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INVESTIGATING THE PREVALENCE AND PATHOGENESIS OF *HELICOBACTER* INFECTIONS IN SOUTHERN SEA OTTERS (*ENHYDRA LUTRIS NEREIS*)

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by

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Abstract

Investigating the prevalence and pathogenesis of *Helicobacter* infections in southern sea otters (*Enhydra lutris nereis*)

Francesca Batac

While it is known that *Helicobacter* species in humans and ferrets can cause gastritis and gastric ulcers, it is unknown if the sea otter bacterium, '*H. enhydrae*' sp. nov., causes similar gastric diseases. Southern sea otters (*Enhydra lutris nereis*) are marine mustelids that commonly have gastric ulcers as a significant contributing cause of death. Determining whether *Helicobacter* infection promotes gastric diseases might aid population recovery by recognizing disease presentation and facilitating treatments. As a first step, we investigated the prevalence of *Helicobacter* infections in southern sea otters using DNA. *Helicobacter* DNA was amplified using quantitative polymerase chain reaction from formalin-fixed and fresh-frozen gastric body and pyloric tissues from 46 sea otters using *Helicobacter* genus 16S rRNA primers. Postmortem examination data and histology were statistically analyzed to uncover associations between *Helicobacter* presence and gastric diseases. Enrolled sea otters had an 85% *Helicobacter spp.* prevalence, which is comparable to *H. mustelae* in ferrets. Fresh frozen tissues showed a higher amount of *Helicobacter* qPCR-positive samples than did formalin-fixed tissues. Gastric ulcers at the postmortem exam were significantly associated with *Helicobacter* positivity, while other analyzed factors such as sex, melena, and shark trauma were not correlated. Antibiotic sensitivity analysis show that '*H. enhydrae*' was sensitive to tetracycline and clarithromycin *in vitro*. Our data suggest that *Helicobacter* infections are

prevalent in southern sea otters and are associated with gastric ulcers. Our data represents a first step in the study of this potential pathogen, supporting that further work is warranted to assess whether '*H. enhydrae*' causes or is simply associated with gastric ulcers.

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Introduction

Sea otters (*Enhydra lutris*) are the second smallest marine mammal but the heaviest member of the family Mustelidae, with a unique metabolism. These animals have twice the metabolic rate of other marine mammals, and 2-3 times the rate of a comparably-sized terrestrial animal; as a result they require food consumption equivalent to 25% of their body weight each day (Iversen 1972; Morrison et al. 1974; Yeates et al. 2007). With such high caloric demands, gastrointestinal health is critical for the wellbeing of sea otters. A known component of gastric health are bacteria in the *Helicobacter* genus. In humans, gastric infection by the bacterium *Helicobacter pylori* is found in 50% of people worldwide and is associated with gastritis, gastric ulcers, and gastric cancers (Marshall and Warren 1984; Cover and Blaser 1996). In other mammals, infection with non-*pylori Helicobacter* species have been found and in some cases shown to co-occur with gastritis, gastric ulcer and/or gastric adenocarcinoma development. Specifically, *H. cetorum* in marine mammals and *H. mustelae* in ferrets (Fox et al. 1990, 1997; Harper et al. 2002, 2003b).

Recently, scientists isolated and characterized a novel *Helicobacter* species, '*H. enhydrae*' sp. nov. (strain MIT 01-6242, GenBank No. AY203901) from the gastric mucosa of southern sea otters (*Enhydra lutris nereis*) (Shen et al. 2017). Based on 16S rRNA PCR, 58% of 31 tested sea otters were positive for gastric *Helicobacter* DNA. In addition, the *'H. enhydrae*' sp. nov. strain demonstrated close phylogeny to *H. mustelae* from domestic ferrets (*Mustela putorius furo*), based on *Helicobacter* 16S and 23S rRNA gene sequences (Shen et al. 2017). A prior study from the same

researchers found, but did not characterize, two southern sea otter *Helicobacter* isolates (MIT 01-5923 and MIT 01-5924) that shared phylogeny with *Helicobacter* spp. from a sea lion (MIT 02-5519-C), and a harp seal (*Phoca groenlandica*) (MIT 01-5529-A) (Harper et al. 2003b). These results suggest that southern sea otters may be carriers of two *Helicobacter* spp. or strains although only one has been definitively characterized.

As part of a recent retrospective study, gastric ulcers were found to be a common contributing cause of death (COD) for wild southern sea otters (Miller et al. unpublished). The study was based on detailed postmortem examination, images, histology, and laboratory results (e.g. protozoal serology). The work specifically found that gastric ulcers were a contributing COD for 42.3% (n = 237) of 560 examined sea otters (Miller et al. unpublished). While gastric ulcer had been reported as a cause of otter mortality (Kreuder et al. 2003), the high prevalence of this condition was not appreciated until this more recent work. In prior reports, gastric ulcers and melena (i.e. gastrointestinal bleeding) in sea otters have often been attributed to captivity-associated stress, or were considered as a sequela to oil contamination (Lipscomb et al. 1993; Loughlin 1994; Williams and Davis 1995; Reimer and Lipscomb 1998). However, another potential cause of gastric ulcers and melena that has not been systematically assessed for sea otters are *Helicobacter* spp. gastric infections.

As a federally-listed threatened species numbering just over 3100 animals (Tinker and Hatfield 2017), it is important to determine whether associations exist for

gastric pathology in sea otters with *Helicobacter* infections. In this study, we performed the first step toward this goal, by assessing prevalence and disease stated in southern sea otter samples using quantitative PCR with *Helicobacter* 16S rRNA primers. We compared fresh frozen gastric regions and matching fixed-tissue preparations. Our results suggest that 85% of southern sea otters carry *Helicobacter* in their stomachs, with the pylorus being more often positive than the body. We round that fresh frozen tissue is needed to accurately assess prevalence. Postmortem examination data and histology were statistically analyzed and revealed *Helicobacter* infections is associated with gastric ulcers, but not with other factors including sex, melena, and shark trauma. We assessed *in vitro* antibiotic efficacy for '*H. enhydrae*' to amoxicillin, chloramphenicol, clarithromycin, kanamycin, and tetracycline. Our findings suggest that *Helicobacter* infection is common in southern sea otters, and infection may be associated with development of gastric ulcer pathology. We have also confirmed that the sea otter *'H. enhydrae'* is susceptible to common antibiotics *in vitro*.

Materials and Methods

Tissue collection and processing procedures

Samples were collected from fresh, necropsied sea otters collected by staff of the California Department of Fish and Wildlife (CDFW) in the course of his or her duties as an official or state employee. All work was performed in accordance with Section 109(h) of US Marine Mammal Protection Act (MMPA), US Fish and Wildlife Service (Service) regulations implementing the MMPA at 50 CFR 18.22(a),

and in accordance with Service regulations implementing the US Endangered Species Act at 50 CFR 17.21(c)(3).

Postmortem examinations and histology sampling protocols for the 46 enrolled sea otter carcasses were performed as described (Miller et al. unpublished; Kreuder et al. 2003). In brief, minimally decomposed sea otters were refrigerated at 7-10°C and examined at CDFW – Marine Wildlife Care and Research Center (CDFW - MWVCRC) (Santa Cruz, CA) from 2015 - 2017. During the postmortem exam, gastric body and pylorus samples were collected in cryovials using scalpel blades for DNA purification, and also placed in 10% buffered formalin for histology (microscopic examination). After collection, cryovial gastric tissues were placed in a -80°C freezer until processed for DNA purification. Gastric ulcers were defined based on the presence of lesions that penetrated deeply through the muscularis mucosae of the stomach, while gastric erosions were defined as superficial lesions not penetrating into the muscularis mucosae (Tarnawski et al. 1995). For simplicity, both gastric erosions and ulcers were pooled for analysis, and were referred to as "ulcers" in this study. Other than gastric ulcers, metadata analyzed from the enrolled sea otters included sex, age, melena, and causes of death (such as shark trauma and parasitic infections).

Histology

All histological evaluations performed by the first author (F. Batac) were reviewed by a veterinary pathologist (M. Miller, DVM, PhD, MS). Current human and sea otter gastric histology grading systems were referenced in conducting sea otter histology

evaluations (Supplemental Material 1). We used hematoxylin & eosin stained slides (H&E) to evaluate the fixed gastric body and pylorus for features including melena, gastritis, inflammatory cell composition, and presence of *Helicobacter*-like organisms (Supplemental Material 2).

DNA isolation and qPCR

DNeasy Blood & Tissue Kit (Qiagen) and QIAamp DNA FFPE Tissue Kit (Qiagen) were used for DNA extraction of the fresh-frozen ("frozen") gastric body and pylorus samples (25 mg/sample) and the formalin-fixed, paraffin-embedded ("fixed") samples (60-80 µm/sample), respectively, per Qiagen's extraction protocols. Primer sequences (Table 1), constructed by Geneious R10.1.3 (Biomatters Ltd.), amplified a 117-122 bp product of the 16s rRNA gene from the *Helicobacter* genus. Quantitative polymerase chain reaction (qPCR) was performed on the CFX Connect™ Thermal Cycler (Bio-Rad Laboratories, Inc.) using 25 µM primer concentration and a 20 µl reaction mix final volume. The following qPCR conditions were specific for SensiMix™ SYBR® No-ROX and primer sequences in Table 1: 1 cycle of polymerase activation at 95°C for 10 min, then 40 cycles of denaturing at 95°C for 15 sec, annealing at 57°C for 15 sec, and elongation at 72°C for 15 sec. The following were used as qPCR controls: no-template control (NTC) with nuclease-free water, negative control with *Escherichia coli* DH10B genomic DNA, positive control with '*H. enhydrae*' genomic DNA, isolated as above (provided by Shen et al. 2017). All frozen and fixed samples had 2-5 technical replicates (Supplemental Materials 3 and 3). For the frozen samples (n= 92), we were able to obtain separate gastric body

and pylorus for all 46 enrolled sea otters. For the fixed samples (n= 59), gastric body and pylorus were either in separate paraffin blocks or combined. The quantification cycle (Cq) values stated in this study were from qPCR runs where the NTCs were non-detectable. Frozen and fixed samples with a non-detectable Cq value were assigned the lowest value of 40 representing an absence of *Helicobacter* DNA amplification. A dilution series with known concentrations of '*H. enhydrae*' showed low detection rates and/or inconsistent results for Cq value above 35 (Table 2). Thus "*Helicobacter* qPCR-positive" samples were defined as those with Cq values <35, and "*Helicobacter* qPCR-negative" samples were defined as those with Cq values ≥35.

'*H. enhydrae*' culture and ETEST® antibiotic sensitivity testing

'*H. enhydrae*' was grown on solid media consisting of Columbia agar with 5% defibrinated horse blood (Hemostat Laboratories) containing 10 µg/mL vancomycin, 50 µg/mL cycloheximide, 5 µg/mL cefsulodin, and 2.5 Units/mL polymxin B (CHBA), or liquid media containing Brucella Broth with 10% heat-inactivated fetal bovine serum (BB10). For either growth mode, '*H. enhydrae*' was cultured at 37°C under microaerophilic conditions (10% CO_2 and 5% O_2, balance N_2). For the antibiotic sensitivity testing, '*H. enhydrae*' was cultured on solid CHBA media, with subculture and passage to a fresh plate every 3 days for 6 days. For BB10 overnight incubation, and small amount of the plate-grown sample was transferred to the liquid. After overnight growth, the absorbance (OD_{600}) was determined and cultures were used when the absorbance was between 0.199 - 0.785. Then, 100 µl of

this broth was spread evenly onto separate CHBA plates, The ETEST® strips (each impregnated with a separate antibiotic, at a concentration gradient from 0.016 - 256 µg/mL), were placed on the surface of individual plates immediately after bacterial spread, and the plates were incubated as above. The zone of bacterial growth inhibition around the ETEST® strips was recorded visually and photographically following 2-days of incubation.

Statistical analyses through JMP Pro 13.0

JMP Pro 13.0 software (SAS Institute Inc.) was used to statistically analyze the relationships between sea otter *Helicobacter* infections to their demography, postmortem data, and histologic evaluations to determine associations. The Goodness-of-Fit test assessed the normal probability of a continuous variable, such as Cq value. The Fisher's exact test evaluated the association between two variables, such as *Helicobacter* infections and gastric ulcers. Pearson and the Likelihood Ratio chi-squared (χ^2) tests examined whether two categorical variables were independent. The significance level or critical probability (p) value was set at $p < 0.05$.

Results

High *Helicobacter* prevalence in fresh-frozen sea otter gastric tissues

Our first goal was to expand upon previous research into *Helicobacter* prevalence in sea otters (Shen et al. 2017). We tested 92 frozen gastric body and pylorus tissue samples from 46 southern sea otters examined at CDFW-MWVCRC in 2015-2017, and used qPCR of purified DNA with *Helicobacter* spp. primers to quantify the amount of *Helicobacter* DNA. DNA amount is presented as Cq values;

lower Cq values indicate greater abundance of target DNA. We first ran a standard curve with purified '*H. enhydrae'* DNA (Table 2). With this approach, we could detect '*H. enhydrae*' DNA concentrations as low as $8x10^{-6}$ ng/µL. Samples containing no or unrelated template had Cqs of 35 - 40, and so we assigned samples with Cq ≥ 35 as *Helicobacter* qPCR-negative. Samples with a Cq <35 were considered *Helicobacter* qPCR-positive.

After establishing our positive and negative cut-off values, we then analyzed our set of frozen tissues. Most (74%, 68/92) of these samples had Cq values <35 (Figure 1); 83% of gastric pylorus (n= 38) and 65% of gastric body (n= 30) samples were *Helicobacter* qPCR-positive. The qPCR-positive gastric samples represented 85% (39/46) of enrolled sea otters (Supplemental Material 3), and gastric pylorus contained approximately 4-fold more *Helicobacter* DNA than gastric body samples from the same sea otters, with mean Cqs of 33.6 and 31.1, respectively (Figure 2).

Formalin-fixed tissue analysis underrepresents *Helicobacter* status

We next compared whether fixed tissues would give similar results to those obtained from the location-matched frozen tissue analysis. Our subsample of fixed tissues had been formalin-fixed for varying lengths of time (from 2 days to 2-3 weeks post-collection) prior to paraffin-embedding. The qPCR of fixed tissues yielded much lower *Helicobacter* detection that we found with frozen tissue (Figure 2). The Cqs were lower in both the gastric body and pylorus (Figure 2). Analysis of fixed tissues suggested that only 40% (18/45, fixed samples unavailable for one animal) of enrolled sea otters were positive for *Helicobacter*. The fixed samples produced a

mean Cq of 4.2-4.7 higher than frozen samples, pushing them above the *Helicobacter* qPCR-positive Cq of <35. The differing frozen and fixed tissue Cq values may be due to creation of abasic sites and DNA fragmentation by formalin fixatives, which can lower DNA integrity and quantity and thus affect its ability to be PCR amplified (Do and Dobrovic 2015). This outcome suggests that sea otter *Helicobacter* spp. analysis using fixed gastric tissues would have provided false negatives and not a true infection frequency for this study. Goodness-of-Fit analyses determined the fixed samples ($p< 0.001$) rejected the null hypothesis that the data has a normal distribution, when compared to frozen samples ($p =0.1441$), which accepted it. This analysis suggests that the fixed qPCR Cq values may not be representative of the true distribution, and additionally appear to not reflect the actual *Helicobacter* DNA quantity at time of initial sampling.

Positive association between *Helicobacter* infections and gastric ulcers in sea otters

To investigate possible connections between *Helicobacter* spp. infection and gastric pathology, we evaluated frozen gastric body and pylorus samples from a subset of 41 sea otters where gross gastric evaluations had been performed as part of the detailed postmortem exam (n= 82). Approximately 45% (37/82) of the frozen samples were gastric ulcer-positive, representing >51% (21/41) of sea otters with available postmortem ulcer data. Nearly 90% of the gastric ulcer-positive frozen samples (33/37) were also *Helicobacter* qPCR-positive (Figure 3). The probability of a *Helicobacter* qPCR-positive result is greater for gastric ulcer-positive sea otters than ulcer-negative, based on the Fisher's exact test ($p= 0.0026$), Pearson χ^2 ($p=$

0.003) and the Likelihood Ratio χ^2 (p= 0.0021). When looking at all 46 enrolled sea otters, 6 of the 7 *Helicobacter* qPCR-negative sea otters did not have visible gastric ulcers on either the stomach body or pylorus. Collectively, these findings suggest that *Helicobacter* qPCR-positive sea otters are more likely to have gastric ulcers than *Helicobacter* qPCR-negative animals (Figure 3).

In addition, a less robust, but significant association was noted for *Helicobacter* qPCR-positive result in relation to gastric sample area, with pylorus (p= 0.048) significantly more likely to be *Helicobacter* qPCR-positive than gastric body (p= 0.984). The antrum/pylorus is also the preferred *Helicobacter* colonization site for ferrets, with higher bacterial abundance and more significant gastritis in this region (Vargas et al. 1991; Yu et al. 1995; Fox et al. 1997).

The following postmortem attributes were not significantly associated with *Helicobacter* infection on Fisher's exact and χ^2 tests: melena (all p> 0.1), sex (all p> 0.1), shark trauma as a COD (all p> 0.7), and parasitic CODs (e.g. acanthocephalan or protozoal infections) (all p> 0.1). We were unable to assess an association with age due to a low sample size for pup (age 6 months – 1 year) and immature (1-3 years) sea otters (Supplemental Material 3).

Histologic assessments did not reflect postmortem assessments

We next asked whether findings from review of histological samples could accurately reflect grossly apparent ulcer status in sea otters (Supplemental Material 1). H&E determined a 25% gastric ulcer presence as opposed to the 45% ulcer presence recorded at the postmortem examinations (Supplemental Materials 2 and 3).

Based on only the histologic evidence, gastritis nor melena were not statistically significant indicators for *Helicobacter* infections (Fisher's exact test both p= 0.3). Some of the reasons for this variation are that we had fewer otters sampled for the gastric histology assessment: 37 of the 46 enrolled sea otters versus 41 for the gross and assessment. This deficit was in part due to autolysis and varying gastric tissue sizes on the microscope slides. Additionally, the gastric histology only represents a small percent of the total, so seems more likely to miss ulcerous regions.

Histologic findings for individual *Helicobacter* qPCR-positive southern sea otters

While different histologic attributes were graded, the main characteristics assessed were gastric ulcers, gastritis, and melena (Supplemental Materials 2). Figure 4 shows severe gastric ulcers from a *Helicobacter* qPCR-positive (pylorus Cq 28.9) subadult female. Severe pyloric gastric mucosal ulcers with severe perilesional mucosal hemorrhage were confirmed at gross necropsy, based on a dark brown to black appearance due to red blood cell hemolysis (Figure 4a). Histopathology of these lesions revealed mucosal coagulation necrosis associated with thrombosis of underlying blood vessels and perilesional hemorrhage (Figure 4b).

Figure 5 shows mucosal lesions from a *Helicobacter* qPCR-positive (pylorus Cq 33.5) adult male. Grossly this otter had severe pyloric gastric mucosal ulcers, severe perilesional mucosal hemorrhage and melena (Figure 5a). Histopathology revealed focal expansion of pyloric lamina propria by nonsuppurative inflammation and mild stromal collapse (Figure 5b). A *Helicobacter*-like organism was present in

the pyloric gland of a *Helicobacter* qPCR-positive (pylorus Cq 26.5) aged adult female with no gastric ulcers (Figure 6).

Antimicrobial sensitivity tests on '*H. enhydrae*' reveal sensitivity to clarithromycin and tetracycline *in vitro*

To investigate antimicrobial sensitivity of sea otter *'H. enhydrae'* strains, we chose gradient strips recommended for *H. pylori* to assess minimum inhibitory concentration (MIC) for common antibiotics using an *in vitro* bacterial model. We assessed MICs for the following: amoxicillin, clarithromycin, chloramphenicol, kanamycin, and tetracycline. These broad-spectrum antibiotics exhibited varying MICs (Table 2) based on their respective zones of inhibition of bacterial growth (Figure 7). Antibiotics effective at low dosages create a large 'zone of inhibition' *in vitro*. Bacterial isolates that can withstand a higher antibiotic dosage exhibit a smaller zone of inhibition around the ETEST® strip, and are described as more resistant. Tetracycline, an inexpensive antibiotic effective against gram-positive and gram-negative bacteria (Chopra and Roberts 2001), exhibited one of the largest zones of inhibition, with an MIC between 0.047 - 0.094 µg/mL; thus this antibiotic may be effective for treating clinical '*H. enhydrae*' infections in sea otters. Amoxicillin, chloramphenicol, and kanamycin were less effective antibiotics, with MIC ranges from 0.5 to 4 µg/mL (Table 3). Clarithromycin may also be effective for treatment of clinically apparent '*H. enhydrae*' infection, but additional confirmatory testing is needed.

Discussion

Our work here suggests that ~85% of sea otters are carriers of gastric *Helicobacter*. The only other mustelid analyzed are captive domestic ferrets with these animals highly colonized by *H. mustelae* (Fox et al. 1990; Forester et al. 2000). Previous analysis found that 100% of 67 sampled ferrets from U.S. research facilities and veterinary practices in a two-year span were *H. mustelae*-positive via culture from gastric biopsies (Fox et al. 1991a). This prevalence is somewhat higher than we report for *'H. enhydrae'*. Possible reasons for the higher prevalence include that *H. mustelae* is known to inhibit acid secretion by parietal cells, increasing gastric pH and creating a favorable environment for bacterial proliferation (Fox et al. 1991b; Vargas et al. 1991), possibly explaining the high levels of ferret infection. Additionally, the analyzed ferrets were not wild animals, and were raised in confined conditions that might promote transmission. *H. mustelae* is the only non-*pylori Helicobacter* reported to cause gastric ulcers and cancer in its native host (O'Toole et al. 2010), and lesion development was confirmed through fulfillment of Koch's postulates (Fox et al. 1991b).

There are several other marine *Helicobacters* that have been analyzed. In the U.S., 53/56 gastric samples from wild and captive common bottlenose dolphins (*Tursiops truncates*) were positive for one or more *Helicobacter* spp. via Sanger-sequencing and pyrosequencing of the 16S rRNA gene (Bik et al. 2016). Infection by *H. cetorum* can be associated with gastritis (Harper et al. 2002, 2003a; Oxley et al. 2005) and gastric ulcer (Harper et al. 2000), but has also been found in clinically

normal dolphins (Bik et al. 2016). With *H. cetorum* present in both healthy and clinically ill populations, it may be that dolphins have a high infection rate with much of its population being asymptomatic, similar to humans infected with *H. pylori*.

In this study, we found that 85% *Helicobacter* prevalence in southern sea otters (n= 39/46) tested from 2015-2017 based on qPCR on frozen gastric tissues. Our 85% prevalence is higher than the 58% reported in a prior study (Shen et al. 2017) that used conventional PCR. This may be because qPCR provides greater detection sensitivity when compared with conventional PCR. This study and Shen et al. 2017 reported higher *Helicobacter* detection in the gastric pylorus (83% and 45%, respectively) than the stomach body (65% and 10%, respectively). We tested matching fixed tissues from the enrolled otters to see whether we could use the archived formalin-fixed gastric tissues to determine *Helicobacter* DNA presence. Fixed tissue analysis under-represented *Helicobacter* status and produced an average overall Cq of 4.2-4.7 higher than frozen samples, thus more fixed tissues were falsely *Helicobacter* qPCR-negative.

Prior to initiation of this research, southern sea otters were known to be infected with '*H. enhydrae*' sp. nov., which is related to the pathogenic species *H. mustelae* from ferrets. In addition, gastric ulcers were recognized as a common gastric lesion in sea otters, often contributing to morbidity and mortality. However, any associations between '*H. enhydrae*' infection and sea otter gastric ulcers were undetermined. In this study, we found *Helicobacter* infections were positively associated with gastric ulcers with nearly 90% of the gastric ulcer-positive frozen

samples (33/37) also *Helicobacter* qPCR-positive (Figure 3). These findings support a possible connection between the presence of *Helicobacter* and sea otter gastric ulcers (Shen et al. 2017). However, these results should be interpreted with caution because sea otter *Helicobacter* pathogenesis has not been confirmed *in vivo*.

There are likely multiple gastric ulcer etiologies in wild and captive sea otters, including the stress caused by oil spill contamination, captivity and as we suggest here, possibly the presence of *Helicobacter* spp. Stress ulcers are defined as multiple superficial erosions of the gastric mucosa. Stress ulcers are typically thought of as independent of *Helicobacter* spp. infection and arise instead in humans from damage to the mucosal barrier that is secondary to some other systemic stress, refluxed bile, and/or high gastric acid secretion. Stress elevates glucocorticoids that can cause increase vasoconstriction, promote clotting, and thus ulcer development (Loughlin 1994). Glucocorticoids also have potent anti-inflammatory and immunosuppressive effects (Munck et al. 1984; Sapolsky et al. 2000), potentially allowing pathogens like *Helicobacter* to proliferate and cause disease. Stress ulcers and sea otter *Helicobacter* ulcers may not be mutually exclusive. Our data suggest that southern sea otter *Helicobacter* infections might be a risk factor for gastric ulcers. The following were not statistically correlated: sex, shark-related mortality, parasite co-infection, and melena, which suggests these factors were not strongly influencing or influenced by *Helicobacter* infections.

A prior study reported '*H. enhydrae*' sensitivity towards cephalothin and nalidixic acid at 30 µg each (Shen et al. 2017). We were unable to obtain ETEST®

cephalothin strips because they were discontinued. We will conduct MICs for ETEST® nalidixic acid strips in future experiments. The next step is to expand upon the antibiotic profile in this study to verify the best antibiotic choices and dosages that could be used against '*H. enhydrae*'.

Gastric ulcers have been reported in southern sea otters since the 1960s and were treated medically (Mattison and Hubbard 1969; Williams and Davis 1995). Gastric ulcers were observed in 2/13 of otters necropsied from 1968-1969, with Kaopectate used as treatment on captive sea otters with suspected gastric lesions (Mattison and Hubbard 1969). During the 1989 Exxon Valdez Oil Spill in Alaska, oiled northern sea otters (*Enhydra lutris kenyoni*) with suspected gastrointestinal ulcers were treated via stress reduction (i.e. reduced human interactions) and cimetidine, an antacid (5-10 mg/kg tid IM or PO) (Williams and Davis 1995). Sick or injured southern sea otters in rehabilitation have been treated with histamine H_2-receptor antagonists (e.g. Famotidine) or nonsteroidal anti-inflammatory drugs (NSAIDs) (e.g. Meloxicam) to prevent gastric ulcers (Monterey Bay Aquarium, pers. comm.). In other *Helicobacter* infections, antibiotics and gastric relief medications are used in tandem to treat humans and ferrets with *Helicobacter* infections and gastric illnesses. A study investigating antimicrobial treatments for ferret *H. mustelae* determined that a combination of drugs was the most effective mode of bacterial eradication. There was a 71% (5/7) success rate in clearing *H. mustelae* in adult ferrets when using a triple antimicrobial therapy consisting of amoxicillin (antibiotic), metronidazole (antibiotic), and bismuth subsalicylate (antacid) (Otto et al. 1990).

Triple antimicrobial therapy is commonly used to treat human *H. pylori* infections with an increasing shift to quadruple antimicrobial therapy due to antibiotic resistance (Graham and Fischbach 2011). If sea otter treatment was to be pursued in the future, it would make sense to explore similar triple antibiotic therapies as those used for *H. pylori* and *H. mustelae*.

Although our work has added new knowledge about sea otter *Helicobacter* infections, there remain several gaps in our knowledge. This information would benefit the sea otter population, biologists, and animal rehabilitators. First and foremost, there remain significant gaps in our understanding of the putative other sea otter *Helicobacter* species that was identified only based on 16s rRNA sequencing (Harper et al. 2003b). A good first step would be to isolate and biochemically characterize this other sea otter *Helicobacter* species. Additionally, it is unknown if '*H. enhydrae*' has multiple strains with varying virulence and antibiotic sensitivity like *H. pylori*. There have been no *in vivo* studies on '*H. enhydrae*' to date and it is unknown if this bacterium can be used in murine models to experimentally validate gastric pathogenesis. Although histologic stains, like H&E and Warthin-Starry stains, are useful in finding *Helicobacter*-like organisms, they are non-specific and not confirmatory. An immunohistochemistry stain to specifically label '*H. enhydrae*' would aid in bacterial visualization, location, and abundance. Fecal-oral transmission of *Helicobacter* spp. through poor sanitation and water quality are a concern with humans and might be a route of exposure for sea otters in close quarters in aquaria and/or rehabilitation. PCR analysis on sea otter fecal matter and survival of *'H.*

enhydrae' in sea water will help elucidate whether this is a potential route of transmission.

Overall, our results indicate that most southern sea otters are infected with gastric *Helicobacter* species. Based on qPCR, sea otter *Helicobacter* spp. prevalence in this study were comparable to the other mustelid analyzed, ferret *H. mustelae*. Using formalin-fixed tissues would have underrepresented the true prevalence, so one main finding from this work is that formalin samples should be used with caution. We furthermore detected an association between *Helicobacter* infections and gastrointestinal disease, specifically gastric ulcers. Furthermore, we show here that these bacteria are sensitive to several antibiotics, so if future studies suggest treatment is warranted, this information can be used to guide that approach.

Tables

Primer	Sequence (5'-3')
116F	AGTAATGCATAGGTTATGTGCCCT
121F	TGCATAGGTTATGTGCCCTTTAGT
237R	CAAGCTGATAGGACATAGGCTGAT

Table 1: Primer sequences used for amplification of 16s rRNA in the *Helicobacter* genus.

Positive and Negative Control Templates	qPCR #1 (Cq)	qPCR #2 (Cq)	qPCR #3 (Cq)	qPCR #4 (Cq)	qPCR #5 (Cq)
Nuclease-Free H2O	38.03	38.03	37.43	37.08	40
mqH2O	nd	nd	36.70	nd	nd
E. coli 7.6 - 8.6 ng/µL	40	40	38.32	37.10	40
no template	nd	nd	35.64	nd	nd
Helicobacter enhydrae 89 ng/µL	10.08	9.94	10.66	nd	10.51
H. enhydrae 8.9 ng/µL	11.66	11.7	13.83	nd	nd
H. enhydrae 0.8 ng/µL	14.84	15.68	17.38	nd	nd
H. enhydrae 8x10-3 ng/µL	20.8	20.15	20.85	nd	nd
H. enhydrae 8x10-4 ng/µL	24.16	24.76	24.23	nd	nd
H. enhydrae 8x10-5 ng/µL	nd	nd	27.69	28.05	29.36
H. enhydrae 8x10-6 ng/µL	nd	nd	31.17	31.28	32.65
H. enhydrae 8x10-7 ng/µL	nd	nd	33.16	36.97	37.86
H. enhydrae 8x10-8 ng/µL	nd	nd	35.64	40	37.22

Table 2: Quantitative PCR results for known negative controls and positive control dilutions from '*Helicobacter enhydrae*' sp. nov. (MIT 01-6242). Nucleic acid concentration measured in ng/µL by NanoDrop 2000 (Thermo Fisher Scientific). Nd = not done. Cq = Quantification cycle value.

	Antimicrobial Agent	MIC by ETEST® (0.016-256 µg/mL)
'*H. enhydrae*'	Amoxicillin (AC)	0.5 - 2.0
	Clarithromycin (CH)	0.016*
	Chloramphenicol (CL)	1.5 - 2.0
	Kanamycin (KM)	3.0 - 4.0
	Tetracycline (TC)	0.047 - 0.094

Table 3: Minimum inhibitory concentrations (MIC) for '*Helicobacter enhydrae*' sp. nov. on antimicrobial agents targeted towards bacteria like *H. pylori*. *Preliminary results that require additional confirmatory trials.

Figures

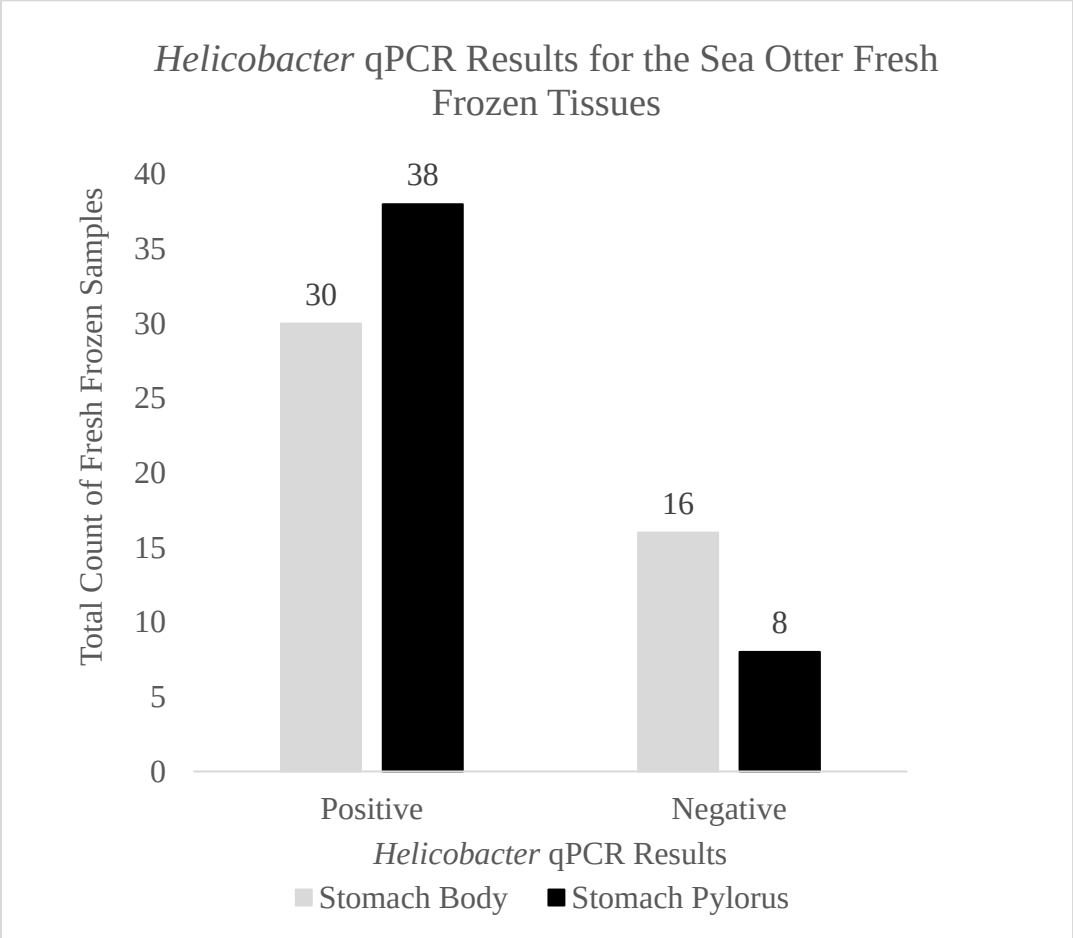

Figure 1: *Helicobacter* PCR results for the fresh-frozen southern sea otter gastric tissue samples (n= 92).

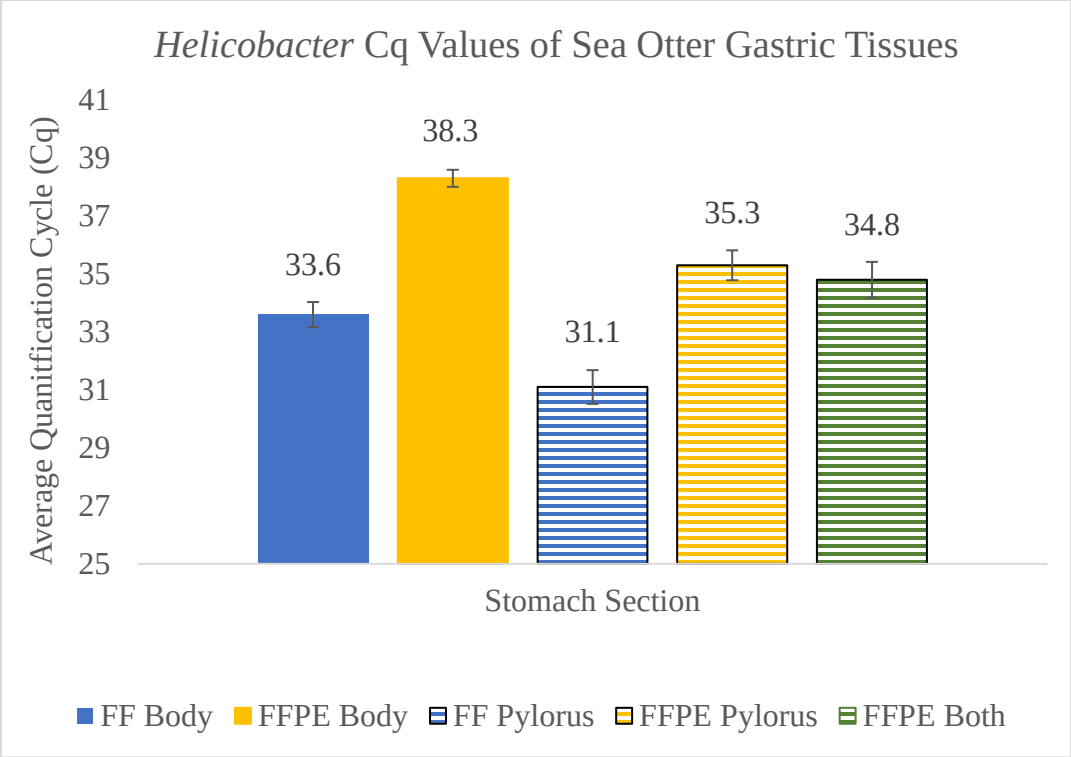

Figure 2: Comparison of the average quantification cycle for sea otter gastric tissues as fresh-frozen (FF) (n= 92) and formalin-fixed, paraffin-embedded (FFPE) (n= 59) samples. FFPE both = gastric body and pylorus in same sample. Error bars = standard error of the mean.

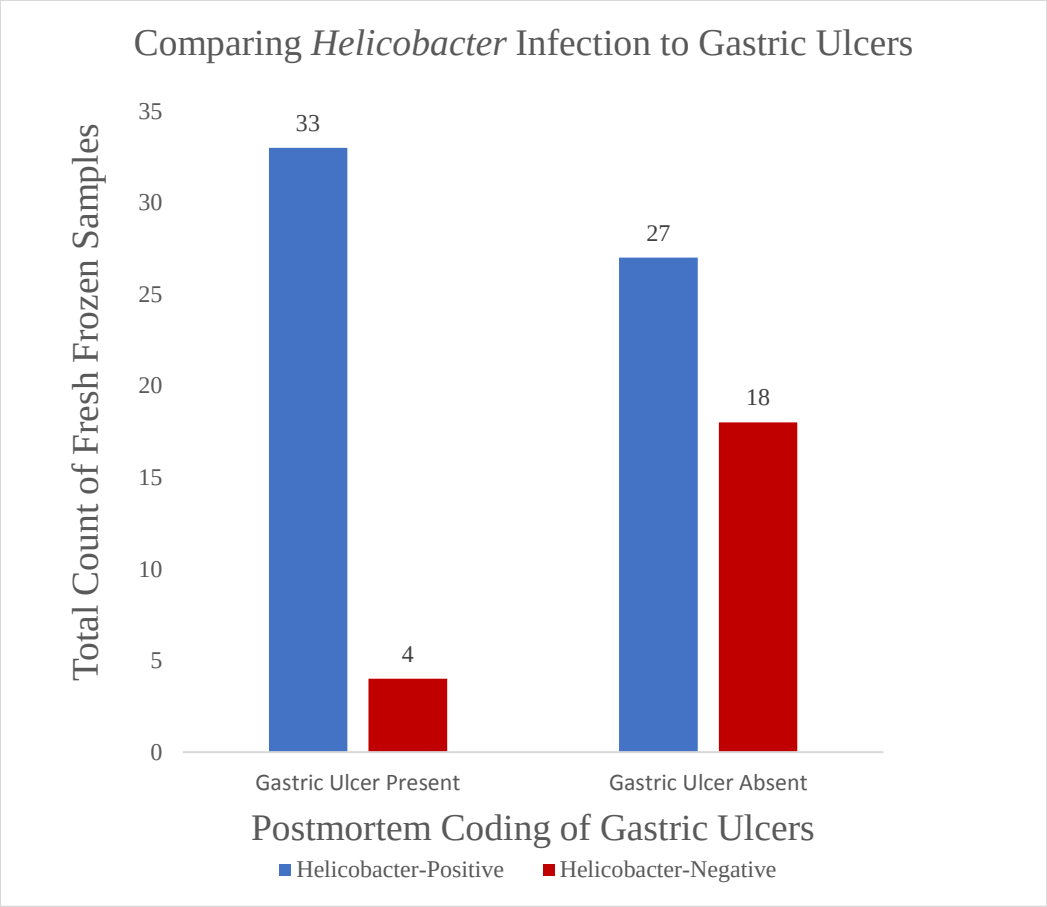

Figure 3: Comparison of southern sea otter *Helicobacter* spp. infections and postmortem coding of gastric ulcers (n= 82, 10 sea otters with unknown ulcers removed from this analysis).

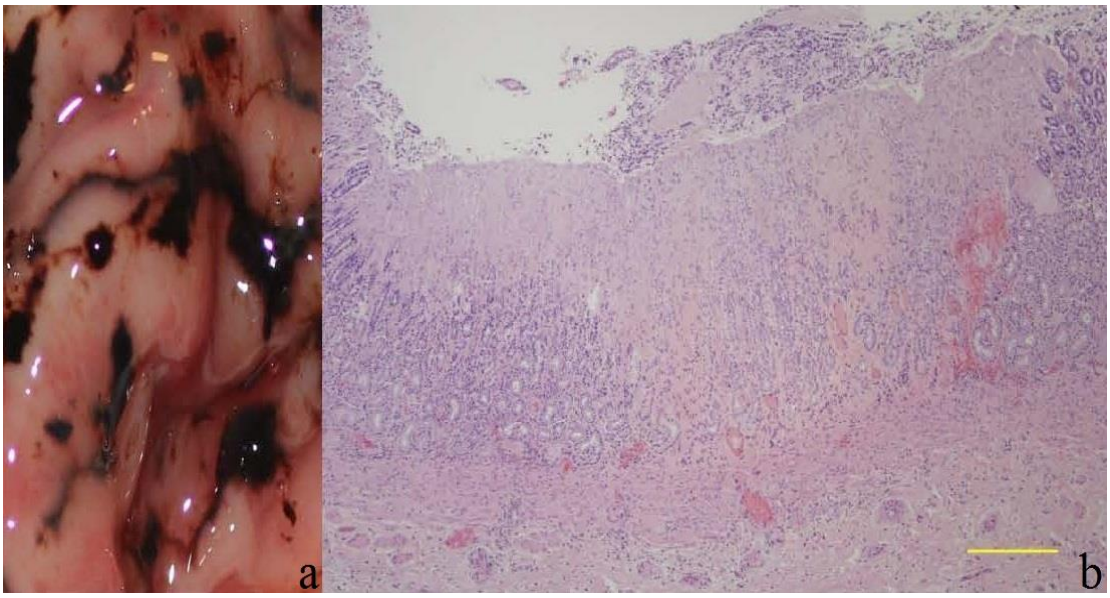

Figure 4: Gross and Hematoxylin & Eosin (H&E) images from a *Helicobacter* qPCR-positive, gastric ulcer-positive subadult female southern sea otter. (a) Gross image of severe pyloric gastric mucosal ulcers with severe perilesional mucosal hemorrhage. (b) Microscopic view of a pyloric mucosal infarct from the same animal as in A. Focally extensive mucosal coagulation necrosis is associated with thrombosis of underlying blood vessels and perilesional hemorrhage. Scale bar = 500 µm

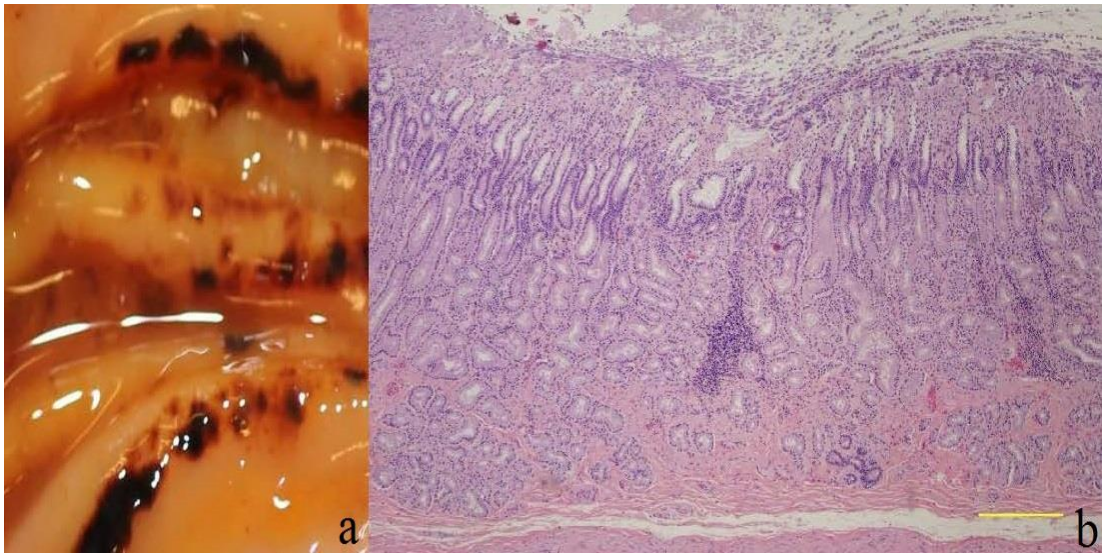

Figure 5: Gross and Hematoxylin & Eosin (H&E) images from a *Helicobacter* qPCR-positive, gastric ulcer-positive adult male southern sea otter. (a) Severe pyloric mucosal erosions and ulcers with perilesional mucosal hemorrhage and melena. Most of the lesions are aligned along the tips and edges of rugal folds. (b) Microscopic view of pylorus, showing focal expansion of pyloric lamina propria by nonsuppurative inflammation, in association with mild stromal collapse. H&E. Scale bar = 500 µm

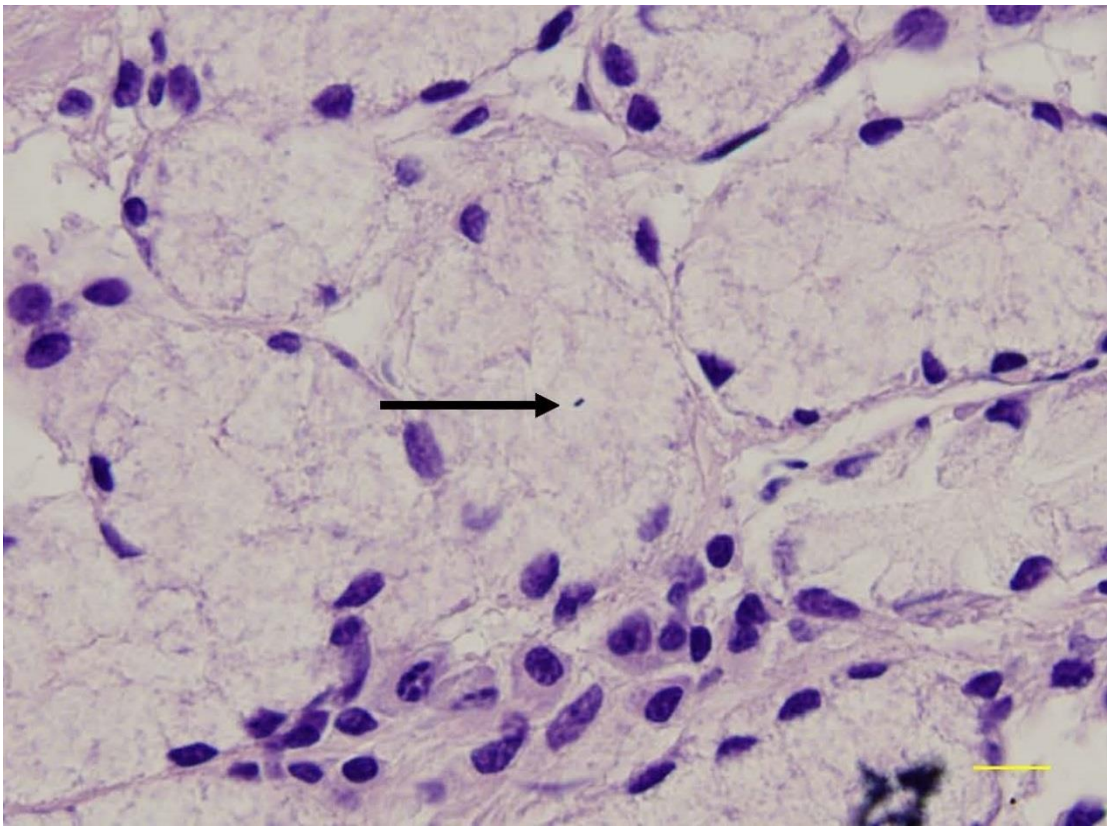

Figure 6: *Helicobacter*-like organism (black arrow) found in a pyloric gland of a *Helicobacter* qPCR-positive, gastric ulcer-negative, aged adult female southern sea otter. H&E. Scale bar = 20 µm

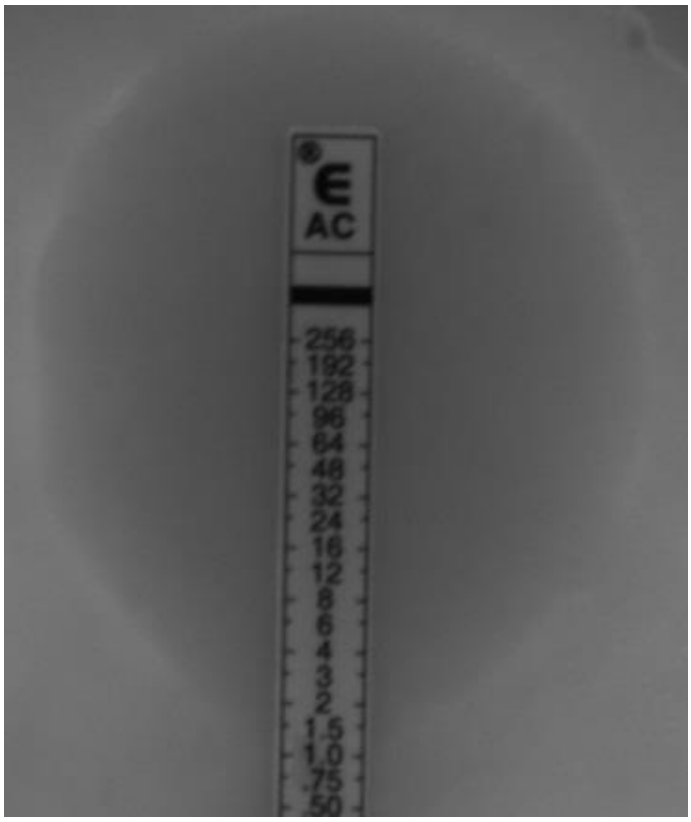

Figure 7: A visible zone of '*H. enhydrae*' growth inhibition around an ETEST® amoxicillin (AC) gradient strip on CHBA media showed this trial with a minimum inhibitory concentration at 1.0 µg/mL.

Supplemental Materials

Supplemental Material 1 - Guides and references for sea otter gastric histology grading.

Supplemental Material 2 – Histologic coding for the hematoxylin & eosin microscope slides for the southern sea otter formalin-fixed, paraffin-embedded gastric tissues (n= 57).

Supplemental Material 3 - Demographic data and Quantitative PCR results for the fresh frozen southern sea otter gastric tissue samples.

Supplemental Material 4 - Quantitative PCR results for the formalin-fixed, paraffin-embedded southern sea otter gastric tissue samples.

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