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Low Occurrence of *Helicobacter* DNA in Tropical Wild Birds, Venezuela

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ABSTRACT: Enteric *Helicobacter* species (*Helicobacter pullorum*, *Helicobacter pametensis*, *Helicobacter canadensis*, *Helicobacter anseris*, and *Helicobacter brantae*) have been found in birds from temperate latitudes. We evaluated the occurrence of *Helicobacter* spp. in terrestrial wild birds from Venezuela. A fragment of 16S rRNA gene was amplified by PCR with *Helicobacter* genus–specific primers. *Helicobacter* spp. were detected in four of 80 fecal and in three of 42 intestinal tissue samples. Analyses of 16S and 23S rRNA gene sequences confirm for the first time the presence of *Helicobacter* in tropical terrestrial wild birds. However, the occurrence of *Helicobacter* was low, suggesting these bacteria may be uncommon in the populations we studied.

Key words: Feces, intestine, *Helicobacter*, PCR, tropical wild birds.

Helicobacter spp. are gram negative, microaerophilic, spiral-shaped bacteria that colonize the gastrointestinal tracts of humans and animals. The genus contains 33 formally named species (Euzeby, 1997). *Helicobacter pullorum*, *Helicobacter pametensis*, *Helicobacter canadensis*, *Helicobacter anseris*, and *Helicobacter brantae* are enteric species commonly found in birds (Whary and Fox, 2004; Fox et al., 2006). Two of these (*H. pullorum* and *H. canadensis*) are associated with gastroenteritis in humans, suggesting that birds may act as reservoirs for the transmission of *Helicobacter* (Fox et al., 2000; Waldenstrom et al., 2003; Ceelen et al., 2005). Although *H. pullorum* has been detected in 4% of healthy humans, its colonization and virulence in humans are poorly understood (Ceelen et al., 2005). *Helicobacter* infection in wild birds has been reported only in temperate regions of Europe and the United States (Dewhirst et al., 1994; Seymour et al., 1994; Waldenstrom et al., 2003, 2007; Fox et al., 2006). We evaluate the occurrence of *Helicobacter* spp. in the gastrointestinal tracts and feces of tropical wild birds from Venezuela.

Fieldwork was conducted in seven locations in Venezuela, September 2007 to July 2009: Margarita Island, Nueva Esparta State (11°02′17″N, 63°50′44″W; elevation 100 m); Altos de Pipe (Instituto Venezolano de Investigaciones Científicas [IVIC]), Miranda State (10°23′58″N, 66°58′44″W; 1,500 m); Caracas, Capital District (10°27′42″N, 66°50′22″W; 1,100 m); Sierra de Tirgua (9°57′42″N, 68°40′19″W; 1,392 m) and Sierra de Aroa (10°21′56″ N, 68°50′11″W: 1,765 m), Yaracuy State; Ramal de Calderas, Barinas State (8°52′42″N, 70°29′28″W; 1,250 m); and Rio Uey, Maloca, Bolívar State (06°04′12″N, 61°28′08″W; 123 m). Birds were sampled under Venezuelan Ministry of Environment permits 3864 and 0665.

Fecal samples were obtained from 80 individuals belonging to five wild bird species (*Columbina passerina* [*n*=28], *Columbina squammata* [*n*=17], *Leptotila verreauxi* [*n*=17], *Mimus gilvus* [*n*=14] and *Cardinalis phoeniceus* [*n*=4]) trapped using mist nets. Live birds were placed in individual plastic boxes to collect feces, which were immediately transferred to 1.5-mL tubes containing 100% ethanol and kept at room temperature until DNA extraction in the laboratory at IVIC.

Additionally, 42 individuals of 20 wild bird species were sacrificed and transported to the laboratory on ice. These included 10 *Columbina passerina*, six *Columbina talpacoti*, one *Columbina squammata*, one

Aulacorhynchus sulcatus, one *Icterus icterus*, one *Notiochelidon cyanoleuca*, one *M. gilvus*, one *Catharus minimus*, one *Platycichla leucops*, two *Platycichla flavipes*, one *Turdus albicollis*, one *Turdus nudigenis*, two *Turdus olivater*, one *Coereba flaveola*, three *Ramphocelus carbo*, one *Thraupis glaucocolpa*, one *Thraupis palmarum*, one *Tiaris bicolor*, two *Zonotrichia capensis*, and four *Saltator maximus*. In the laboratory, samples of crop (*Columbina* spp. only), gizzard, and intestine were removed, transferred to 1.5 mL tubes containing 100% ethanol, and kept at room temperature until DNA extraction. Dissection scissors were washed with detergent and alcohol and flamed between samples to prevent cross contamination.

DNA from 80 fecal samples was purified using the UltraClean® Fecal DNA Isolation Kit (MO BIO Inc., Carlsbad, California, USA) and DNA from 42 tissue samples was obtained using the QIAamp DNA Mini Kit (Qiagen, Valencia, California, USA). To confirm the presence of bacterial DNA in all samples, we used 16S rRNA gene primers to amplify the domain bacteria (8F and 1525R; Contreras et al., 2007). *Helicobacter* spp. DNA was detected by PCR using *Helicobacter* genus–specific primers for the 16S rRNA gene (Germani et al., 1997). The *Helicobacter* 23S rRNA gene was amplified using PCR primers O20 and V64 (Dewhirst et al., 2005) to confirm *Helicobacter* species in the PCR-positive samples for the 16S rRNA gene.

The PCR reactions were performed using the Ready-To-Go PureTaq PCR kit (Amersham Biosciences, Piscataway, New Jersey, USA) in a PCR thermal cycler (GeneAMP PCR System 9700, Applied Biosystems, Foster City, California, USA). Each reaction mixture contained 6 μL of extracted DNA, 3 μL of mix primers (5 μmol/L) and sterile water to 25 μL. The negative control for PCR was prepared by adding water to reaction mixture instead of DNA. The positive control was prepared by adding 1 μL of a *Helicobacter pylori* DNA from a previously identified clinical isolate.

Helicobacter genus–specific fragments of the 16S and 23S rRNA genes were amplified and purified for sequencing using the QIAquick PCR Purification Kit (Qiagen), according to the manufacturer's recommendations. Purified 16S rRNA amplicons (~300 base pairs [bp]) and 23S rRNA amplicons (~1,400 bp) were sequenced at Macrogen Inc., Seoul, Korea. Purified 23S rRNA amplicons were sequenced using five primers (O20, M92, V62, M86, V64; Dewhirst et al., 2005). The *Helicobacter* 16S and 23S rRNA gene sequences were deposited in GenBank under the accession numbers JN635701–JN635707 and JX515552–JX515556. We aligned our *Helicobacter* sequences with closest GenBank matches using the SINA software (Pruesse et al., 2012) and constructed two phylogenetic trees using the neighbor-joining method and the Jukes-Cantor model provided in Molecular Evolutionary Genetics Analysis 2.1 software (version 5.0; Tamura et al., 2011).

All samples analyzed were PCR positive for the eubacteria 16S rRNA gene, confirming the presence of bacterial DNA and, consequently, the absence of PCR inhibitors. The *Helicobacter* spp. 16S rRNA gene was detected in four of 80 fecal DNA (5%) and in three of 42 intestinal tissues DNA (7%) accounting for 6% in total, considering samples from all sources together. All crop and gizzard samples were negative, suggesting that *Helicobacter* DNA was present only in intestine. This prevalence is lower than the 15–40% reported in wild aquatic birds such as geese (*Branta* spp.), Common Terns (*Sterna hirundo*) and gulls (*Larus* spp.; Dewhirst et al., 1994; Fox et al., 2006; Waldenstrom et al., 2007), but similar to values reported in urban Rock Pigeons (*Columba livia*) and Passeriformes (0–8%) from temperate regions (Seymour et al., 1994; Robino et al., 2010).

Three intestine samples, belonging to one Bare-eyed Thrush (*Turdus nudigenis,*

FIGURE 1. Phylogenetic tree of partial 16S rRNA sequences of the genus *Helicobacter* obtained from intestinal samples of tropical wild birds in Venezuela. The tree was constructed using neighbor joining. Bootstrap values are based on 10,000 replicates. No values are given for groups with bootstrap values less than 50%. The scale bar represents 0.009 (0.9%) nucleotide sequence difference.

ID COP91) and two Black-hooded Thrushes (*Turdus olivater*, ID COP32 and COP124), and four fecal samples, belonging to three Common Ground Doves (*Columbina passerina*, ID CP15, CP18, and CP20) and one Scaled Dove (*Columbina squammata,* ID SS32) were sequenced and the phylogenetic relationship of these sequences with other *Helicobacter* species based on 16S rRNA sequence data is shown in Figure 1. The dove samples clustered with *Helicobacter equorum* (DQ307736; 98% homology) and with an *H. canadensis* strain (AY323505; 99% homology) from Barnacle Geese (*Branta leucopsis*), but distinct from other *H. canadensis* and *H. pullorum* sequences. The amplicons from Black-hooded Thrushes (COP32 and COP124) had identical sequences and clustered with *H. anseris* (99% homology) and *Helicobacter* Bird-C (M88144; 99% homology), whereas the amplicon from the Bare-eyed Thrush (COP91) clustered with sequences related to *H. pametensis* (97% homology) and *H. brantae* (98% homology; Fig 1). Several authors have suggested that the 16S rRNA gene is not an adequate for identification of *Helicobacter* species (Dewhirst et al., 2005; Hannula and Hanninen, 2007). Dewhirst et al. (2005) suggested that the 23S rRNA gene sequence data are significantly more reliable for the identification and classification of *Helicobacter* because of the threefold-higher number of informative bases. Therefore,

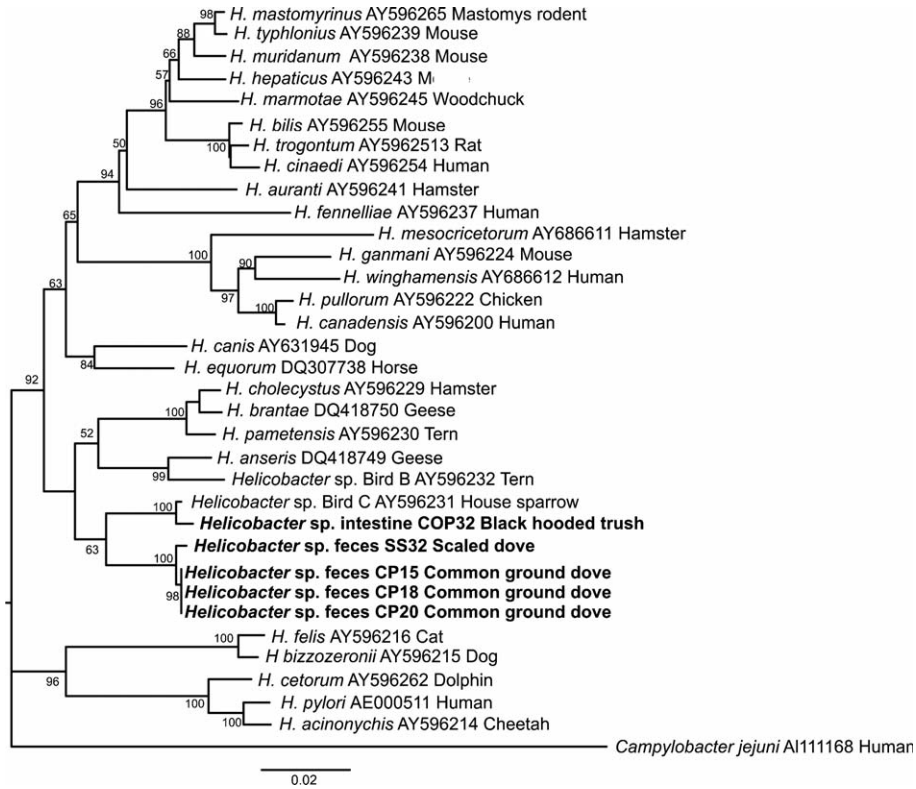

FIGURE 2. Phylogenetic tree of partial 23S rRNA sequences of the genus *Helicobacter* obtained from intestinal samples of tropical wild birds in Venezuela. The tree was constructed using neighbor joining. Bootstrap values are based on 10,000 replicates. No values are given for groups with bootstrap values less than 50%. The scale bar represents 0.02 (2%) nucleotide sequence difference.

we also amplified the 23S rRNA gene in the seven PCR-positive samples for 16S rRNA. The expected band was found in all fecal samples and in only one intestinal sample (COP32). The COP91 and COP124 intestinal samples were probably excluded by these 23S rRNA primers because the primers' targets are not present in their 23S rRNA gene. Similar results have been reported in environmental samples comparing 16S rRNA and 23S rRNA gene libraries, where some phyla showed low abundance with 23S rRNA primers because of mismatches observed in their sequences (Hunt et al., 2006). In the 23S rRNA gene tree (Fig. 2), the sequences form a cluster with *Helicobacter* sequences related to bird hosts. The Black-hooded Thrush (COP32) sequence clustered with a *Helicobacter* species from the House Sparrow (*Passer domesticus*; AY596231; 99% homology), as it is observed in the 16S rRNA tree (Fig. 1). The *Helicobacter* sequences from doves form their own cluster, where Common Ground Doves and Scaled Doves are in two clades, distinct from the Black-hooded Thrush and House Sparrow sequences (Fig. 2). The 16S rRNA sequence analyses suggest that all dove sequences belong to the same species and are closely related to *H. canadensis* and *H. equorum*. Although the 16S and 23S rRNA sequences can be discordant, the 23S rRNA information appears to be more congruent.

We captured wild birds with *Helicobacter* infection in localities far from human settlements, which suggests that tropical wild birds are unlikely to serve as transmission carriers to humans, as had been

suggested in other studies (Dewhirst et al., 1994; Waldenstrom et al., 2003; Fox et al., 2006; Robino et al., 2010). Nevertheless, the possibility of a zoonotic transmission of *Helicobacter* to humans cannot be discarded because doves and thrushes frequent habitats near human settlements, including parks, gardens, or plantations (Restall et al., 2006), and we do not know if these *Helicobacter* species are commensal or pathogens of the gastrointestinal tract of wild birds.

This is the first report of *Helicobacter* spp. in tropical wild birds, in particular in Venezuela. Even though their occurrence is low, more studies are necessary to establish the role of these enteric *Helicobacter* species as potentially emerging pathogens or commensals.

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