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Prevalence and Distribution of *Vibrio* spp. in Wild Aquatic Birds of the Southern Caribbean Sea, Venezuela, 2011–12

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ABSTRACT: *Vibrio* spp. are associated with waterbirds mainly in temperate latitudes. We evaluated the prevalence and distribution of *Vibrio* spp. from fecal samples of resident and migratory aquatic birds collected during October 2011 and March 2012 at two coastal sites in the tropical southern Caribbean Sea. We amplified DNA by PCR in 40% of samples, resulting in 47% and 36% estimated prevalence for resident and migratory birds in Cuare Wildlife Refuge, and 33% and 44% in Margarita Island, respectively. We found nontoxigenic *Vibrio cholerae* in Cuare Wildlife Refuge with a higher prevalence in resident birds (18%). Our PCR results for *Vibrio* and *V. cholerae* were not significantly different between sites or bird migratory status. The 16S rRNA phylogenetic analysis sequences from fecal samples from Cuare Wildlife Refuge were highly similar to *V. cholerae* and *Vibrio vulnificus*, whereas sequences from Margarita Island samples formed clusters with species related to the Harveyi clade. Our findings indicate that several species of *Vibrio* are common in aquatic birds along the southern Caribbean Sea and contribute to our understanding of the role of birds as possible reservoirs of potentially pathogenic bacteria.

Key words: Aquatic birds, Caribbean Sea, feces, migration, *Vibrio*.

The genus *Vibrio* consists of more than 100 species associated with aquatic organisms, birds, mammals, and humans, in commensal or pathogenic relationships (Thompson et al. 2004). Although data on the association between *Vibrio* and birds is limited, the main human pathogenic species (*Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*) have been reported in aquatic birds, mainly from temperate latitudes (Miyasaka et al. 2006; Vezzulli et al. 2010); limited studies of *Vibrio* occurrence in tropical aquatic birds have been conducted. We evaluated the prevalence and distribution of *Vibrio* in aquatic bird feces from the southern Caribbean Sea.

Fieldwork was conducted at two protected coastal areas in Venezuela from October 2011 through March 2012: Cuare Wildlife Refuge (10°57′N; 68°21′W) in the northwest, and Margarita Island (10°52′N; 64°03′W) in the northeast (Fig. 1). Both areas have substantive bird richness and abundance. We took fresh fecal samples (n=160) from resident, migratory (regional and long-distance), and nonidentified birds from mangroves and soils from shallow waters (Table 1). Nonidentified bird feces were collected on soils where no specific groups were identified. Soils close to feces were collected as controls. Long-distance migratory individuals: Semipalmated Sandpiper (*Calidris pusilla*; n=2), Least Sandpiper (*Calidris minutilla*; n=1), and Semipalmated Plover (*Charadrius semipalmatus*; n=1) were captured using mist nets. Live birds were placed in boxes to collect feces and then released. Samples were collected with Venezuelan Ministry of Environment permits; specimens were transported frozen to the laboratory, and kept at –80 C.

We purified DNA with Power soil DNA kit (MO BIO Inc., Carlsbad, California, USA). The *Vibrio* 16S rRNA gene was amplified using genus-specific primers (Liu et al. 2006). We tested *V. cholerae* and cholera toxin using the 16S-23S rRNA intergenic spacer region (ISR; Chun et al. 1999) and the *ctxA* (Lipp et al. 2003), respectively. *Vibrio parahaemolyticus* thermolabile haemolysin (*tl*; Rizvi and Bej 2010) and *V. vulnificus* haemolysin/cytolysin (*vvhA*; Warner and Oliver 2008), were eval-

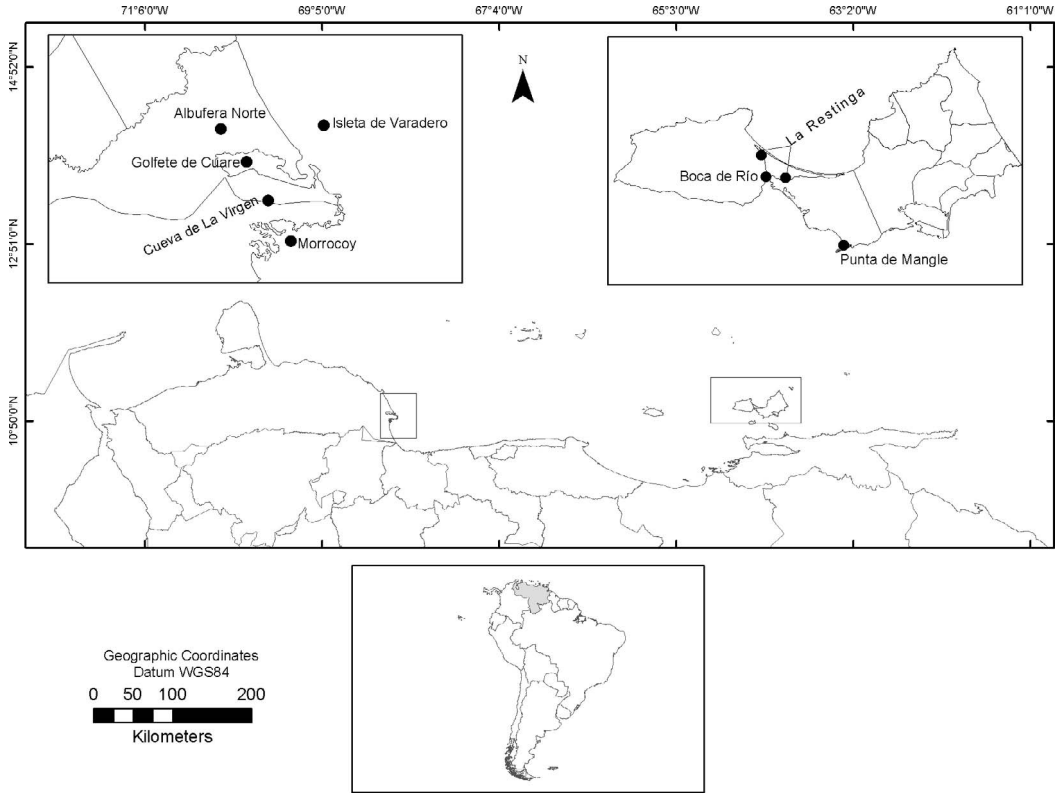

FIGURE 1. Sites map of Cuare Wildlife Refuge and Margarita Island, Venezuela. The sampling sites are represented by solid black circles. Images were provided by the Geographical Information System from Centro de Ecología, Instituto Venezolano de Investigaciones Científicas and processed with ArcGIS 10.0 Software.

uated. We performed PCR using Ready-To-Go PureTaq PCR kit (Amersham Biosciences, Piscataway, New Jersey, USA), containing 2–8 μL of DNA, 3 μL of primers (5 μmol/L), and sterile water to 25 μL in a Gen AMP 9700 thermal cycler (Applied Biosystems, Foster City, California, USA).

Genus-specific 16S rRNA fragments (~500 base pairs [bp]) were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, California, USA) and sequenced at Macrogen Inc. (Seoul, Korea). All fecal sequences were 99–100% homologous to *Vibrio* 16S rRNA sequence and were deposited in GenBank (KP762347–KP762357). The sequences were aligned with the closest matches using SINA Software (Pruesse et al. 2012). We constructed a phylogenetic tree using the neighbor-joining algorithm and the Jukes–Cantor model in Molecular Evolutionary Genetics Analysis 5.0 (Tamura et al. 2013). The stability of grouping was estimated by bootstrap analysis (10,000 replicates).

We used the nonparametric Mann-Whitney test for two independent samples to determine differences ($P<0.05$) among sites and bird migratory status in the *Vibrio* and *V. cholerae* PCR results using the PAST program (Hammer et al. 2001).

Vibrio was amplified by PCR in 40% of samples collected from both sites. In Cuare Wildlife Refuge, the prevalence of *Vibrio* was 47% of resident, 36% of migratory, and 32% of nonidentified birds. In Margarita Island, the prevalence was 33% of resident, 44% of migratory, and 47% of nonidentified birds. A higher prevalence of *Vibrio* was detected for migratory birds at Margarita Island than Cuare Wildlife Refuge; however, the difference between sites was not statistically

TABLE 1. Prevalence of *Vibrio* spp. and *V. cholerae* in fecal samples of aquatic birds from Cuare Wildlife Refuge and Margarita Island, Venezuela.

Sampling site	Date	Sample origin	Bird identification (*n*)	Migration status[a]	PCR 16S *Vibrio* (%)	PCR ISR *V. cholerae* (%)[b]
Cuare Wildlife Refuge	October/2011, March/2012	Soil	Sandpipers (*Tringa* spp.) (4)	M	0	ND
			Sandpipers/Plovers (*Calidris* spp./*Charadrius* spp.) (31)	M	7 (23)	1 (3)
			Herons (*Ardea* spp.) (3)	R	0	ND
			Large-billed Tern (*Phaetusa simplex*) (1)	R	1 (100)	1 (100)
			Ibises (*Eudocimus* spp.) (3)	R	3 (100)	0
			Black Skimmer (*Rynchops niger*) (3)	MRe	1 (33)	0
			American Flamingo (*Phoenicopterus ruber*) (13)	MRe	10 (77)	2 (15)
			Wattled Jacana (*Jacana jacana*) (6)	R	3 (50)	2 (33)
			Nonidentified (23)		8 (35)	3 (13)
		Mangrove leaves	Magnificent Frigatebird (*Fregata magnificens*) (1)	R	0	ND
			Black Skimmer (*R. niger*) (1)	MRe	0	ND
			Neotropic Cormorant (*Phalacrocorax brasilianus*) (3)	R	0	ND
			Nonidentified (2)		0	ND
		Captured birds	Semipalmated Sandpiper (*Calidris pusilla*) (2)	M	2 (100)	1 (50)
			Least Sandpiper (*Calidris minutilla*) (1)	M	1 (100)	0
			Semipalmated Plover (*Charadrius semipalmatus*) (1)	M	0	ND
			Total resident birds (17)		8 (47)	3 (18)
			Total migratory birds (56)		20 (36)	4 (7)
			Total nonidentified birds (25)		8 (32)	3 (12)
			Total birds (98)		36 (37)	10 (10)
Margarita Island	February/2012	Soil	Herons (*Ardea* spp.) (3)	R	1 (33)	0
			Sandpipers/Plovers (*Calidris* spp./*Charadrius* spp.) (4)	M	3 (75)	0
			American Flamingo (*P. ruber*) (21)	MRe	8 (38)	0
			Nonidentified (34)		16 (47)	0
			Total resident birds (3)		1 (33)	0
			Total migratory birds (25)		11 (44)	0
			Total birds (62)		28 (45)	0
Both sites	2011–12		Total resident birds (20)		9 (45)	3 (15)
			Total migratory birds (81)		31 (38)	4 (5)
			Total nonidentified birds (59)		24 (41)	3 (6)
			Total birds (160)		64 (40)	10 (6)

[a] M = long-distance migratory; MRe = regional migratory; R = resident.

[b] ISR = intergenic spacer region; ND = not determined.

significant (P=0.485; Table 1). Additionally, there were no statistically significant differences in the prevalence of *Vibrio* between resident and migratory birds (P>0.05). Soil controls did not have detectable *Vibrio* DNA. These results are consistent with previous findings related to the association of *Vibrio* spp. with aquatic birds worldwide (Miyasaka et al. 2006; Vezzulli et al. 2010).

We did not detect *V. parahaemolyticus* and *V. vulnificus* genes in any samples. The ISR of *V. cholerae* was detected only in Cuare Wildlife Refuge with a prevalence of 18% in residents, 7% in migratories, and 12% for nonidentified birds; no significant differences were found between resident and migratory birds (P=0.205; Table 1). Although we did not detect the *ctxA* gene for cholera toxin, the emergence of new toxigenic clones has been reported from marine environments (Thompson et al. 2004). Few investigators have found *V. cholerae* in aquatic bird feces (pelicans [*Pelecanus* spp.], herons [Ardeidae], gulls [*Larus* spp.], cormorants [*Phalacrocorax* spp.], ducks [Anatidae], and geese [Anatidae]; Lee et al. 1982; Ogg et al. 1989). To our knowledge, this is the first report of *V. cholerae* in Wattled Jacana (*Jacana jacana*), terns (*Phaetusa* spp.), sandpipers (*Calidris* spp.), and flamingos (*Phoenicopterus* spp.). The increasing number of *Vibrio* spp. in these sites suggests their spread in the digestive tract of aquatic birds in the Neotropics.

As inferred from the 16S rRNA partial sequencing and phylogenetic analysis, most fecal sequences from Cuare Wildlife Refuge formed clusters with *V. cholerae* and only one with *V. vulnificus*, whereas sequences from Margarita Island were grouped with *Vibrio alginolyticus* and *Vibrio harveyi* (Fig. 2). Accurate identification of vibrios at the family and genus levels is obtained by 16S rRNA sequencing. However, exclusive comparisons of 16S rRNA at the species levels might provide limited resolution for identifying *Vibrio* spp. (Thompson et al. 2004). In Cuare Wildlife Refuge, four *Vibrio* sequences formed a clade with *V. cholerae* in agreement with most ISR results by PCR, which confirms its presence in Large-billed Tern (*Phaetusa simplex*), American Flamingo (*Phoenicopterus ruber*), and Semipalmated Sandpiper. In particular, the detection of *V. cholerae* in a sequence from a Semipalmated Sandpiper (KP762351) demonstrates the presence of this bacterium in the gastrointestinal tract.

Another sequence from an American Flamingo (KP762350) clustered with *V. vulnificus*, which has only been reported in gulls and ducks from Japan (Miyasaka et al. 2006). On the other hand, the sequences from Margarita Island grouped mainly with species related to the Harveyi clade (*V. harveyi*, *V. alginolyticus*, *Vibrio campbellii*, and *V. parahaemolyticus*). However, these bacteria have not been reported in aquatic wild birds before. Although a previous study identified *V. cholerae* by biochemical and serologic tests in Wilson's Plover (*Charadrius wilsonia*) and Greater Yellowlegs (*Tringa melanoleuca*) samples from a coastal lagoon close to Margarita Island, this report should be confirmed by molecular tests (Rodriguez et al. 2010). There is a surprising clear distribution of *Vibrio* species between sites (Fig. 2), considering that aquatic birds are very mobile animals. Long-distance migrants such as sandpipers or flamingos are able to move hundreds of kilometers in one day (Halpern et al. 2008).

Vibrio presence in bird feces could be explained by their feeding habits as a source of bacteria in the bird's gut. This is supported by a previous report of *V. cholerae* in seawater and plankton from Cuare Wildlife Refuge (Fernández-Delgado et al. 2009), whereas species related to the *V. alginolyticus*, *V. harveyi*, and *V. campbellii* group have been found in the Cariaco water column close to Margarita Island (Bozo-Hurtado et al. 2013). Fish have been demonstrated to be reservoirs of *Vibrio* spp. (Chatterjee and Haldar 2012), including *V. cholerae* (Halpern et al. 2008; Senderovich et al. 2010). Herons, Large-billed Terns, Black Skimmers (*Rynchops niger*), Magnificent Frigatebirds (*Fregata magnificens*), and Neotropic Cormorants (*Phalacrocorax brasilianus*) feed on fish (Hilty 2003). Although our PCR results do not allow us to establish if the *Vibrio* we identified came from the bird's diet or their intestinal microbiota,

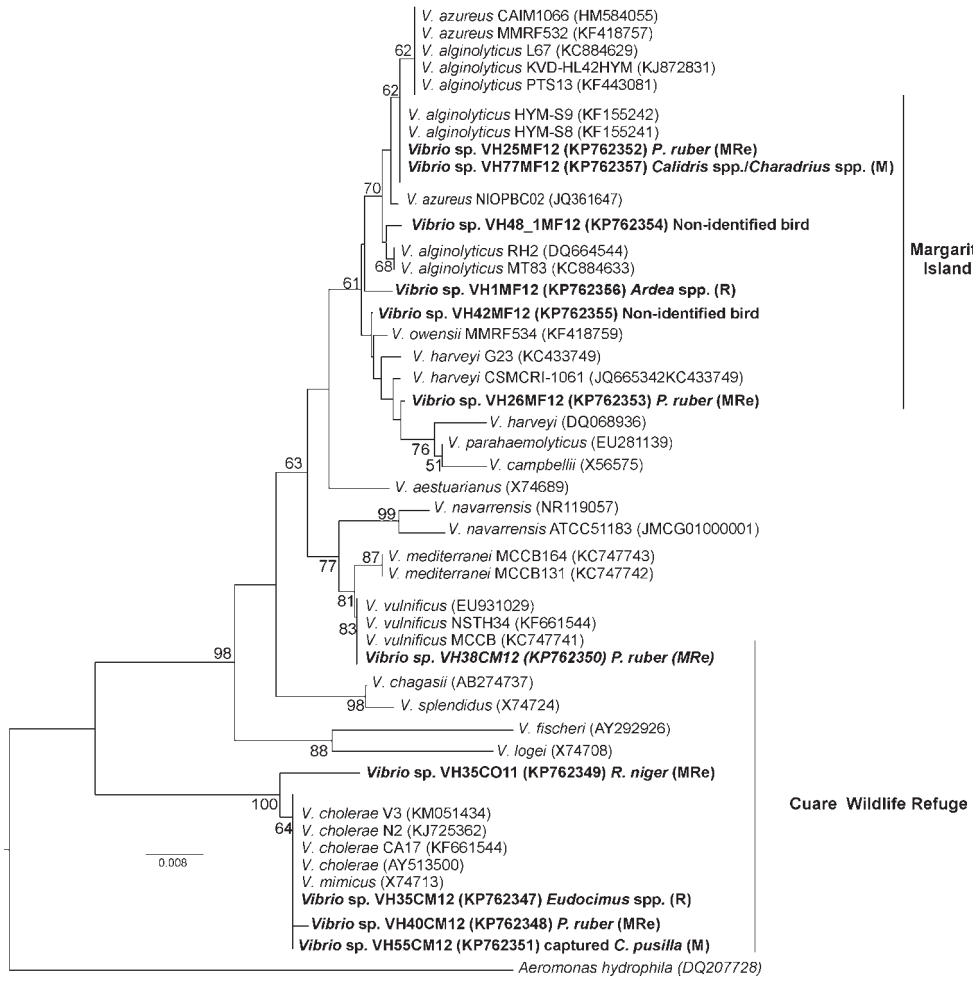

FIGURE 2. Phylogenetic tree of partial 16S rRNA sequences of *Vibrio* obtained from aquatic bird fecal samples from Cuare Wildlife Refuge and Margarita Island, Venezuela. Tree was constructed using neighbor-joining algorithm and the Jukes–Cantor model. Bootstrap values are based on 10,000 replicates, and no values are given for groups with bootstrap values <50%. The scale bar represents 0.008 (0.8%) nucleotide sequence difference. M=long-distance migratory; MRe=regional migratory; R=resident.

most reports indicate that invertebrates—mainly copepods (Crustacea), chironomids (Diptera), crabs (Crustacea), protozoa, and bivalves (Bivalvia)—and fishes—tilapia (*Oreochromis niloticus*), salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), and eels (Anguilliformes)—are consumed by aquatic birds (Halpern et al. 2008; Vezzulli et al. 2010; Chatterjee and Haldar 2012). More studies are necessary to understand the *Vibrio* dynamics and interactions with birds and aquatic reservoirs. The ubiquity of certain resident birds (herons, terns, skimmers, jacanas, and ibises) along coastlines, and their association with human activities, such as fishing, shellfish beds, and tourism, might pose significant risk for the spread of vibrios to areas of ecologic and human health importance (Miyasaka et al. 2006; Vezzulli et al. 2010). Our data indicate that several species of *Vibrio* are common in aquatic birds along the southern Caribbean Sea. Culture and isolation of *Vibrio* is needed to gain more information regarding the species, the strains, and their pathogenic potential. Our results

contribute to understanding the role of birds as reservoirs of pathogenic bacteria.

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