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Mycobacteriosis Associated with *Mycobacterium peregrinum* Infection in Red-Crowned Cranes (*Grus japonensis*) in China

Huimin Liu,[1,2,5] Jing Yan,[1,5] Jing Luo,[1] Ruoqian Yan,[3] Hao Chen,[4] Hai Cheng,[4] Dawei Liu,[4] and Hongxuan He[1,6] [1]National Research Center for Wildlife-Borne Diseases, Institute of Zoology, Chinese Academy of Sciences, No. 1-5 Beichenxi Road, Chaoyang District, Beijing, People's Republic of China 100101; [2]University of Chinese Academy of Sciences, No. 1-5 Beichenxi Road, Chaoyang District, Beijing, People's Republic of China 100101; [3]Henan Center for Animal Disease Control and Prevention, Animal Husbandry Bureau of Henan Province, No. 1 Minan Road, Erqi District, Zhengzhou city, Henan province, People's Republic of China 450001; [4]Yancheng National Nature Reserve, Dafeng city, Yancheng, Jiang Su province, People's Republic of China 224000; [5] these authors contributed equally to this work; [6]Corresponding author (email: hehx@ioz.ac.cn)

ABSTRACT: We describe mycobacteriosis caused by *Mycobacterium peregrinum* in Red-crowned Cranes (*Grus japonensis*) in China. Isolates were identified by bacteriology, molecular identification methods, and phylogenetic analysis. This study shows that *M. peregrinum* is an important pathogen for mycobacteriosis and could represent a threat to conservation efforts of endangered species.

Mycobacteriosis of birds is a chronic progressive disease caused by *Mycobacterium* species, with *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium genavense* being most commonly identified (Tell et al. 2001). Other mycobacterial species such as *Mycobacterium fortuitum* and *Mycobacterium terrae* have also been implicated as potential causes of mycobacterial infections in birds (Soler et al. 2009). Wild birds play an important role in the ecology and movement of mycobacterial species. *Mycobacterium peregrinum*, a fast-growing nontuberculous mycobacteria (NTM), belongs to the *M. fortuitum* group, and can cause mycobacteriosis in humans (Brown-Elliott and Wallace 2002) and birds (Vitali et al. 2006). We describe the identification of *M. peregrinum* as the cause of mycobacteriosis in Red-crowned Cranes (*Grus japonensis*). The Red-crowned Crane is a large East Asian crane and one of the most endangered crane species; only about 1,500 birds remain in the wild.

In May 2012, five adult Red-crowned Cranes aged 5–8 yr were found dead in Yancheng National Nature Reserve, Jiang Su, China. For several weeks previously, the cranes had exhibited signs of illness, including fever, moist rales, shortness of breath, and loss of appetite. Oral administration of erythromycin, amoxicillin, and cephalosporin incorporated into their feed was unsuccessful. Postmortem examination revealed white nodules of various sizes in the lungs and livers. A fast-growing *Mycobacterium* was isolated from these tissues after 7 days of incubation at 37 C under aerobic conditions. Colonies on modified Lowenstein-Jensen medium were yellow, dry, irregular, and rough. The bacteria were long, rod-shaped, gram-positive, and acid-fast, and adhered in an arrangement similar to branched hyphae. Spores, capsules, and true branching were not observed. Histopathologic examination revealed granulomatous inflammations with abundant macrophages and multinucleated giant cells.

Biochemical characterization of the strain was performed using the Vitek 2 identification system (bioMérieux, Marcy l'Étoile, France), which identified the isolates as *Mycobacterium peregrinum* (probability 99.3%). We tested the bactericidal activity of 13 antibiotics, alone and in two- and three-drug combinations, against *M. peregrinum* clinical isolates. An inoculum of approximately 10^5 colony-forming units was cultured in 10 mL Mueller Hinton broth with individual drugs or drug combinations and incubated at 37 C for 4 days (Santos et al. 2008). Of the 13 antibiotics tested, moxifloxacin showed the highest bactericidal activity against *M. peregrinum*, either alone or in combination with other antibiotics tested (Table 1).

To further characterize the isolates, molecular identification using 16S rRNA,

TABLE 1. Antibiotics tested in combination against *Mycobacterium peregrinum* isolated from a Red-crowned Crane (*Grus japonensis*) in China (number of isolates on which bactericidal activity was observed/number of isolates tested).[a]

Combination of two antibiotics	Bactericidal activity	Three antibiotics	Bactericidal activity
M + LZ	1/2	+AK	2/2
		+GT	1/2
		+T	2/2
		+D	2/2
		+I	2/2
		+E	2/2
		+INH	1/2
M + CL	1/2	+RP	1/2
		+LZ	2/2
		+N	2/2
		+AK	2/2
		+GT	2/2
		+T	2/2
		+D	2/2
		+I	2/2
		+E	2/2
		+LZ	2/2
M + RP	1/2	+AZ	2/2
		+LZ	2/2
M + N	2/2		
M + AZ	2/2		
M + AK	2/2		
M + GT	2/2		
M + T	2/2		
M + D	2/2		
M + I	2/2		
M	2/2		

[a] M = moxifloxacin; LZ = linezolid; CL = clarithromycin; AZ = azithromycin; RP = rifampicin; AK = amikacin; T = tobramycin; GT = gentamycin; D = doxicyclin; I = imipenem; E = ertapenem; N = neomycin; INH = isoniazide.

rpoB, and *hsp*65 genes were performed (Aranaz et al. 2008). The isolates were confirmed as belonging to the genus *Mycobacterium* by PCR amplification and sequencing of the 16S rRNA and *rpoB* genes. These PCR assays target a 1,030-bp sequence specific to 16S rRNA of *Mycobacterium* spp. and a 136-bp sequence of the *rpoB* gene (Table 2), which can discriminate between the *Mycobacterium tuberculosis* complex and NTM (Kim et al. 2004). Identification of isolates to species level was carried out by PCR amplification and sequencing of the 16S rRNA and the 65-kDa heat-shock protein (*hsp*) genes (Table 2). The isolates were designated as JS-201205, according to their origin, and the 16S rRNA sequence was deposited in GenBank (accession KC292269). According to a Basic Local Alignment Search Tool search (NCBI 2013), strain JS-201205 was 100% similar to previously deposited sequences of *M. peregrinum* (accessions JX266704, HE575962, and AM884581). The strain was further confirmed as *M. peregrinum* according to the 441-bp product by *hsp*65, which was used for the identification of fast-growing mycobacteria to species level (Aranaz et al. 2008).

Sequence alignment was performed using the Clustal W multiple alignment in the MegAlign program of DNASTAR (DNASTA, Inc., Madison, Wisconsin, USA). Phylogenetic analyses were performed based on the 16S rRNA and *hsp*65 genes by the neighbor-joining method as implemented in

TABLE 2. Primers used for PCR amplification of the 16S rRNA, *rpoB*, and *hsp*65 genes of *Mycobacterium peregrinum* isolated from a Red-crowned Crane (*Grus japonensis*) in China.

Gene	Sequence (5′–3′)	Size (bp)	Target
16S rRNA	AGAGTTTGATCCTGGCTCAG	1030	*Mycobacterium* spp.
	AGAGTTTGATCCTGGCTCAG		
rpoB	GGAGCGGATGACCACCCAGGACGTC	136	Nontuberculous mycobacteria
	CAGCGGGTTGTTCTGGTCCATGAAC		
16S rRNA	GAGAGTTTGATCCTGGCTCAGGA	1500	Species level
	AAGGAGGTGATCCAGCCGCA		
*hsp*65	ACCAACGATGGTGTGTCCAT	441	Species level
	CTTGTCGAACCGCATACCCT		

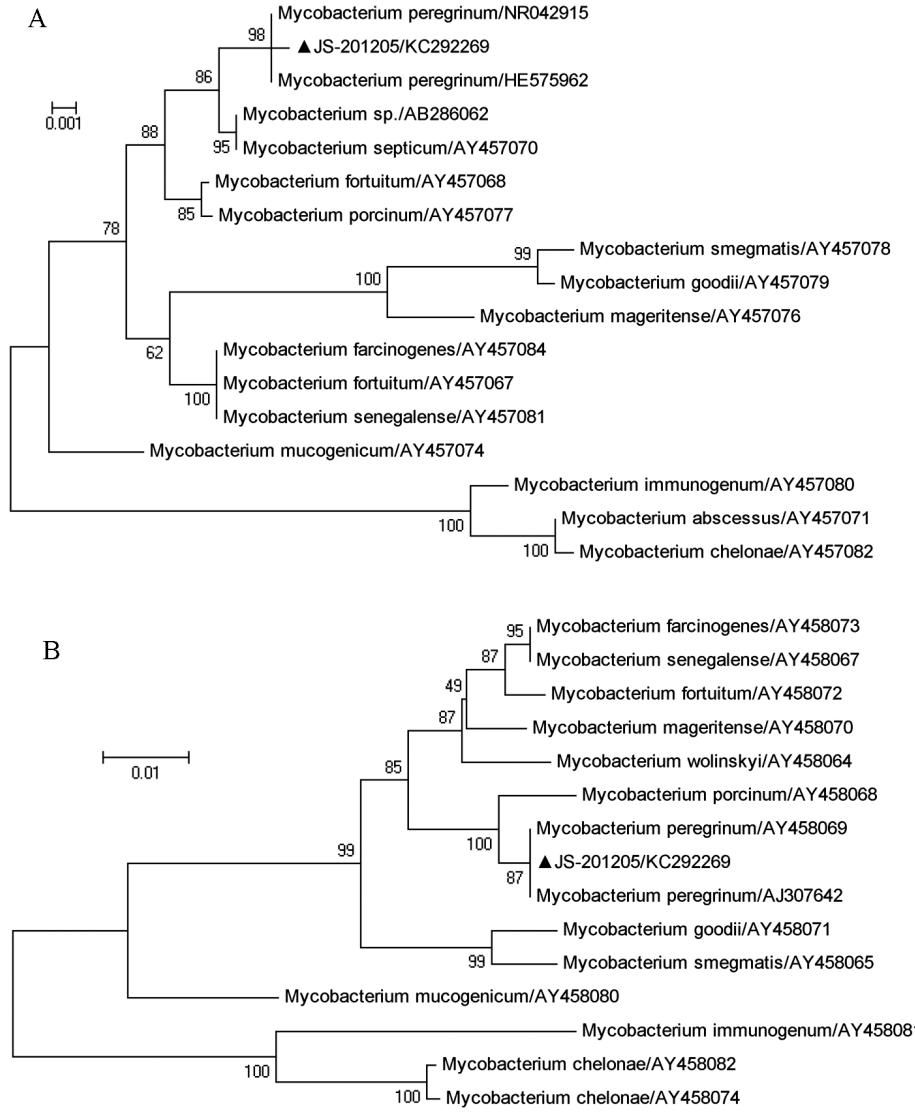

FIGURE 1. Phylogenetic analysis of (A) 16S rRNA and (B) *hsp*65 genes for the *Mycobacterium peregrinum* isolate JS-201205 and other closely related *Mycobacterium* species as conducted in MEGA 5.2 (Tamura et al. 2011).

MEGA 5.2 (Tamura et al. 2011). Phylogenetic analysis of existing *M. peregrinum* isolates revealed that strain JS-201205 clustered with other *M. peregrinum* isolates, with a 98% bootstrap value with 16S rRNA and 87% with *hsp65* (Fig. 1).

Red-crowned Cranes were kept in semicaptivity in the nature winter habitat reserve and allowed to come and go freely. How Red-crowned Cranes become infected with *M. peregrinum* is unclear, but the bacteria are free-living in water and soil, so waterborne transmission among cranes seems likely.

Mycobacterium peregrinum can also cause infections in humans. These infections often involve the skin, are difficult to treat, and require long-term antibiotic therapy. Mycobacterial infections in humans are associated with exposure to fish

or contaminated water (Aranaz et al. 2008). Over the last decade, a small but increasing number of sporadic human infections with *M. peregrinum* have been reported (Ishii et al. 1998; Rodríguez-Gancedo et al. 2001; Short et al. 2005). Although the transmission of *M. peregrinum* to humans through exposure to contaminated water is very likely, the potential for accidental infections of breeders from handling infected cranes is of concern.

We have demonstrated that *M. peregrinum* causes mycobacteriosis in wild birds. This infection could have important implications for the conservation of endangered species, and possible public health risks should be considered.

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