). Human infection with *C. psittaci* can induce psittacosis, a disease that occasionally leads to severe pneumonia, headache, chills, malaise, and myalgia (Fraeyman *et al.*, 2010; Osman *et al.*, 2013). Human psittacosis is a notifiable disease in the USA, Australia, and most European countries (Harkinezhad *et al.*, 2009a). There have been 6500 cases reported from 1996 to 2007 in these regions (Harkinezhad *et al.*, 2009b). However, no vaccines or valid agents are available against *C. psittaci*.

Yunnan is the richest in bird species in China on account of its special geographical location and climate. It is reported that about 1288 species of birds inhabit China, most of which can be found in Yunnan (http://avibase.bsc-eoc.org). *C. psittaci* infection in birds is prevalent throughout the world (Geigenfeind *et al.*, 2012). However, there is limited information on the prevalence and the genotypes of *C. psittaci* in China. The aim of this study was to assess the

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prevalence and the genotypes of *C. psittaci* in the faeces of birds from zoos and pet markets in Kunming City of Yunnan Province, China.

2 Materials and methods

2.1 Ethics statement

Before specimen collection, we contacted the administrators of the zoos and pet markets and obtained their permission to investigate their animals. All animal work followed guidelines in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals, and was approved by the Animal Ethical Committee of Kunming University of Science and Technology, China.

2.2 Sample collection and DNA extraction

A total of 136 fresh fecal samples from psittacine birds were collected from two zoos (n=60) and five different pet markets (n=76) in Kunming, Yunnan, China. Potassium dichromate was washed off fecal specimens with distilled water by centrifugation at 1500*g* for 10 min 4 times at room temperature. DNA was extracted from 200 mg feces using QIAamp DNA Stool Mini kit (catalog No. 51304; Qiagen, Inc.) according to the manufacturer's instructions. The DNA was eluted into 200 μl of Qiagen elution buffer and stored at −70 °C.

2.3 Real-time PCR

C. psittaci was detected by the TaqMan minor groove binder (MGB) fluorescent real-time quantitative polymerase chain reaction (qPCR) assay that targeted the *OmpA* gene. The primers and probe were: forward, 5'-TGTGATTCACAAACCAAGAGGCTATA-3'; reverse, 5'-CGAGGCCTACTTGCCATTCA-3'; probe, (FAM)5'-TATGTTTAGGCATCTAAAAC-3' (MGB). In brief, the mixture for one reaction contained 12.5 μl of 2× TaqMan universal PCR master mix, final concentrations of 900 nmol/L each of the forward and reverse primers, 250 nmol/L of MGB probe, and 1 pmol of the template DNA, and nuclease-free water (catalog No. P1193; Promega) was added to give a final volume of 25 μl. Real-time PCR was performed with a Corbett Rotor-Gene 6000 (catalog No. 65H0; Corbett Life Sciences) under the following cycling conditions: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

2.4 Amplification of *OmpA* gene and sequencing

All the *C. psittaci*-positive DNAs identified by TaqMan MGB real-time PCR were used to perform the amplification of major outer-membrane protein gene A (*OmpA*) fragment as previously described (de Freitas Raso *et al.*, 2006). The specific primers were as follows: 5GPF, 5'-ACGCATGCAAGACACTCCAAAGCC-3' (forward primer); 3GPB, 5'-ACAAATTCCTAGGTTCTGATAGCGGGA-3' (reverse primer), generating a product of about 1460 bp. PCR reaction was carried out using the Premix Taq™ Hot Start version (TaKaRa, Dalian, China) under the following conditions: 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min, with a final extension 72 °C for 5 min. Lastly, the obtained products were analyzed by agarose gel electrophoresis, the positive PCR products were purified using PCR Product Gel Extraction Kit (Tiangen, Beijing, China) and then were sequenced by Invitrogen Biotechnology Co. (Guangzhou, China).

2.5 Phylogenetic analysis and genotyping

To determine the genotype of *C. psittaci*, phylogenetic analysis was performed based on the 1460-bp *OmpA* gene fragments obtained. The representative sequences of *C. psittaci* involving genotypes A, B, C, D, E, E/B, and F were downloaded from the GenBank database. Then, a dataset for the analysis of the *OmpA* gene was composed of the reference sequences and the resulting sequences. The alignment and manual editing of multiple sequences generated were conducted using Clustal 1.8.3 and BioEdit 7.0, respectively. A phylogenetic tree was constructed using the neighbor-joining method based on the Kimura 2-parameter model with 1000 bootstrap replicates and a transition-transversion ratio of 2.0 applied in MEGA 6.02.

2.6 Statistical analysis

Differences in *C. psittaci* infection rates between zoos and pet markets were analyzed using the SPSS software (Release 18.0 standard version, SPSS Inc., Chicago, Illinois, USA). P<0.05 was considered statistically significant.

3 Results

3.1 Prevalence of *C. psittaci*

A total of 136 specimens of psittacine birds were collected from zoos (n=60) and pet markets (n=76). The psittacine birds contained eight different subspecies, including *Ara ararauna*, *Cacatua galerita*, *Agapornis fischeri*, *Psittacula eupatria*, *P. derbiana*, *Moustached parakeet*, *Melopsittacus undulatus*, and *P. cyanocephala*. The infection rates of *C. psittaci* are given in Table 1. The frequency of *C. psittaci* infection was 19.9% (27/136; 13.2%–26.6%) in the psittacine birds. From the subspecies of psittacine birds, *Ara ararauna* had the highest prevalence rate of 41.2% (7/17; 17.8%–64.6%), followed by *Agapornis fischeri* 33.3% (10/30; 16.5%–50.2%), *Melopsittacus undulatus* 20% (4/20; 2.5%–37.5%), *P. eupatria* 18.2% (2/11; 4.6%–40.9%) and *Cacatua galerita* 17.4% (4/23; 1.9%–32.9%). *C. psittaci* was detected in 26.3% (20/76; 16.4%–36.2%) and 11.7% (7/60; 3.5%–19.8%) of the samples from pet birds and zoo birds, respectively. The infection rate of *C. psittaci* in pet birds was significantly higher than that in zoo birds (P=0.034). In particular, in *Agapornis fischeri*, *C. psittaci* infection (10/20; 50%) was significantly higher in pet market birds than in zoo birds (P=0.006). No other statistically significant difference was found between pet and zoo birds.

3.2 Genotype analysis

Among the positive samples of *C. psittaci* detected using real-time PCR, 23 *OmpA* gene fragments (1.4 kb) were successfully amplified and sequenced, with a success rate of 85.2% (23/27). The sequences obtained shared 99.6%–100.0% nucleotide identity with each other, and had the highest similarities (99.5%–99.9%) with the MN Zhang strain derived from human *C. psittaci* infection. Phylogenetic analysis showed that all the 23 strains belonged to genotype A (Fig. 1).

4 Discussion

C. psittaci is an avian pathogen and is generally prevalent in wild birds, pet birds, and poultry. However, it can cause zoonotic disease in humans. Humans have been reported to face a particular risk for the transmission of *C. psittaci* from birds, especially Psittaciformes. The purpose of this investigation was to survey and collect baseline data in China on the prevalence of *C. psittaci* in psittacine birds. To our knowledge, the current study is the first report concerning the molecular epidemiology of *C. psittaci* in psittacine birds in Kunming, Yunnan, China.

In this study, *C. psittaci* was detected in 27 out of 136 fresh fecal samples (19.9%) in psittacine birds using TaqMan MGB real-time PCR, which is a highly sensitive and specific technique for detecting chlamydial DNA. Previous studies have reported the prevalence of *C. psittaci* in pet birds, in market-sold adult chickens, ducks, and pigeons, and in the domestic goose, *Anser domestica.* Cong *et al.* (2013) investigated the frequency of *C. psittaci* infection among birds from markets in adult chickens (13.32%), ducks (38.92%), and pigeons (31.09%). Later, the same group reported a 10.8% infection rate of *C. psittaci* in pet birds by the indirect haemagglutination assay (IHA) in Gansu Province (Cong *et al.*, 2014). A recent study of *C. psittaci* infection among pet parrots

Table 1 Differences of *Chlamydia psittaci* infection between zoo birds and pet birds

Psittacine species	Total (infection rate (%))*	Positive number/negative number		χ^2	P-value#
		Zoo	Pet market		
Ara ararauna	17 (41.2)	7/10	0/0		
Cacatua galerita	23 (17.4)	0/8	4/11	2.58	0.108
Agapornis fischeri	30 (33.3)	0/10	10/10	7.50	0.006
Psittacula eupatria	11 (18.2)	0/6	2/3	2.93	0.087
Psittacula derbiana	9 (0)	0/4	0/5		
Moustached parakeet	9 (0)	0/5	0/4		
Melopsittacus undulatus	20 (20.0)	0/4	4/12	1.25	0.264
Psittacula cyanocephala	17 (0)	0/6	0/11		
Total	136 (19.9)	7/53	20/56	4.52	0.034

* The percentage is the infection rate of *C. psittaci* in the respective species. # Statistical significance was determined by Fisher's exact test

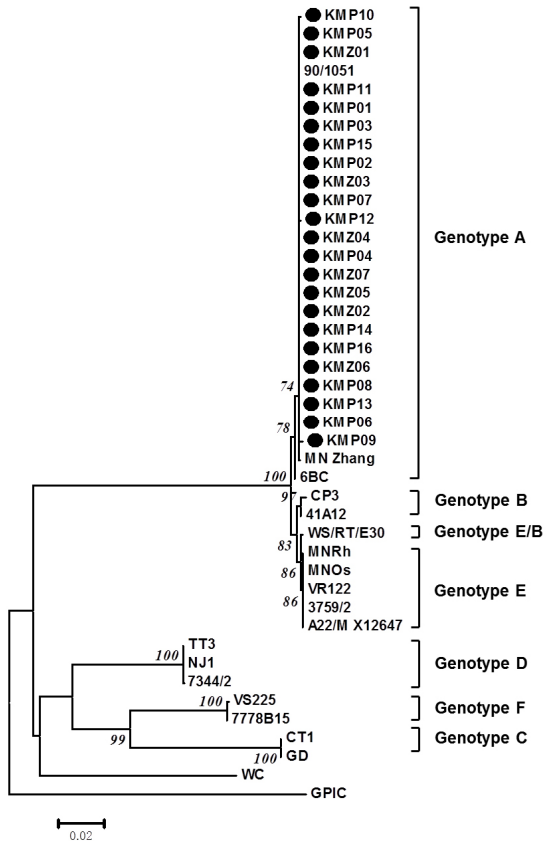

Fig. 1 Phylogenetic tree constructed by the neighbour-joining method based on partial nucleotide sequences of *OmpA* of *C. psittaci* (1054 bp)

The *C. psittaci* groups were indicated by alphabets A–F. Bootstrap value was shown if the reliability was greater than 70%. The *OmpA* gene sequences obtained were submitted to GenBank and assigned accession numbers HM450387–HM450409, respectively. The black circles showed the strains measured in this study

in the cities of Beijing and Weifang reported a prevalence of 35.37% (110/311) (Zhang *et al.*, 2015). In addition, compared with the data of *C. psittaci* infection in other countries, the infection rate of 19.9% observed in psittacine birds in our study was higher than that in the Netherlands (7.9%), but lower than that of 22.9% in Japan (Tanaka *et al.*, 2005; Heddema *et al.*, 2006). Taken together, it is difficult to compare different studies; the reasons may be due to differences in environment, diagnostic methods, feeding conditions, as well as animal husbandry practices and animal welfare.

In comparison, the overall prevalence of *C. psittaci* infection in psittacine birds in Yunnan Province was relatively higher than that in other Chinese provinces. Yunnan has a climate and geographical environment in which factors such as temperature, moisture, wetness, and landscape may make it easy for *C. psittaci* to establish infection and spread among birds.

Another important finding of this study is that the pet market specimens had a higher positive rate (26.3%) of *C. psittaci* than the zoo samples (11.7%) in psittacine birds (P=0.034). This high frequency of *C. psittaci* infection in pet birds may be attributed to group feeding, mixed feeding, stacked cages, and lack of ventilation systems in the pet markets. Furthermore, pet-shop owners or caretakers may not clean up fecal debris or spilled food in a timely manner. The pet birds could be regarded as the potential reservoirs of zoonotic psittacosis and they may increase the risk of human infection in this region. Our results suggested that people who have contact with pets should be made aware of the possibility of contradicting psittacosis from their pets.

Our results indicated that the highest infection rate (41.2%) was identified in *Ara ararauna*, suggesting that this is the most susceptible to *C. psittaci* among psittacine birds investigated. Of note, all the *Ara ararauna* with *C. psittaci* infection were from zoos. Regarding *Ara ararauna* and group feeding in zoos, the birds have limited space and remain as nestlings for longer. Therefore, once an outbreak of psittacosis is initiated, it easily spreads among *Ara ararauna*, in accordance with a previous report that the prevalence of *C. psittaci* is high among macaws (37.8%) (de Freitas Raso *et al.*, 2006). Furthermore, only the subspecies of *Ara ararauna* tested positively for *C. psittaci* DNA in psittacine birds from zoos, implying a single source of *C. psittaci*. Therefore, the relevant managerial staff should adopt effective measures to control the spread of the agent from *Ara ararauna* at the earliest.

Our results reveal a high prevalence of genotype A of *C. psittaci* in the Kunming region, supporting the conclusion that the *C. psittaci* genotype A was the major genotype associated with parrots (Zhang *et al.*, 2015). In particular, previous studies reported an outbreak of psittacosis due to the *C. psittaci* genotype A in humans and birds, indicating that *C. psittaci* genotype A may be a strain capable of bird-to-human transmission (Andersen, 1991; Vanrompay *et al.*, 1997; Heddema *et al.*, 2006). Thus, the high

prevalence of genotype A of *C. psittaci* in this study should raise significant concerns. Although no pet-bird owners or veterinarians have ever presented with symptoms of psittacosis, further studies are necessary to investigate the prevalence and genotype of *C. psittaci* in the human population.

Our study had several limitations. First, comparisons of results from this study with previous *C. psittaci* infection epidemiological observations are difficult because of different test methods. Although the sensitivity of serological methods is low, the IHA was the most common method of determining *C. psittaci* infection in some previous investigations. However, we used a highly sensitive and specific technique of TaqMan MGB real-time PCR method. Second, our study used a small sample size and a single fecal specimen, and lacked some demographic information involving age, gender, and source.

5 Conclusions

The results of the present study revealed a high *C. psittaci* infection in psittacine birds in Yunnan, China, which poses a potential threat to human health in this area. Therefore, it is necessary to take effective measures to prevent the spread of *C. psittaci* among psittacine birds, as well as among employees and customers.

Compliance with ethics guidelines

Yue FENG, Yue-mei FENG, Zhong-hua ZHANG, Shao-xiong WU, Du-bo ZHONG, and Chen-jian LIU declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：云南省昆明市动物园和宠物市场中鸟类鹦鹉热衣原体的流行病学调查及其基因型分布研究

目　的：调查云南省昆明市动物园和宠物市场的鹦形目鸟类中鹦鹉热衣原体的流行情况。

创新点：首次在云南地区开展鹦形目鸟类中鹦鹉热衣原体的流行病学调查，发现该地区鹦形目鸟类中具有较高的鹦鹉热衣原体感染率。

方　法：本研究共采集8种鹦形目鸟类的新鲜粪便样本136份，其中动物园样本60份，宠物市场样本76份。首先，利用高灵敏和高特异性的TaqMan MGB探针荧光定量聚合酶链反应（qPCR）方法检测粪便样品中鹦鹉热衣原体的感染率，然后针对阳性样品，利用PCR技术进行鹦鹉热衣原体*OmpA*基因扩增、纯化、测序以及基因型分析。

结　论：本实验结果显示：鹦鹉热衣原体的感染率为19.9%（27/136），其中宠物市场鸟类中鹦鹉热衣原体的感染率（26.3%；20/76）明显高于动物园（11.7%；7/60）（P=0.034）；金刚鹦鹉中感染率最高，达41.2%（7/17）。同缘关系进化树分析表明，这些鹦鹉热衣原体都属于易于由鸟向人跨物种传播的A型。综上所述，昆明地区鹦形目鸟类中具有较高的鹦鹉热衣原体流行，给饲养人员和观鸟者的健康带来了潜在的威胁。

关键词：鹦鹉热衣原体；基因型；*OmpA*基因；流行病调查；云南