

Short Paper

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Detection of *Chlamydia psittaci* and *Chlamydia ibidis* in the Endangered Crested Ibis (*Nipponia nippon*)

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Abstract

Chlamydia spp. are a group of obligate intracellular pathogens causing a number of diseases in animals and humans. Avian chlamydiosis (AC), caused by *Chlamydia psittaci* (*C. psittaci*) as well as new emerging *C. avium*, *C. gallinacea* and *C. ibidis*, have been described in nearly 500 avian species worldwide. The Crested Ibis (*Nipponia nippon*) is a world endangered avian species with limited population and vulnerable for various infections. To get a better understanding of the prevalence of *Chlamydia* spp. in the endangered Crested Ibis, faecal samples were collected and analysed. The results confirmed that 20.20% (20/99) of the faecal samples were positive for *Chlamydiaceae* and were identified as *C. ibidis* with co-existence of *C. psittaci* in one of the 20 positive samples. In addition, *ompA* sequence of *C. psittaci* obtained in this study was classified into the provisional genotype Matt116, while that of *C. ibidis* showed high genetic diversity, sharing only 77% identity with *C. ibidis* reference strain 10-1398/6. We report for the first time the presence of *C. ibidis* and *C. psittaci* in the Crested Ibis, which may indicate a potential threat to the endangered birds and should be aware of the future protection practice.

Introduction

Chlamydia spp. are a group of obligate intracellular bacteria that cause a number of important diseases in animals and humans. Avian chlamydiosis (AC), caused by *Chlamydia psittaci* (*C. psittaci*), has been shown to occur in nearly 500 avian species worldwide, including domestic, companion and wild birds [1]. Recently, new emerging avian chlamydial pathogens, such as *C. avium*, *C. gallinacea*, *C. ibidis* (*Candidatus* taxon) and *C. buteonis* have been described [2–4]. Birds infection with *Chlamydia* spp. usually show respiratory, ocular and enteric symptoms occasionally with fatal outcome, but asymptomatic, latent infections are also common. The clinical signs vary greatly in severity and depend on the species and age of the bird, as well as the causative strain involved [1]. Shedding of *Chlamydia* spp. through respiratory and intestinal routes occurs intermittently by infected birds, which represent a reservoir of infection [5]. Notably, *Chlamydia* species hosted by birds may present risks of zoonotic infections to humans. Several reports have shown the prevalence and diversity of *Chlamydia* spp. in domestic poultry and pet birds in China. A nationwide survey of *Chlamydia* spp. in domestic birds including chickens, ducks, geese and pigeons has shown that *C. gallinacea* and *C. psittaci* presented in all four avian species investigated, and *C. gallinacea* is the endemic chlamydial species in chickens, whereas *C. psittaci* dominates only in pigeons [6]. Investigation and genotyping of *Chlamydia* spp. in pigeons in Northern China showed that the total prevalence was 20.4% (215/963) and was identified as genotype B of *C. psittaci* [7]. Recently, an outbreak of *Chlamydia*-related egg production declines in domestic duck farms has been reported due to the infection of *C. psittaci* (genotype A) in Southeast of China [8]. In addition, this pathogen has also been detected and isolated occasionally from pet birds such as parrots [9]. These reports suggest domestic and companion birds might be a neglected reservoir of chlamydial pathogens which may threaten the poultry production and present risks to public health in China. However, there is no data about the prevalence of *Chlamydia* spp. in wild birds, which may also be reservoirs for poultry and human infections.

The Crested Ibis (*Nipponia nippon*) is a rare and precious species in the world and is red-listed as 'Endangered' by the International Union for Conservation of Nature. This bird was thought to be extinct in the wild until the last seven individuals were rediscovered in China in 1981 [10]. Since then, great efforts have been made on the protection of Crested Ibises. The total number of Crested Ibis has now increased to over 3000. Most of the Crested Ibis

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inhabit in areas of Qinling Mountains in Shaanxi province, China, while a certain number of the birds are in Sado Island in Japan, which have been introduced from China [11]. However, recovery of the Crested Ibis population is still difficult due to small population size, low genetic diversity as well as habitat loss, winter starvation and persecution [12, 13]. In addition, diseases and undetermined fitness also pose a threat to the Crested Ibis [14]. The wide distribution and broad-host infection of avian chlamydial pathogens may also exhibit a potential healthy risk to the endangered Crested Ibis. To get a better understanding of the prevalence of *Chlamydia* spp. in Crested Ibis, we analysed faecal samples of Crested Ibis collected in a breeding centre in Shaanxi, China.

Materials and methods

The faecal samples were collected from the Crested Ibis in Shaanxi Rare Wildlife Rescue and Breeding Research Center, Louguantai, a Qinling Mountain area in Xi'an, Shaanxi province, China. There were 206 of the captive-bred Crested Ibis living in this area during the sampling time. The living area (about 3600 m²) of Crested Ibis was segregated by nets to avoid the escape of the birds and also to keep away other animals which may threaten them. In this Center, the living area was divided into 44 cages. The birds were fed and given water by specialized breeders of the Center every day. When the birds were routinely fed, a clean plastic sheet was put on the floor nearby the feeding site in each cage. The plastic sheet was removed after the Crested Ibis finished their meal and get away. Only fresh faecal samples were collected from the sheet. Faecal samples were collected only once from each cage. A total of 99 faecal samples were random collected from this population. A formal ethical approval is not required for this kind of study and sampling was performed by a specialized breeder of the Crested Ibis during routine feeding activities. At the time of sampling, all the birds showed no evident signs of disease. The faeces were transported on ice to the laboratory and stored at -80 °C until use.

DNA extraction from faeces was performed using the commercial QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's protocol. Extracted DNA from faeces was initially screened by using a *Chlamydiaceae*-specific real-time polymerase chain reaction (PCR) targeting the 23S rRNA gene fragment [15]. A plasmid containing a fragment of 23S rRNA gene of *C. abortus* was used as a positive control. All *Chlamydiaceae*-positive DNA were retested with genus-specific real-time PCR assays to identify avian *Chlamydia* species including *C. psittaci* [16], *C. avium* [17], *C. gallinacea* [17] as well as *C. abortus* [16], which was also detected in birds [18]. For the detection of *C. ibidis*, a pair of primers and probe were designed according to the *ompA* sequences (GenBank access no: MN106768) obtained from this study. The oligo sequences were synthesized as a forward primer (5'-TCTTTGGGAATGTGGTTGG-3'), reverse (5'-GGTATCCTTCTC CGTCCCAG-3') and the probe (5'-FAM-CCGCAGCGCAA-TTCAAGTGCACAMRA-3'). Positive DNA samples were further confirmed by amplification and sequencing of specific fragments of *ompA*, 16S rRNA and 16S rRNA-23S rRNA intergenic spacer together with 23S rRNA domain I (IGS-23S rRNA) as described previously [19–21]. PCR amplicons were sent to an external company (Shanghai Sangon Biotech, China) for sequencing. The obtained sequences were deposited in the GenBank database.

The data were analysed using MEGA 5.05 software [22]. Amplicons were subjected to BLAST analysis against the

GenBank database (NCBI) to identify related entries and aligned with a panel of *Chlamydia* reference strains. To assess the phylogenetic relationship between *Chlamydia* spp. and the tested samples, phylogenetic trees for 16S rRNA (1358 bp) and IGS-23S rRNA (990 bp) as well as *ompA* (940 bp) were constructed by the neighbour-joining method with 1000 replicates' bootstrap using the Maximum Composite Likelihood model with MEGA 5.05.

Results and discussion

The results of real-time PCR assay showed 20 of the 99 collected faecal samples were positive for *Chlamydiaceae* giving a positive rate of 20.20%. One of the 20 positive DNA samples was identified as *C. psittaci* by the species-specific real-time PCR, while no *C. avium*, *C. gallinacea* or *C. abortus* was detected. To further identification and confirmation of the *Chlamydia* species in the faeces of Crested Ibis, all *Chlamydiaceae*-positive DNA were subjected to amplification and sequencing for the specific fragments of 16S rRNA, IGS-23S rRNA and *ompA* genes. Out of the 20 positive samples, six amplicons for 16S rRNA (MN093379-MN093384), seven for IGS-23S rRNA (MN096559-MN096565) and six for *ompA* (MN106768-MN106773) were successfully acquired and sequenced. BLAST analysis against the GenBank database led to the identification of *C. ibidis* and confirmation of *C. psittaci* existence in the faeces of Crested Ibis. Based on the *ompA* sequences of *C. ibidis* obtained in this study, a pair of primers and probe for real-time PCR detection were designed and the *Chlamydiaceae*-positive DNA was retested. The results showed that all the 20 positive DNA samples were identified as *C. ibidis*. Furthermore, dendrograms were constructed on the basis of the obtained 16S rRNA, IGS-23S rRNA and *ompA* gene fragments aligning with *Chlamydia* species reference sequences available in GenBank and altogether showed a similar topology. All sequences obtained in this study were assigned to either *C. psittaci* or *C. ibidis* within the *Chlamydiaceae* family (Fig. 1). These results suggested that the Crested Ibis was a host of this novel *Candidatus Chlamydia* species with *C. psittaci* co-existence occasionally.

The *ompA* gene, coding the major outer membrane protein (MOMP), is one of the sources of diversity among chlamydial genomes in some species including *C. psittaci* [23, 24]. Genotyping using *ompA* sequencing was employed commonly to detection and identification of *C. psittaci* strains. Based on the sequences of *ompA*, nine classical genotypes were described in *C. psittaci* (A to F, E/B, M56 and WC), along with a number of provisional genotypes (CPX0308, YP84, R54, Matt116, 6N, 1V, I and J) which were untypable so far [23, 24]. The *ompA* sequence of *C. psittaci* we obtained in this study was identical to a parrot isolated strain Matt116 (GenBank access: AB284058.1), which was classified into the provisional genotype Matt116 (Fig. 1d) [24]. It was evident that *C. psittaci* strains of each genotype were related to a certain range of hosts and pathogenicity [25]. Considering that the strain Matt116 was isolated among psittacine birds during an AC outbreak, *C. psittaci* strains classified into this genotype might be of high virulence. Therefore, the presence of this pathogen should be aware, especially in the endangered Crested Ibis, even though it was identified only in one sample.

In this study, the data provided evidence of the presence of *C. ibidis* in the faecal samples of Crested Ibis. To further characterization of the prevalent strains, we analysed the obtained *ompA* sequences (MN106768, MN106770–MN106773). The results showed that these sequences matched totally, while shared only

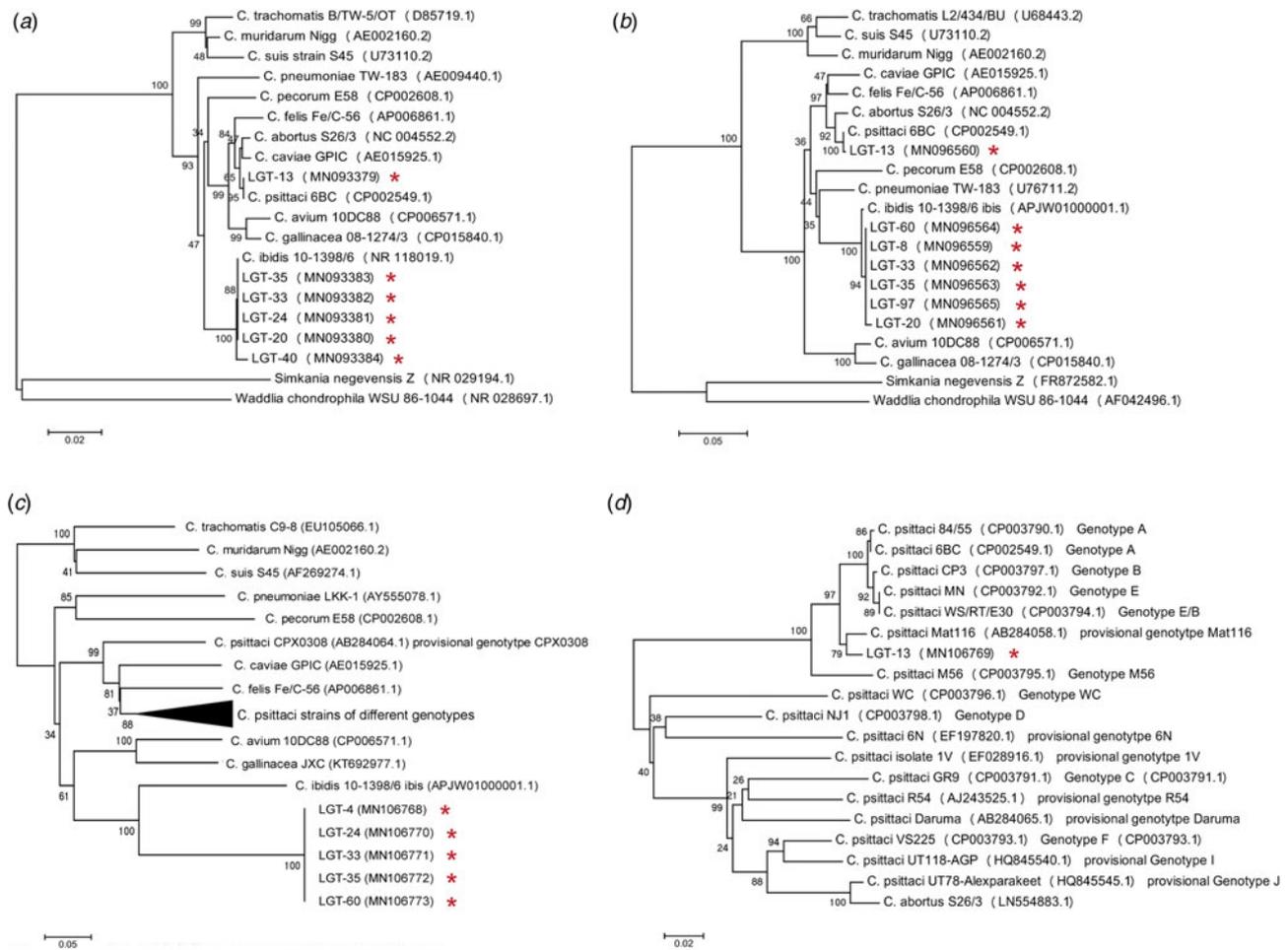


Fig. 1. Phylogenetic tree based on 16S rRNA gene fragment (1356 bp) (a), 16S-23S intergenic spacer and full length of 23S rRNA domain I fragment (923 bp) (b) and *ompA* gene fragment (720 bp) of all *Chlamydiaceae* members (c) and *C. psittaci* strains of different *ompA* genotypes (d). Representative sequences of established *Chlamydiaceae* species as well as the sequences obtained from this study (GenBank accession number was marked by red asterisks) were included. *S. negevensis* strain Z and *Waddlia chondrophila* strain WSU 86-1044 were used as an outgroup. Based on these alignments, phylogenetic trees were constructed by the neighbour-joining method using the Maximum Composite Likelihood model with MEGA 5.05.

77% identity with that of the *C. ibidis* reference strain 10-1398/6 (data not shown). As described above, *ompA* sequences variation was common among strains in a chlamydial species, which represented by the variations of amino acid sequences of MOMP coded by *ompA* genes [26]. To determine whether the nucleotide mismatches were random or with regularity between the obtained and reference sequences, the putative amino acid sequences of MOMP coded by *ompA* genes were also analysed. It presented four regions of amino acid sequences variations among the obtained and reference sequences, namely VD1–VD4, with a few amino acid substitutions between VD2 and VD3 (Fig. 2). These data were coincidence with the previous works that the amino acid sequences of MOMP were interspersed by four short variable domains (VD1–VD4) whose sequences vary among different strains of genotypes or serotypes within the species of *C. trachomatis* or *C. pecorum* [27, 28]. In addition, when aligning the MOMP sequences of *C. trachomatis* strains belonging to different biovars (trachoma biovar, genital tract infection biovar and lymphogranuloma venereum biovar), it also presented four variation regions, which were almost the same at positions and in sizes as that shown in Figure 2 (data not shown). These data suggested that *C. ibidis* was a species of high genetic diversity.

It also implied that the *C. ibidis* strains circulated in Crested Ibis presented a novel *ompA* genotype that differed from the reference strain isolated previously.

C. ibidis was first identified in feral African Sacred Ibises (*Threskiornis aethiopicus*) in western France and was confirmed as a new candidatus species of the family *Chlamydiaceae* [3]. However, this *Chlamydia* species has not yet been detected in any other birds or elsewhere since its discovery. In this study, we provided evidence for the presence of *C. ibidis* in the Crested Ibis. It seemed that *C. ibidis* was a species of highly genetic diversity, and the genetic diversity might be related to the hosts and/or geographic origin of the strains. Although no evidence showed the pathogenicity of *C. ibidis* in either feral African Sacred Ibises or Crested Ibises yet, due to the less isolated strains and few known infected hosts, intensive investigate is still needed to uncover the prevalence, transmission and pathogenicity of this new emerging *Chlamydia* species. However, *C. psittaci*, the major caused agent of AC, is a highly pathogenic species with various genotypes, knowing for its wide distribution and high prevalence in birds. The *C. psittaci* strains of different genotypes present relative host specificity, and most of them have been identified in cases of zoonotic transmission [25]. The harbouring and

<i>Chlamydia ibidis</i> 10-1398/6	1	MKLLKSVLLSTAMGSALSLQALPVGNPAPESLLIDGTMWEGAAGDPCPCATWCDAISV	60
LGT-24	1	MKLLKSVLLSTAMGSALSLQALPVGNPAPESLLIDGTMWEGAAGDPCPCATWCDAISV	60
VD 1			
<i>Chlamydia ibidis</i> 10-1398/6	61	RAGFYGDYVDFRVLKVDVAKTIQMGPAPTASNTSGIVATANKTQVD--RNLLAYGKHLQD	119
LGT-24	61	RAGFYGDYVDFR+LK D KT MG A T +N S V+T + R N AYGKHLQD	120
VD 2			
<i>Chlamydia ibidis</i> 10-1398/6	120	AEWCTNAAFALALNIWDRFDVFCFLGACSGYFKGNSAAFNLVGLLGIQGTDLSDQPPNIT	178
LGT-24	121	AEWCTNAAFALALNIWDRFDVFCFLGACSGYFKGNSAFNL+GL G+ T ++ + PN	180
<i>Chlamydia ibidis</i> 10-1398/6	179	LSQAVVEMYTDTSTFASVSGARGSLWECGCATLGAEFQYAAQAKPKVEELNAICGVAQFTVH	238
LGT-24	181	L+Q VEMYTDTSTF WSVGARG+LWECGCATLGAEFQY+QAKPK+EELNAIC AQFTVH	240
VD 3			
<i>Chlamydia ibidis</i> 10-1398/6	239	KPKGYKNQAFPLPADAGTSAAGTKSATVDYHEWQVGMALSYRLNMLVPYVGVKWSRASY	298
LGT-24	241	KPKGYKN PLP DAG A TK+A++DYHEWQVGMALSYRLNMLVPYVGVKWSRASY	300
VD 4			
<i>Chlamydia ibidis</i> 10-1398/6	299	DADTIRIAQPKL--SEYILNLTALNPITLLGQATTVDSSNKLEDFMQLVSLQINKMKSRSKS	356
LGT-24	301	DADNIHIAQPRLDPAESILGLTAWNPTFQSSNDV-TSNKLETFMQIASLQINKMKSRSKS	359

Fig. 2. Alignment of the two amino acid sequences of MOMP from *C. ibidis* reference strain (10-1398/6) and a field prevalent strain (LGT-24) in the Crested ibis in China.

shedding of *C. psittaci* by the Crested Ibis may pose a potential risk to the whole population and as a source of environmental contamination.

Finally, there are some limitations to this study. The faecal samples tested in this work were collected randomly from the living area of the Crested Ibis. It is possible that some of the faecal samples may be from the same bird, and therefore the chlamydial prevalence of 20% may be overestimated in this population. On the other hand, the faecal sample could not be linked to a specific individual so that it remained unclear about the intrinsic characteristic related to the prevalence of this chlamydial species in this bird. Nevertheless, it was still evident that this endangered avian species harboured *C. psittaci* and *C. ibidis*. This study not only identified new a chlamydial host but could also help to gain deeper insights into the evolution of *Chlamydiaceae*. In addition, the presence of *C. psittaci* and *C. ibidis* in Crested Ibis might indicate a potential threat to the endangered birds, which should be aware in the future protection practice.

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Conflict of interest. The authors declare that there is no conflict of interest.

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