Comparison of Diagnostic Techniques for *Helicobacter cetorum* Infection in Wild Atlantic Bottlenose Dolphins (*Tursiops truncatus*)

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Helicobacter cetorum sp. nov. has been cultured from the stomach of Atlantic white-sided dolphins (*Lagenorhynchus acutus*) and the feces of Pacific white-sided (*L. obliquidens*) and Atlantic bottlenose (*Tursiops truncatus*) dolphins and a beluga whale (*Delphinapterus leucas*). *H. cetorum* has high homology to *Helicobacter pylori* as shown by 16S rRNA sequencing, and *H. cetorum* infection has been associated with gastritis and clinical signs in cetaceans. Because the prevalence of *H. cetorum* in wild populations is unknown, minimally invasive techniques for detecting *H. cetorum* were compared for 20 wild bottlenose dolphins sampled as part of a long-term health study. Fecal samples were tested for helicobacter by culture, Southern blotting, and PCR using genus-specific and *H. cetorum*-specific primers. An enzyme-linked immunosorbent assay (ELISA) was developed to measure *H. cetorum* immunoglobulin G (IgG). *H. cetorum* was cultured from 4 of 20 fecal samples, 7 samples were positive using *Helicobacter* sp. PCR, and 8 samples were positive for *H. cetorum* using species-specific primers. Two additional fecal samples were positive by *Helicobacter* sp. Southern blotting, suggesting infection with another helicobacter. All 20 sera contained high levels of IgG antibodies to *H. cetorum* that were significantly lowered by preabsorption of the sera with whole-cell suspensions of *H. cetorum* ($P < 0.02$). Until the specificity of the serum ELISA can be determined by testing sera from dolphins confirmed to be uninfected, PCR and Southern blot screenings of feces are the most sensitive techniques for detection of *H. cetorum*, and results indicate there is at least a 50% prevalence of *H. cetorum* infection in these dolphins.

Helicobacter species are microaerobic, motile, fusiform, or slightly curved-to-spiral, gram-negative bacteria that over the last decade have been detected in a wide variety of animals and humans (3). The growing diversity of *Helicobacter* species that have been isolated is matched by the diversity of ecological niches that these bacteria colonize within mammalian and avian hosts. The majority of *Helicobacter* species naturally colonize the lower intestinal tract and may cause typhlitis or colitis. Some of these enterohepatic helicobacters have also been associated with chronic hepatitis in rodents, dogs, nonhuman primates, ferrets, birds, and humans based on recovery by culture, PCR evidence, or direct observation of helicobacters in histologic sections of liver. Other helicobacters have adapted to colonizing the stomach, specifically *Helicobacter pylori*, the well-established type species that causes gastritis, peptic ulcer disease, and gastric cancer in humans (18); *H. mustelae* as a cause of chronic gastritis in the ferret (4); and the recently reported *H. cetorum*, which was associated with gastritis in cetaceans (10). Clinical manifestations of helicobacter infection vary from subclinical inflammation in the majority of naturally infected hosts to significant inflammation and the risk of cancer in a smaller percentage. Development of disease is influenced by the immune status and genetic background of the host, the infected target organ, and the expression of helicobacter virulence factors, such as *H. pylori* cagA, which is associated with a higher risk for atrophic gastritis and progression to cancer in humans (18).

H. cetorum has been previously isolated from the main stomachs and feces of stranded and captive cetaceans, and it was suggested that this helicobacter may be involved in the development of gastritis in cetaceans (10). *H. cetorum* infection has been demonstrated in clinically healthy captive cetaceans and others with signs including chronic regurgitation, intermittent inappetance, weight loss, and lethargy, and in some cases infections were associated with gastritis and the presence of spiral-to-curved bacteria in inflamed tissue. Since its first isolation in 1999 (11), *H. cetorum* infection has been detected by PCR of feces or gastric fluid in 9 of 14 captive cetaceans and 13 of 18 stranded wild cetaceans, including Atlantic bottlenose dolphins (*Tursiops truncatus*), Atlantic white-sided dolphins (*Lagenorhynchus acutus*), Pacific white-sided dolphins (*Lagenorhynchus obliquidens*), and a captive beluga whale (*Delphinapterus leucas*). No intestinal *Helicobacter* species have been reported in dolphins to date.

The Sarasota Dolphin Research Program in Florida has conducted a long-term study (>32 years) of the health, biology, population dynamics, social structure, and life history of a resident population of 140 bottlenose dolphins (24). Because the dolphins were only briefly restrained for examination, the objective of this study was to compare several minimally invasive diagnostic techniques for diagnosing *H. cetorum* infection.

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TABLE 1. Primers used to amplify 16S rRNA sequence[a]

Primer	Position	Orientation	PCR specificity	Sequence
C97	276–291	Forward	*Helicobacter* spp.	GCTATGACGGGTATCC
C05	1477–1495	Reverse	*Helicobacter* spp.	ACTTCACCCCAGTCGCTG
Hce F	128–146	Forward	*Helicobacter cetorum*	GTTATGTGCCTCTTAGTTTG
Hce R	1133–1150	Reverse	*Helicobacter cetorum*	AGAGTTCTCAGCATAACCT

[a] C97 and C05 are used for amplification to the genus (*Helicobacter*) level, and Hce F and Hce R are used for amplification to the species (*H. cetorum*) level; the former produce a 1,200-bp amplicon, while the latter generate a 1,022-bp product.

Fecal samples were tested for helicobacter by culture, Southern blotting using a *Helicobacter* genus-specific probe, and PCR using genus-specific and *H. cetorum*-specific primers. An enzyme-linked immunosorbent assay (ELISA) was developed to measure *H. cetorum* immunoglobulin G (IgG) and preabsorption studies were performed to demonstrate its specificity.

MATERIALS AND METHODS

Animals and sample collection. Feces and serum were collected from 20 wild Atlantic bottlenose dolphins by the Sarasota Dolphin Research Program in Sarasota Bay, Fla. Sample collection was conducted under NOAA Fisheries Scientific Research Permit 522-1569-01 issued to R. Wells. For sampling purposes, dolphins were encircled with a 500-m-long seine net in shallow water (<2 m deep) and moved via sling to the shaded, padded deck of a veterinary examination vessel (23). Gender was determined and age was estimated either from monitoring offspring since birth or from examination of growth layer patterns in teeth (13). Twelve dolphins were females estimated to be from 3 to 43 years of age, and 8 were males estimated to be from 3 to 25 years of age. All animals appeared to have normal body condition. Fecal samples were obtained with rectal swabs, and serum was collected from the ventral fluke vasculature (1). Following sampling and measurements, the dolphins were released at their capture sites. Blood and fecal samples were kept on ice during collection and then stored at −70 or −80°C pending assays.

Microaerobic culture. Fecal samples were placed in 3 ml of 20% glycerol in brucella broth and stored at −70°C prior to culture. Culture media were Trypticase soy agar with 5% sheep blood and TVP (trimethoprim, vancomycin, and polymyxin) and CVA (cefoperazone, vancomycin, and amphotericin B) antibiotic-impregnated media (Remel Laboratories, Lenexa, Kans.). Additional selective antibiotic media contained a blood agar base of 5% horse blood (Remel), amphotericin B (50 μg/ml), vancomycin (100 μg/ml), polymyxin B (3.3 μg/ml), bacitracin (200 μg/ml), and nalidixic acid (10.7 μg/ml) (Sigma Chemical Co., St. Louis, Mo.). Approximately 100 μl of homogenized sample was applied to the plates and incubated at 37°C for up to 3 weeks under microaerobic conditions. Microaerobic conditions were maintained in vented GasPak jars (Becton Dickinson, Franklin Lakes, N.J.) without a catalyst by evacuation to −20 mm of Hg and then repressurization with a gas mixture consisting of 80% N_2, 10% H_2, and 10% CO_2 to yield a final O_2 concentration of 5% (6).

Specificity and sensitivity of *Helicobacter* spp. and *H. cetorum* primers. The specificity of the primers used to generate 1,200-bp amplicons of *Helicobacter*-genus specific 16S rRNA was previously reported (5) (Table 1). Additional primers were designed to amplify *H. cetorum*-specific 16S rRNA. Amplicons were the expected size of a 1,022-bp product and by sequencing were found to be >99% identical to *H. cetorum*. Specificity of the primers for *H. cetorum* was tested against DNA extracted from *Escherichia coli*, *Enterococcus faecalis*, *Proteus mirabilis*, *H. pylori*, and several novel helicobacters isolated from harp seals, sea otters, and sea lions. The sensitivities of the two primer sets were compared using two different assays. To determine the minimum detection limit of *H. cetorum* DNA required to produce a detectable PCR band, each primer set was used to amplify 10-fold serial dilutions (50 ng to 0.05 fg) of DNA extracted from a pure culture of *H. cetorum*. Potential for assay interference from inhibitory substances in feces was assessed by addition of 10-fold serial dilutions of *H. cetorum* DNA (50 ng to 0.05 fg) to 100 ng of DNA extracted from dolphin feces demonstrated to be helicobacter-free by culture and genus-specific PCR (data not shown). These samples were then tested using both primer sets under the PCR conditions described below.

PCR of fecal samples. DNA was extracted from feces using the Mini QIAamp DNA kit (QIAGEN Inc., Valencia, Calif.). The PCR mixture contained 10 μl of sample, 1× high-fidelity buffer, a 0.5 μM concentration of each of the two

primers, a 200 μM concentration of each deoxynucleoside triphosphate, and 200 μg of bovine serum albumin per ml. The samples were heated at 94°C for 4 min, centrifuged briefly, and cooled to 58°C, and then 2.5 U of high-fidelity polymerase (Roche Molecular Biochemicals, Indianapolis, Ind.) was added. Amplification conditions were denaturation at 94°C for 1 min, annealing at 58°C for 2 min, and elongation at 72°C for 3 min. Thirty-five cycles were completed before a final elongation step at 72°C for 8 min. A 15-μl aliquot of the PCR product was electrophoresed through a 1% agarose gel separation matrix prior to ethidium bromide staining and viewing under a UV light.

Southern blot analysis. Southern blot analysis was performed with a horse-radish peroxidase-labeled 1,200-bp 16S rDNA helicobacter genus-specific probe generated using primers C97 and C05 (Table 1) on a sample of *H. pylori* DNA as previously described (5). Fifteen-microliter aliquots of 1,200-bp amplicons from extracted DNA were electrophoresed through a 1% agarose gel, transferred onto a Hybond N nylon membrane as outlined by the manufacturer (Amersham Biosciences, Arlington Heights, Ill.), and then UV cross-linked. The fixed DNA was then hybridized overnight at 42°C with the labeled probe, and this was followed by exposure in the presence of luminol to Hyperfilm-ECL as outlined by the manufacturer (Amersham Biosciences).

ELISA. Checkerboard titration methods were used to optimize antigen coating concentration and dilution of the secondary detection antibody. Immulon II plates (Dynax Technologies, Chantilly, Va.) were coated with outer membrane protein antigens (25) of *H. cetorum* at 1 μg/ml overnight at 4°C. Plates were then blocked with phosphate-buffered saline–2% bovine serum albumin for 1 h at 37°C. Serum samples diluted 1:200 to 1:12,800 were added for 1 h at 37°C, and this was followed by incubation with peroxidase-labeled rabbit anti-bottlenose dolphin IgG (10 μg/ml; Bethyl Laboratories, Montgomery, Tex.) diluted 1:8,000 for 1 h at 37°C. Optical density (OD) readings at 405 nm were obtained 30 min after addition of ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)]substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Md.). To demonstrate antigen specificity of the ELISA, aliquots of all serum samples were diluted to 1:400 in phosphate-buffered saline–1% bovine serum albumin and then preabsorbed overnight at 4°C with 4×10^6 organisms/ml of whole-cell suspensions of *H. cetorum*, mouse-adapted *H. pylori* (SS1), two novel but distinct *Helicobacter* isolates (MIT 01-5229-A and 01-5229-B) from the gastric mucosa of a harp seal (*Phoca groenlandica*) (C. G. Harper, S. Xu, A. B. Rogers, Y. Feng, Z. Shen, N. S. Taylor, F. E. Dewhirst, B. J. Paster, M. Miller, J. Hurley, and J. G. Fox, unpublished data), and murine *H. hepaticus*. Preabsorbed and unmanipulated sera were then compared in an *H. cetorum* antigen ELISA using the same conditions as described above.

RESULTS

Microaerobic culture. *H. cetorum* was cultured from the feces of 4 of the 20 bottlenose dolphins (Table 2). Identity of the isolates as *H. cetorum* was confirmed by consistent morphology under phase microscopy; Gram staining; and uniform profiles of urease-, catalase-, and oxidase-positive biochemistry. Also PCR assays using both *Helicobacter* genus- and *H. cetorum*-specific primers were positive. No other *Helicobacter* spp. were recovered by culture.

Specificity and sensitivity of *Helicobacter* spp. and *H. cetorum* primers. For each set of primers, amplification products of the expected size (1,200 bp for genus-specific and 1,022 bp for *H. cetorum*-specific primers) were detected in ethidium bromide-stained gels. The specificity of the primers for *H. cetorum*

TABLE 2. Analysis of fecal and serum samples of 20 Atlantic bottlenose dolphins[e]

Accession no.	Patient gender[a]	Estimated age (yr) of patient	Result of:				
			Culture	Southern blotting[b]	*Helicobacter* genus-specific PCR[c]	*H. cetorum* PCR[d]	ELISA
6095	F	17	−	−	−	−	+
6097	F	13	−	−	−	−	+
6098	M	20	−	−	−	−	+
6101	F	43	−	−	−	−	+
6103	M	9	−	−	−	−	+
6104	M	9	−	−	−	−	+
6106	F	5	−	−	−	−	+
6111	M	25	−	−	−	−	+
6112	M	5	−	−	−	−	+
6113	F	6	−	−	−	−	+
6102	F	31	−	+	−	−	+
6114	F	12	−	+	−	−	+
6108	F	30	−	+	−	+	+
6099	F	3	−	+	+	+	+
6105	M	13	−	+	+	+	+
6109	F	10	−	+	+	+	+
6096	F	10	+	+	+	+	+
6100	F	38	+	+	+	+	+
6107	M	3	+	+	+	+	+
6110	M	14	+	+	+	+	+

[a] Abbreviations: F, female; M, male.
[b] *Helicobacter* genus-specific probe.
[c] With primers C05 and C97.
[d] With primers Hce F and Hce R.
[e] Southern blotting was the most sensitive technique for detecting helicobacter DNA, and all serology results appeared to be strongly positive.

was confirmed by lack of amplification when tested against DNA extracted from *E. coli*, *E. faecalis*, *P. mirabilis*, *H. pylori*, and several novel helicobacters isolated from harp seals, sea otters, and sea lions (data not shown). Using DNA extracted from a pure culture of *H. cetorum*, the lower detection limit for *H. cetorum* primer amplification was 5 fg of *H. cetorum* DNA, whereas the lower detection limit for the genus-specific primers was 10-fold higher (50 fg) (Fig. 1A). This increased sensitivity of the *H. cetorum* primers was also observed when known quantities of *H. cetorum* DNA were added to 100-ng samples of DNA extracted from helicobacter-free dolphin feces (Fig. 1B). The lower detection limit for *H. cetorum* primer amplification was 50 fg of *H. cetorum* DNA, and the lower detection limit was 500 fg of *H. cetorum* DNA with the genus-specific primers.

PCR and Southern blot analysis. Seven of the 20 fecal samples from the bottlenose dolphins were PCR positive for *Helicobacter* sp. using the genus-specific primers, and 8 samples were PCR positive using the *H. cetorum*-specific primers. Ten of the 20 fecal samples were positive for *Helicobacter* sp. by Southern blot when probed with the *Helicobacter* genus-specific probe (Fig. 2 and Table 2). Samples yielding four of the most intense bands on the Southern blot (lanes 2, 6, 13, and 16) were from animals that also tested positive for *H. cetorum* by culture and by both genus- and species-specific PCR. An additional intense Southern blot band (lane 15) was from a culture-negative sample that was positive by genus- and species-specific PCR. The less intense Southern blot bands (lanes 8, 11, 14, and 20) reflect low copy numbers of helicobacter in the sample and were from culture-negative samples. Two of these samples were also PCR negative (lanes 8 and 20), one was PCR positive for both genus- and *H. cetorum*-specific primer sets (lane 11), and one was PCR positive for *H. cetorum* only

(lane 14). Seven of the 10 animals testing positive for *Helicobacter* sp. by Southern blot were females (58% of females surveyed), and 3 were males (38% of males surveyed); the age range was estimated to be from 3 to 38 years.

ELISA. To analyze the ELISA data, the dolphin serum samples were first grouped by Southern blot results which indicated whether helicobacter DNA was detected in the fecal samples. All 20 dolphins appeared to be strongly seropositive to *H. cetorum* irrespective of Southern blot results ($P = 0.41$) (Fig. 3A and Table 2). Thus, a cutoff value to objectively discriminate between positive and negative serum samples could not be determined, because all 20 dolphins appeared to have titers of at least 1:1,600 or more. Antigen preabsorption with a whole-cell suspension of *H. cetorum* significantly lowered antibody levels ($P < 0.02$), whereas preabsorption with whole-cell suspensions of *H. pylori*, the two harp seal isolates, and *H. hepaticus* did not lower levels of antibody to *H. cetorum* significantly when considered as a group (analysis of variance, $P = 0.49$) (Fig. 3B). Preabsorption of the dolphin sera with a whole-cell suspension of *H. pylori* did not reduce the level of IgG to *H. cetorum* to a significant extent ($P = 0.12$) but the effect was greater compared to preabsorption of the sera with whole-cell suspensions of the other helicobacters ($P = 0.89$).

DISCUSSION

The number of recognized novel helicobacters continues to expand because of improved isolation and diagnostic methods and considerable interest in the scientific community regarding the pathogenic potential of the emerging *Helicobacter* genus (2, 3, 10; Harper et al., unpublished data). The association between gastritis in cetaceans and *H. cetorum* infection has been reported, but the opportunities to strengthen an epidemiologic
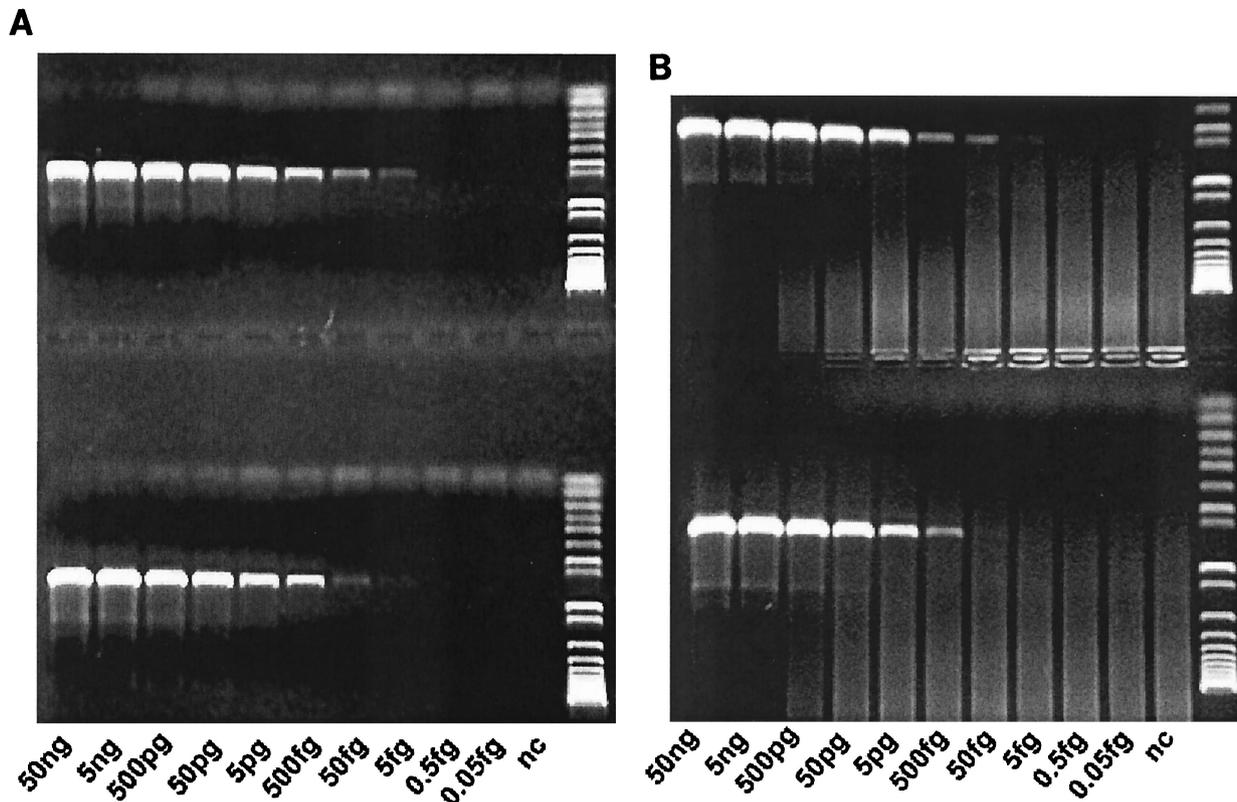
FIG. 1. (A) Sensitivities of primers for *Helicobacter* spp. and *H. cetorum* (Table 1) were compared by assaying serial 10-fold dilutions of DNA (50 ng to 0.05 fg) extracted from a pure culture of *H. cetorum*. A band of the top gel in the eighth from the left represents *H. cetorum*-specific primer amplification of 5 fg of *H. cetorum* DNA, and a band of the bottom gel in the seventh lane from the left represents amplification of 50 fg of *H. cetorum* DNA when the genus-specific primers were used. *H. cetorum*-specific primers were more sensitive. (B) Sensitivities of primers for *Helicobacter* spp. and *H. cetorum* (Table 1) were compared in the presence of potential inhibitory substances in feces. Tenfold serial dilutions of *H. cetorum* DNA (50 ng to 0.05 fg) were added to 100 ng of DNA extracted from helicobacter-free dolphin feces followed by PCR with each primer set. A band of the top gel in the seventh lane from the left represents *H. cetorum*-specific primer amplification of 50 fg of *H. cetorum* DNA, and a band of the bottom gel in the sixth lane from the left represents amplification of 500 fg of *H. cetorum* DNA when the genus-specific primers were used. *H. cetorum*-specific primers were more sensitive. nc, negative control.

link by assessment of biopsy samples are limited. Thus, the goal of this project was to compare several minimally invasive diagnostic techniques for detecting *H. cetorum* infection so that wildlife biologists and veterinarians would have additional

tools to assess the health status of wild populations. *H. cetorum* was recovered by microaerobic culture from only 4 of the 20 fecal samples. Molecular techniques identified another six infected animals, and serology was positive for significant anti-
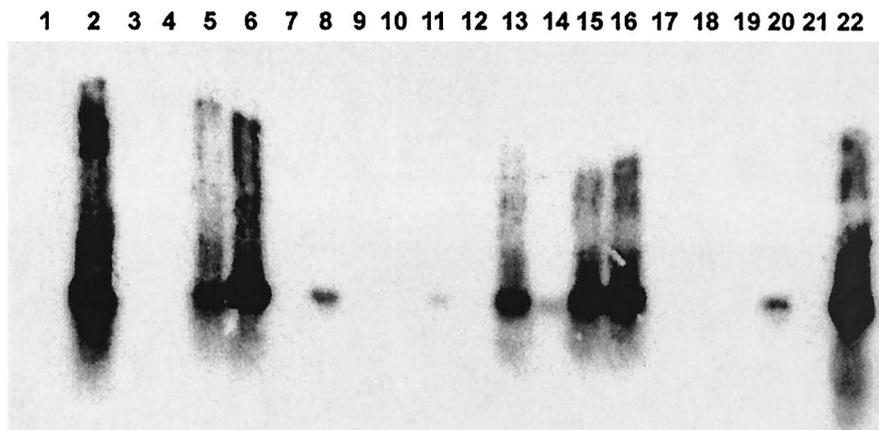

FIG. 2. Southern blot of DNA extracted from dolphin fecal samples using a *Helicobacter*-genus specific probe. Lanes 21 and 22 contain negative and positive controls, respectively.
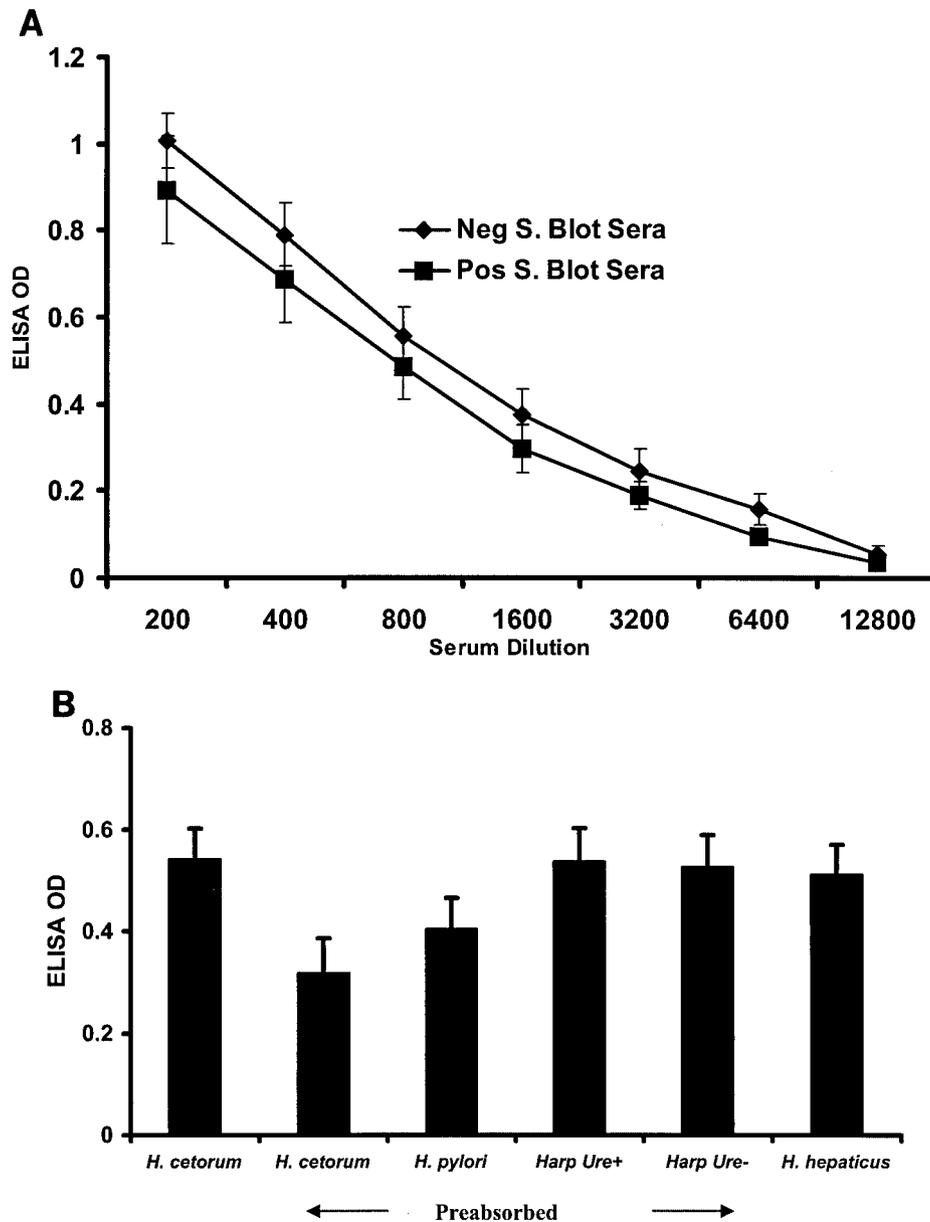
FIG. 3. (A) Serially diluted dolphin sera were tested against *H. cetorum* antigens by ELISA. Results were categorized by Southern blotting (S. Blot) as positive (Pos) or negative (Neg) assay results. Lack of sera from animals confirmed to be uninfected prevented traditional analysis of seroconversion, which typically is defined as an OD value that exceeds the mean plus 3 standard deviations of values obtained from antigenically naïve animals. Data represent a mean OD ± standard error (error bars) of 10 animals per group. (B) Dolphin sera diluted 1:400 was preabsorbed against whole-cell suspensions of *H cetorum*, *H pylori*, two novel isolates (Harp Ure+, urease-positive MIT 01-5229-A; Harp Ure−, urease-negative MIT 01-5229-B) of helicobacter isolated from a harp seal, or *H. hepaticus* (which infects mice). Unmanipulated and preabsorbed sera were then tested by ELISA for IgG to *H. cetorum*. Antibody binding to *H cetorum* was reduced significantly ($P < 0.02$) by preabsorption to *H. cetorum*, whereas preabsorption with other helicobacter whole-cell suspensions did not have a significant effect. Data represent mean ODs + standard errors (error bars) of 20 dolphin serum samples.

body levels in all 20 dolphins. Collectively, these diagnostic techniques suggest a prevalence of *H. cetorum* in at least 50% of the dolphins in this population. The apparent high prevalence of *H. cetorum* in this population is consistent with the high prevalence of other helicobacter infections found in nature. Notable examples include *H. pylori* in humans, *H. mustelae* in the ferret, and "*H. heilmannii*" in nonhuman primates, all of which colonize upwards of 100% of the natural host population (2). Once the ELISA is validated by testing sera from

dolphins confirmed to be uninfected with *H. cetorum* through analysis of gastric biopsy samples by PCR and culture, the prevalence of *H. cetorum* infection may be much higher based on our finding of high ELISA OD values to *H. cetorum* in the present study.

Definitive evidence of gastric helicobacter infection relies on culture recovery of bacterial isolates from gastric tissue, often obtained by endoscopic biopsy or postmortem tissue collection. The nature of this study precluded tissue collection either

at postmortem or endoscopy. Cetaceans have a three-chambered stomach composed of the nonglandular forestomach and the main and pyloric stomachs. *H. cetorum* has been reported to colonize the main and pyloric stomachs and not the forestomach (11). Additional evidence that *H. cetorum* colonizes the stomach in dolphins is supported by its close homology to *H. pylori* in 16S rRNA sequence and its production of urease (10), an important adaptation of all known gastric helicobacters for survival in the low-pH environment of the glandular stomach. Because technical and research permit limitations usually prevent routine endoscopic examination of the dolphin distal gastric compartments, this study relied on sera and fecal samples to screen for helicobacter infection, both of which are minimally invasive methods (12, 17, 20).

H. cetorum was cultured from only 4 of the 20 fecal samples, but this result is consistent with similar studies that have attempted to isolate other gastric helicobacters from feces (16). Factors that may lower the success rate and influence the number of false negative fecal cultures include the abundance of competing microorganisms that inhibit in vitro growth of fastidious helicobacters, the unknown potential for only periodic shedding of viable gastric helicobacters in the fecal stream and as suggested for *H. pylori*, *H. cetorum* may be shed as nonculturable coccoid forms in feces (16). The low viability of *H. pylori* in the fecal stream has been associated with bile exposure during transit through the gastrointestinal system (14). In ferrets confirmed to be experimentally infected with *H. mustelae* by culture of gastric biopsies, isolation of *H. mustelae* from feces was successful in only 11 of 36 ferrets (7). This suggests that shedding of *H. mustelae* may have corresponded to periods of transient hypochlorhydria from the associated gastritis, or *H. mustelae* may have been shed in feces intermittently. The difficulty in recovering gastric helicobacters through fecal culture was also supported by a study in which *H. pylori* was cultured from stools obtained from known-infected humans only when a cathartic was used (21). Low shedding of viable gastric helicobacters in feces is also supported by epidemiologic evidence that oral-oral transmission of *H. pylori* through saliva (15) or vomitus (21) is a significant risk. The mode of transmission for *H. cetorum* has not been documented, but *Helicobacter* sp. DNA was detected in dental plaque from two captive dolphins (8), suggesting that *H. cetorum* could be shed from the upper as well as the lower gastrointestinal tract into the water column. Thus, the social grouping of these dolphins would be conducive to horizontal transmission of *H. cetorum*.

PCR is a sensitive and specific technique for the detection of target DNA in various clinical specimens, but fecal samples may contain substances that are inhibitory to PCR. PCR of human fecal samples for diagnosis of *H. pylori* infection is considered less optimum than other minimally invasive techniques, which include fecal antigen detection, serology, and urea breath testing (22). Compared to histology, serology, and gastric tissue DNA analyses, PCR of human fecal samples for *H. pylori* has been reported to be 73% sensitive, with a specificity of 100% (9). In our study, genus-specific PCR primers detected 7 positive animals and *H. cetorum*-specific PCR primers detected 8 positive animals out of the 20 dolphins. The difference in results between PCR assays may be explained by the increased sensitivity of the *H. cetorum* primers. Southern

blot results identified two other animals as infected with helicobacter that tested negative for helicobacters by PCR using both genus- and species-specific primer sets. These dolphins may be infected with *H. cetorum* or another *Helicobacter* species, which could only be substantiated by repeated sampling. Notably, enteric species of helicobacters have not been isolated from wild or captive dolphins to date, but more animals are required for testing before conclusions can be made about the potential for infection with helicobacters other than *H. cetorum*. Thus, Southern blotting was the most-sensitive molecular technique to detect helicobacter DNA in the dolphin fecal samples, and PCR using the *H. cetorum* primers was more sensitive than PCR using genus-specific primers.

The limitations of detecting a gastric helicobacter infection through culture or PCR of fecal samples may partially explain why all 20 dolphin sera were apparently seropositive for *H. cetorum* by ELISA. The high anti-*H. cetorum* IgG levels in these dolphins is consistent with observations made in ferrets in which 100% were found to be infected with *H. mustelae*, with seroconversion occurring soon after weaning (26), and the correlation of positive serology and prevalence of *H. pylori* infection in humans, which varies from low to 90% or higher in some populations (19). Serology kits used for screening humans for *H. pylori* infection have reported a median sensitivity and specificity of 92 and 83%, respectively (22). The lack of serum samples from dolphins confirmed to be uninfected with *H. cetorum* by gastric biopsy prevented traditional evaluation of seroconversion, which relies on statistical analysis of assay values on samples obtained from antigenically naïve animals. Reduction in the level of *H. cetorum* IgG in serum by preabsorption of the dolphin sera with a whole-cell suspension of *H. cetorum* and not with cell suspensions of the other helicobacters supports the specificity of the ELISA for detecting serum IgG directed against *H. cetorum*. Notably, although preabsorption of the dolphin sera with a whole-cell suspension of *H. pylori* did not reduce the level of IgG to *H. cetorum* to a significant extent, the effect was greater compared to preabsorption of the sera with whole-cell suspensions of the other helicobacters, which is consistent with the close homology of *H. cetorum* and *H. pylori* in their 16S rRNA sequences (10).

In conclusion, the testing of fecal samples from this dolphin population indicated a 50% prevalence of *H cetorum* infection. Given that *H. cetorum* is a gastric helicobacter and testing feces and sera have limitations, the true prevalence of *H. cetorum* is probably higher. Testing fecal samples for *H. cetorum* offers a minimally invasive diagnostic technique for dolphins exhibiting clinical signs consistent with gastritis but for whom endoscopy or gastric biopsy is unavailable or contraindicated. As expected, PCR and Southern blotting were more sensitive than culture, and in particular, the *H. cetorum* primers identified more animals that were infected with *H. cetorum* than did use of the genus-specific primers. Until further studies validate the utility of the ELISA based on testing sera from dolphins known to be uninfected, our recommendation is to survey for helicobacter infection in wild dolphins using each of the techniques described in the present study; that is, PCR-based assays of fecal samples, serology, and concurrent attempts to recover *Helicobacter* spp. by culture.

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