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Tracking the Source of Helicobacter pylori in Watersheds of San Juan, Puerto Rico

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Chapters

Abstract

Helicobacter pylori is a pathogenic bacterium that infects more than half of the world's population. The large number of *H. pylori* infections in Puerto Rico could be related to the waterborne transmission of the pathogen. While the San Juan Bay Estuary (SJBE) system is home to over three million people, water quality studies in this area of Puerto Rico are lacking. The goal of this study is to determine seasonal and yearly (2020-2021) shifts and relationships between the presence of *H. pylori* and host-specific (human and dog) *Bacteroides* in streams that flow through the northern coastal zone of Puerto Rico and streams that drain into the SBJE waters using quantitative polymerase chain reaction (qPCR) methods and microbial source tracking (MST) techniques. The 16s rRNA gene fragment of *H. pylori* was detected in twenty-nine (16.86%) of the 172 water samples collected over the two-year period with higher detection rates in the wet seasons (17.65%) than the dry seasons (16.09%). Microbial source tracking of human- and dog-specific *Bacteroides* in *H. pylori* positive samples over the two-year period resulted in equal detection rates (51.72%) of both host sources. Human- and dog-specific *Bacteroides* were detected in eighty-three (48.26%) and seventy-six (44.19%) of the 172 samples collected, respectively. A total of eleven samples were positive for all three markers (*H. pylori*, human, dog). Five of these samples were collected during the 2020 sampling event from two sample sites at subbasin Juan Méndez and one sample site from the Blasina, Rio Piedras Norte, and Dona Ana subbasins. The remaining six samples were collected during the 2021 sampling event from two sample sites located at the Dona Ana subbasin, and one sample site from subbasins Juan Méndez, Río Herrera, Blasina, and Río Canovanillas.

Introduction

Puerto Rico

The San Juan Bay Estuary (SJBE) area in Puerto Rico is home to approximately 3.2 million people (United States Census Bureau 2020). The island of Puerto Rico receives an abundant amount of rainfall each year and averages about one tropical cyclone per year (Pielke *et al.* 2003). With average water temperatures of approximately 25°C in both the wet and dry seasons, the climate of this area is optimal for the growth and survival of aquatic enterococci and potential pathogens (Truitt *et al.* 2020). Fecal contamination from human or animal sources poses a serious health risk when detected in surface waters. Septic tanks introduce approximately 165 million gallons per day into the streams of the Rio Piedras Watershed alone (Sodenberg 2008, Quiñones 2012, Garcia-Montiel *et al.* 2014). Human fecal pollution from septic tanks has previously been identified as having a correlation to general public health of tropical regions (Lamparelli *et al.* 2015). This risk remains present whether the population is using the surface water for drinking, food production, or recreational use (Soller *et al.* 2010, Kauppinen *et al.* 2019).

It is not uncommon for the presence of potentially harmful pathogens to correlate with locations high in enterococci number (Korajkic *et al.* 2018). *H. pylori* is a human pathogen that largely impacts the health of people living in developing countries, such as Puerto Rico (González-Pons *et al.* 2017). *Helicobacter pylori* infections have previously been correlated with gastric carcinomas (Ernst & Gold 2000, Peek & Crabtree 2006). Gastric cancer was listed as the fifth leading cause of death for men living in Puerto Rico from the years 2008 to 2012 (Zavala-Zegarra *et al*. 2015). A 2017 study of a Puerto Rican population found that 33% of the population was infected with *H. pylori* (González-Pons *et al.* 2017).

Helicobacter pylori

Helicobacter pylori is a gram-negative, flagellate, microaerophilic bacterium that was first identified as pathogenic in 1984 (Marshall & Warren). This bacterium can be found in numerous morphological forms including spiral, curved/straight, filamentous, and coccoid forms (Martinez *et al.* 2017, Sycuro *et al.* 2010, Sycuro *et al.* 2012). The variability of *H. pylori* forms as a rapid response to environmental changes may facilitate its pathogenicity and allow for survival in a particular niche. *H. pylori* has previously been isolated from human hosts as well as aquatic environments (Idowu *et al.* 2019, Li *et al.* 2021). *H. pylori* employs an array of virulence factors to achieve its goal of attacking host cells. The pathogen's motility is mediated by flagella as it moves toward the epithelial cells of the host's stomach where it will eventually penetrate the mucus lining (Kao *et al.* 2016). *H. pylori* can attach to the epithelial cells of the host through the production of adhesins (Huang *et al.* 2016). The pathogen exits the host through the host's feces which can eventually be found in nearby aquatic environments that may be involved in the transmission route of the pathogen (Vale & Vitor 2010).

Previous literature provides evidence for *H. pylori* infections through oral-oral, fecal-oral, or gastro-oral routes. Indirect transmission routes such as food or water have also been suggested (Khalifa *et al.* 2010, Goh *et al.* 2011). Water was first suggested as a route of transmission in 1991 and *H. pylori* has since been detected in water sources through PCR amplification and media culturing (Klein *et al*. 1991, Hulten *et al.* 1996, Moreno *et al.* 2007, McDaniels *et al.* 2005, Nayak & Rose 2007). Following this suggestion, *H. pylori* infections have also been linked to lack of clean drinking water and proper sanitation (Baker & Hegarty 2001, Brown 1999, Kusters *et al.* 2006, Soto *et al.* 2003, Ramirez-Ramos *et al.* 1997).

After the detection of *H. pylori* in patients with gastric ulcers, duodenal ulcers, and duodenal cancer, Marshall and Warren (1984) identified *H. pylori* as an infectious pathogen. *H. pylori* has since been identified as the source of infection in human patients diagnosed with gastric carcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Crowe 2019, McColl 2010, Gravina *et al.* 2018). It has been shown that the number of coccoid forms of *H. pylori* is higher in patients that have been diagnosed with gastric adenocarcinoma than in patients that have been diagnosed with peptic ulcer diseases. This suggests the coccoid bacteria's potential involvement in carcinogenesis (Zhao *et al.* 2017). Prompted by this discovery, the International Agency for Research on Cancer (IARC) (1994) classified *H. pylori* as a class I carcinogen. It has been shown that both the spiral and coccoid forms of *H. pylori* are crucial for the colonization of the human stomach (Worku *et al.* 1999).

Helicobacter pylori possesses the ability to adapt and survive in harsh or unfavorable conditions. This bacterium is capable of decreasing metabolic activity by transforming into a coccoid form during stressful conditions (Saito *et al.* 2003, Sõrberg *et al.* 1996, Kusters *et al.* 1997). The coccoid form of *H. pylori* has been divided into the three following categories: dying form, viable and culturable form, and a viable but nonculturable (VBNC) form (Gião *et al.* 2008, Azevedo *et al*. 2007). *H. pylori* may convert to a VBNC form once it experiences an environmental shift, such as a decrease in temperature or the depletion of nutrients (Besnard *et al.* 2002, Cook & Bolster 2007). The conversion of *H. pylori* to a VBNC state can increase its chance of survival by slowing metabolism, reducing nutrient absorption, and decreasing the amounts of cytoplasm and proteins which gives it a higher resistance to physical and chemical factors (Kryżek & Gosciniak 2018, Zhao *et al.* 2017). VacA and UreA mRNA genes have previously been detected through reverse transcription-polymerase chain reaction of *H. pylori*

cells in the coccoid, VBNC form suggesting that the bacterium may still be infectious in this form (Nilsson *et al.* 2002).

The conversion of *H. pylori* into a VBNC state can make detection of the pathogen much more difficult. In this state, it is difficult to culture pathogens on typical media, which makes the identification of *H. pylori* increasingly more challenging (Oliver 2005). The detection of *H. pylori* in both human host and aquatic sources is key in eradication of gastric diseases linked with the bacterium. The urea breath test (UBT) and the stool antigen test (SAT) are two most commonly used non-invasive methods of *H. pylori* detection in human hosts (Saxena *et al.* 2020, Idowu *et al.* 2019, Yañez *et al.* 2000, Pellicano *et al.* 2005). In marine samples, the most specific detections of *H. pylori* have been a result of polymerase chain reactio amplification of virulence genes (Idowu *et al.* 2019).

Microbial Source Tracking (MST)

Fecal indicator bacteria (FIB), such as *Escherichia coli*, have often been used as measurement of fecal contamination and water quality in tropical water sources (Kongprajug 2021). This detection method, while useful, is unable to identify the source host of the fecal contamination. Instead, microbial source tracking (MST) is now often used to identify and distinguish fecal contamination from different animal hosts (Sowah *et al.* 2017, Brooks *et al.* 2020, Marti *et al.* 2013, Molina *et al.* 2014). Animal hosts are identified through qPCR amplification of the 16S ribosomal RNA gene of different *Bacteroides* that are specific to different animal hosts, such as humans and dogs (Boehm *et al.* 2013, Layton *et al.* 2013, Bernhard & Field 2000, Kildare *et al.* 2007). The objectives of this study are to identify the presence of *H. pylori* in the SJBE waters of Puerto Rico between the years 2020 and 2021 and to

identify relationships between *H. pylori* and human- and dog-specific fecal pollution through quantitative polymerase chain reaction (qPCR) and microbial source tracking (MST).

Methods

Sampling Sites

A total of one hundred and seventy-two samples were collected from twenty-three different subbasins located in the SBJE. Sample site locations were selected using specific criteria of interest such as agriculture or population. Dry season sampling events (June 2020 and June 2021) and wet season sampling events (August 2020 and August 2021) are listed by sample number and site location in Table 1 and are depicted in Figures 1-6.

Table 1. Watershed, station, latitude, and longitude for each corresponding sample site. (a) June 2020 sampling event. (b) August 2020 sampling event. (c) June 2021 sampling event. (d) August 2021 sampling event.

Sample #	Subbasin	Station	Latitude	Longitude
1a	Rio Piedras Sur	9	18.3435	-66.0598
2a	Rio Piedras Sur	7	18.35853	-66.0656
3a	Rio Piedras Norte	1	18.36687	-66.0633
4a	Rio Piedras Norte	4	18.39435	-66.056
5a	Rio Piedras Norte	7	18.41659	-66.0785
6a	Margarita	9	18.41197	-66.1039
7a	Margarita	11	18.40846	-66.0963
8a	Juan Méndez	4	18.39855	-66.0405
9a	Juan Méndez	10	18.42451	-66.0397
10a	Juan Méndez	11	18.42454	-66.0395
11a	Juan Méndez	12	18.42725	-66.0395
12a	Blasina	8	18.39529	-65.9655
13a	Blasina	9	18.41554	-65.9652

14a	Blasina	10	18.41848	-65.9665
15a	Blasina	17	18.38433	-65.9677
16a	Blasina	19	18.38887	-65.9741
17a	San Antón	8	18.41328	-66.0078
18a	San Antón	11	18.41798	-66.0006
19a	San Antón	12	18.42146	-65.991
20a	Dona Ana	5	18.39349	-66.0906
21a	Dona Ana	11	18.40133	-66.0778
22a	Josefina	3	18.3947	-66.0798
23a	Josefina	5	18.39996	-66.0766
24a	Río Grande de Loíza	2	18.3859	-65.9209
25a	Río Grande de Loíza	14	18.42989	-65.8806
26a	Río Grande de Loíza	16	18.43329	-65.8837
27a	Río Canovanillas	2	18.30439	-65.9103
28a	Río Canovanillas	5	18.3153	-65.9041
29a	Río Canóvanas	3	18.29217	-65.8889
30a	Río Canóvanas	15	18.33826	-65.8884
31a	Río Canóvanas	16	18.34728	-65.8917
32a	Río Canóvanas	17	18.34455	-65.8919
33a	Bocaforma	1	18.37559	-65.9042
34a	Bocaforma	2	18.3775	-65.9053
35a	Bocaforma	5	18.38054	-65.8966
36a	Río Herrera	2	18.33242	-65.867
37a	Río Herrera	3	18.33947	-65.8675
38a	Río Herrera	4	18.3394	-65.8668
39a	Río Herrera	6	18.34865	-65.8661
40a	Río Herrera	11	18.3815	-65.8538
41a	Quebrada Angela	7	18.38796	-65.8446
42a	Quebrada Cambalache	4	18.3815	-65.8623
43a	Canal San Isidro	2	18.39106	-65.892453
44a	Canal San Isidro	4	18.39817	-65.896519
1b	Rio Piedras Norte	3	18.38402	-66.0587
2b	Rio Piedras Norte	5	18.40246	-66.0649

3b	Rio Piedras Norte	6	18.41052	-66.0704
4b	Margarita	1	18.39897	-66.1086
5b	Margarita	2	18.39891	-66.1086
6b	Catano	Toro Greek	18.41919	-66.1281
7b	Catano	Puente Blanco	18.43014	-66.1369
8b	Juan Méndez	3	18.39778	-66.0422
9b	Juan Méndez	7	18.41992	-66.0374
10b	Blasina	5	18.38292	-65.9839
11b	Blasina	7	18.38577	-65.9782
12b	Blasina	19	18.38887	-65.9741
13b	San Antón	7	18.41064	-66.0012
14b	Sabana Llana	6	18.39249	-66.014
15b	Guaracanal	6	18.38432	-66.0574
16b	Guaracanal	1	18.36378	-66.0314
17b	Buena Vista	7	18.39982	-66.0671
18b	Dona Ana	6	18.38989	-66.094
19b	Josefina	2	18.3908	-66.0816
20b	Josefina	4	18.3972	-66.0781
21b	Río Grande de Loíza	4	18.39203	-65.913
22b	Río Grande de Loíza	7	18.41363	-65.8905
23b	Río Grande de Loíza	15	18.43035	-65.8813
24b	Río Grande de Loíza	16	18.43329	-65.8837
25b	Río Canovanillas	4	18.30906	-65.9051
26b	Río Canovanillas	9	18.34989	-65.9236
27b	Río Canovanillas	12	18.37135	-65.9205
28b	Río Canovanillas	13	18.37699	-65.9164
29b	Río Canóvanas	2	18.26704	-65.8751
30b	Río Canóvanas	6	18.31625	-65.8842
31b	Río Canóvanas	8	18.32654	-65.8888
32b	Río Canóvanas	18	18.36137	-65.8877
33b	Río Canóvanas	20	18.37866	-65.8922
34b	Bocaforma	3	18.37585	-65.8972
35b	Bocaforma	6	18.38137	-65.9006

36b	Río Herrera	6	18.34865	-65.8661
37b	Río Herrera	8	18.37807	-65.8588
38b	Río Herrera	10	18.38031	-65.85
39b	Quebrada Angela	4	18.35905	-65.8672
40b	Quebrada Angela	6	18.37815	-65.8616
41b	Quebrada Angela	8	18.38893	-65.8623
42b	Quebrada Cambalache	1	18.36911	-65.8733
43b	Canal San Isidro	1	18.3819	-65.886270
1c	Rio Piedras Norte	2	18.40245639	-66.06490361
2c	Rio Piedras Norte	2	18.41052389	-66.07038472
3c	Juan Méndez	4	18.39778333	-66.04221611
4c	Juan Méndez	4	18.41991889	-66.03736694
5c	Buena Vista	10	18.39982389	-66.06707472
6c	Dona Ana	12	18.38989361	-66.09403417
7c	Josefina	13	18.39080278	-66.08157778
8c	Dona Ana	12	18.3972	-66.07811389
9c	Catano	Toro Greek	18.419188	-66.128051
10c	Catano	Puente Blanco	18.430143	-66.136908
11c	San Antón	7	18.41063833	-66.00124389
12c	Río Grande de Loíza	14	18.39203	-65.913011
13c	Río Grande de Loíza	14	18.413626	-65.890491
14c	Río Grande de Loíza	14	18.430346	-65.881289
15c	Río Grande de Loíza	14	18.43329	-65.883662
16c	Río Herrera	18	18.348647	-65.866104
17c	Río Herrera	18	18.378067	-65.858803
18c	Río Herrera	18	18.380314	-65.849966
19c	Quebrada Angela	19	18.359046	-65.86724
20c	Quebrada Angela	19	18.378148	-65.861579
21c	Quebrada Angela	19	18.38893	-65.86228

22c	Quebrada Cambalache	20	18.369111	-65.873332
23c	Canal San Isidro	21	18.381895	-65.88627
24c	Rio Piedras Norte	2	18.38401583	-66.05871889
25c	Blasina	5	18.38291944	-65.98387972
26c	Blasina	5	18.38577194	-65.97821833
27c	Blasina	5	18.388865	-65.974051
28c	Sabana Llana	8	18.39249472	-66.01400028
29c	Guaracanal	9	18.36378083	-66.03140361
30c	Guaracanal	9	18.38431639	-66.057385
31c	Río Canovanillas	15	18.30906	-65.905053
32c	Río Canovanillas	15	18.34989	-65.923588
33c	Río Canovanillas	15	18.371353	-65.920459
34c	Río Canovanillas	15	18.376992	-65.916357
35c	Río Canóvanas	16	18.267042	-65.875099
36c	Río Canóvanas	16	18.316247	-65.884178
37c	Río Canóvanas	16	18.326537	-65.888797
38c	Río Canóvanas	16	18.361373	-65.887702
39c	Río Canóvanas	16	18.378657	-65.892234
40c	Bocaforma	17	18.375847	-65.89716
41c	Bocaforma	17	18.381368	-65.90062
1d	Rio Piedras Sur	1	18.34349722	-66.05983306
2d	Rio Piedras Sur	1	18.35853028	-66.06557778
3d	Rio Piedras Norte	2	18.36687111	-66.06330722
4d	Rio Piedras Norte	2	18.39434917	-66.05604444
5d	Margarita	3	18.41196722	-66.10391139
6d	Margarita	3	18.40845778	-66.09632861
7d	Juan Méndez	4	18.39854917	-66.040465
8d	Juan Méndez	4	18.42450889	-66.03970111

9d	Juan Méndez	4	18.42454417	-66.03948694
10d	Juan Méndez	4	18.42724694	-66.03953778
11d	Blasina	5	18.39528944	-65.96548
12d	Blasina	5	18.41553833	-65.96521583
13d	Blasina	5	18.41847722	-65.96653333
14d	Blasina	5	18.38433139	-65.96771222
15d	Blasina	5	18.388865	-65.974051
16d	San Antón	7	18.4132825	-66.00784444
17d	San Antón	7	18.41798	-66.000591
18d	San Antón	7	18.421456	-65.990973
19d	Dona Ana	12	18.39349361	-66.09062583
20d	Dona Ana	12	18.40132778	-66.07781917
21d	Josefina	13	18.3947	-66.07979444
22d	Josefina	13	18.39996111	-66.07657222
23d	Rio Piedras Norte	2	18.4165925	-66.07854333
24d	Río Grande de Loíza	14	18.385898	-65.920941
25d	Río Grande de Loíza	14	18.429894	-65.880633
26d	Río Grande de Loíza	14	18.43329	-65.883662
27d	Río Canovanillas	15	18.34989	-65.923588
28d	Río Canovanillas	15	18.371353	-65.920459
29d	Río Canóvanas	16	18.292173	-65.88889
30d	Río Canóvanas	16	18.338262	-65.888405
31d	Río Canóvanas	16	18.347277	-65.891652
32d	Río Canóvanas	16	18.344551	-65.891866
33d	Bocaforma	17	18.375592	-65.904241
34d	Bocaforma	17	18.377496	-65.905261
35d	Bocaforma	17	18.380541	-65.896559
36d	Río Herrera	18	18.332418	-65.866983

37d	Río Herrera	18	18.339469	-65.86748
38d	Río Herrera	18	18.339396	-65.866819
39d	Río Herrera	18	18.348647	-65.866104
40d	Río Herrera	18	18.381499	-65.853813
41d	Quebrada Angela	19	18.387964	-65.844627
42d	Quebrada Cambalache	20	18.381499	-65.862326
43d	Canal San Isidro	21	18.391064	-65.892453
44d	Canal San Isidro	21	18.398167	-65.896519

DNA Extraction

A 0.22-µm-pore-size nitrocellulose membrane filter (Type GS, Millipore, Billerica, MA, USA) was used to filter one hundred milliliters of each water sample. These filters were then frozen at -20°C and shipped on dry ice to Georgia College and State University where a Qiagen PowerSoil Kit (Qiagen, Hilden, Germany) was used to extract DNA from the filters following a modified protocol (Bachoon *et al.* 2010). Extracted DNA was quantified using a Nanodrop ND-1000 Spectrophotometer (Wilmington, DE) and then stored at -20°C until further analysis.

Quantitative Polymerase Chain Reaction (qPCR) Detection of Helicobacter pylori

Quantitative polymerase chain reaction (qPCR) assays targeted an 85-bp segment of the 16S ribosomal RNA gene found in *H. pylori* (Kobayashi *et al.* 2002). Extracted DNA amplification using the Bio-Rad CFX96 (Hercules, California, 94547, USA) was performed in duplicates and followed a modified protocol (Holman *et al.* 2014). Each assay consisted of genomic DNA from *H. pylori* ATTC® 700392D-5 as a positive control, genomic DNA from *E. coli* strain B Sigma® D4889 as a negative control, and a no-template control. The final mixture

of each SsoFast Evagreen (Bio-Rad) reaction had a volume of 20µL and contained 10ng of template DNA, 1.5mM of magnesium, and 500nM of each primer (Table 2). Each qPCR reaction was performed under the following conditions: 95°C for 3 minutes; 40 cycles of 95°C for 10 seconds, and 67°C for 20 seconds (Holman *et al.* 2014).

Quantitative Polymerase Chain Reaction (qPCR) Assays of Microbial Source Tracking (MST)

Both human and dog fecal pollution qPCR assays were performed in duplicate using QuantiTect Probe PCR (Qiagen) reagents. Each assay had a total volume of 20 µl and consisted of 2 µl of extracted DNA, 500nM of each primer, and 200nM of the probe (Table 2). Assays were subjected to thermal conditions beginning with 95°C for 15 minutes, followed by 40 cycles at 95°C for 10 seconds and 60°C for 40 seconds. *Bacteroides* specific to dog and human hosts were used as positive controls and *E. coli* strain B genomic DNA Sigma® D4889 was used as a negative control (Bachoon *et al.* 2010). Specifically, *Bacteroides dorei* and canine fecal DNA were used as positive controls. No-template controls were also performed for both assays.

Table 2. Primer and probe sequences of *Helicobacter pylori* and microbial source tracking qPCR assays.

Target	Primer	Sequence	Reference
16S rRNA gene	HP-F HP-R	CTCATTGCGAAGGCGACCT TCTAATCCTGTTTGCTCCCCA	Kobayashi *et al.* 2002
Human	HF-183-1 BFDRev BFDFam	ATCATGAGTTCACATGTCCG CGTAGGAGTTTGGACCGTGT 6-FAM-CTGAGGAGAAGGTCCCCCACATTGGA-TAMRA	Green *et al.* 2014
Dog	BacCan-545F1 BacUni-690R2 Probe	GGAGCGCAGACGGGTTTT AATCGGAGTTCCTCGTGATATCTA 6-FAM-CTGAGGAGAAGGTCCCCCACATTGGA-TAMRA-MGB	Kildare *et al.* 2007

Results

Helicobacter pylori and MST

A total of one hundred and seventy-two water samples were analyzed for the presence of *H. pylori* and host-specific MST markers during the wet and dry seasons between the years 2020 and 2021 (Table 3). A total of eighty-seven samples were analyzed from the dry and wet seasons during the year 2020. Approximately 15.91% of the samples collected during the 2020 dry season and 20.93% of the samples collected during the 2020 wet season were positive for *H. pylori*. The water samples collected during the 2020 wet and dry sampling events collectively showed an *H. pylori* detection rate of 18.39% (Figure 1).

Microbial source tracking of the samples collected during the dry 2020 sampling event resulted in three samples that were positive for both *H. pylori* and human fecal bacteria (Figure 1 and Figure 3). During the wet 2020 sampling event, six of the samples were positive for both *H. pylori* and human fecal bacteria. During the 2020 sampling season, a total of 5 samples were positive for all markers tested (*H. pylori*, human fecal pollution, and dog fecal pollution). Two of these samples were collected during the dry sampling event from sample sites at Blasina and Juan Méndez, and the remaining three samples were collected during the wet sampling event from sample sites at Rio Piedras Norte, Juan Méndez, and Dona Ana (Figure 1, Figure 3, and Figure 5).

A total of eighty-five water samples were analyzed from the dry and wet seasons during the year 2021. Approximately 19.51% of the samples collected during the 2021 dry season and 11.36% of the samples collected during the 2021 wet season were positive for *H. pylori*. The water samples collected during the 2021 wet and dry sampling events collectively showed an *H. pylori* detection rate of 15.29% (Figure 2).

Microbial source tracking of the samples collected during the dry 2021 sampling event resulted in five samples that were positive for both *H. pylori* and human fecal bacteria (Table 3). During the wet 2021 sampling event, only one sample, collected from the Río Canovanillas subbasin, was positive for both *H. pylori* and human fecal bacteria markers (Table 3, Figure 2, and Figure 4). During the 2021 sampling event, a total of six samples were positive for all markers tested (Table 3). Five of the samples were collected during the dry sampling event from two sample sites at Dona Ana, and sample sites located at the Juan Méndez, Río Herrera, and Blasina subbasins. The remaining sample was collected during the wet sampling event from a sample site located at the Río Canovanillas subbasin (Figure 2, Figure 4, and Figure 6).

Table 3. Positive (+) and negative (-) qPCR results for *Helicobacter pylori* and animal-specific fecal markers in water samples collected during dry (June) and wet (August) seasons over a two-year period. (a) June 2020. (b) August 2020. (c) June 2021. (d) August 2021.

Sample Site	*H. pylori*	Human	Dog
1a	-	-	-
2a	-	-	+
3a	-	+	+
4a	-	+	+
5a	-	+	+
6a	-	+	+
7a	-	+	-
8a	+	+	+
9a	-	-	-
10a	-	+	+
11a	-	+	+
12a	+	+	+
13a	-	-	-
14a	-	-	-
15a	-	-	-
16a	-	-	-
17a	-	+	+
18a	-	-	-
19a	-	+	+
20a	-	+	+
21a	-	+	+
22a	-	+	+
23a	-	+	+
24a	-	-	-
25a	+	+	-
26a	-	-	+
27a	-	-	-
28a	-	-	+
29a	-	-	+
30a	-	-	-
31a	-	-	-

32a	-	-	+
33a	+	-	+
34a	-	-	-
35a	-	-	+
36a	+	-	-
37a	-	-	-
38a	-	-	+
39a	+	-	+
40a	+	-	-
41a	-	+	+
42a	-	+	+
43a	-	+	-
44a	-	+	+
1b	+	+	+
2b	+	+	-
3b	-	+	+
4b	-	+	-
5b	+	+	-
6b	-	+	-
7b	-	-	+
8b	-	+	+
9b	+	+	+
10b	-	+	+
11b	-	+	+
12b	-	+	+
13b	-	+	+
14b	-	+	-
15b	-	+	-
16b	-	-	-
17b	-	+	+
18b	+	+	+
19b	-	-	-
20b	-	+	+
21b	-	-	-
22b	-	-	-
23b	-	-	-

24b	+	-	-
25b	-	-	-
26b	+	-	-
27b	-	+	+
28b	-	+	+
29b	-	-	-
30b	-	-	-
31b	+	-	+
32b	-	-	-
33b	-	-	-
34b	-	-	-
35b	+	+	-
36b	-	-	-
37b	-	-	-
38b	-	+	-
39b	-	-	-
40b	-	+	-
41b	-	-	-
42b	-	-	-
43b	-	-	-
1c	-	+	+
2c	-	+	+
3c	+	+	+
4c	-	+	+
5c	-	+	+
6c	+	+	+
7c	-	-	-
8c	+	+	+
9c	-	+	+
10c	-	+	-
11c	-	+	+
12c	-	-	-
13c	-	+	-
14c	-	-	-
15c	-	-	-
16c	-	-	-

17c	-	-	+
18c	+	+	+
19c	-	-	+
20c	-	+	-
21c	-	-	-
22c	-	-	+
23c	-	-	-
24c	-	-	+
25c	-	+	-
26c	+	+	+
27c	-	+	+
28c	-	+	+
29c	+	-	+
30c	-	+	+
31c	-	-	+
32c	-	-	-
33c	-	+	+
34c	-	-	-
35c	-	+	+
36c	+	-	-
37c	-	-	+
38c	-	-	-
39c	-	-	+
40c	+	-	-
41c	-	+	+
1d	-	-	-
2d	-	-	-
3d	-	-	-
4d	-	-	-
5d	+	-	-
6d	-	+	-
7d	+	-	-
8d	-	-	-
9d	-	+	+
10d	-	+	+
11d	+	-	-

12d	-	-	+
13d	-	-	+
14d	+	-	-
15d	-	-	+
16d	-	+	+
17d	-	-	-
18d	-	+	+
19d	-	+	+
20d	-	-	-
21d	-	-	-
22d	-	-	+
23d	-	+	-
24d	-	-	-
25d	-	+	-
26d	-	-	-
27d	-	-	-
28d	+	+	+
29d	-	-	-
30d	-	+	-
31d	-	+	-
32d	-	+	-
33d	-	+	-
34d	-	-	-
35d	-	+	-
36d	-	-	-
37d	-	-	-
38d	-	+	-
39d	-	-	-
40d	-	+	-
41d	-	+	-
42d	-	+	-
43d	-	+	-
44d	-	+	-

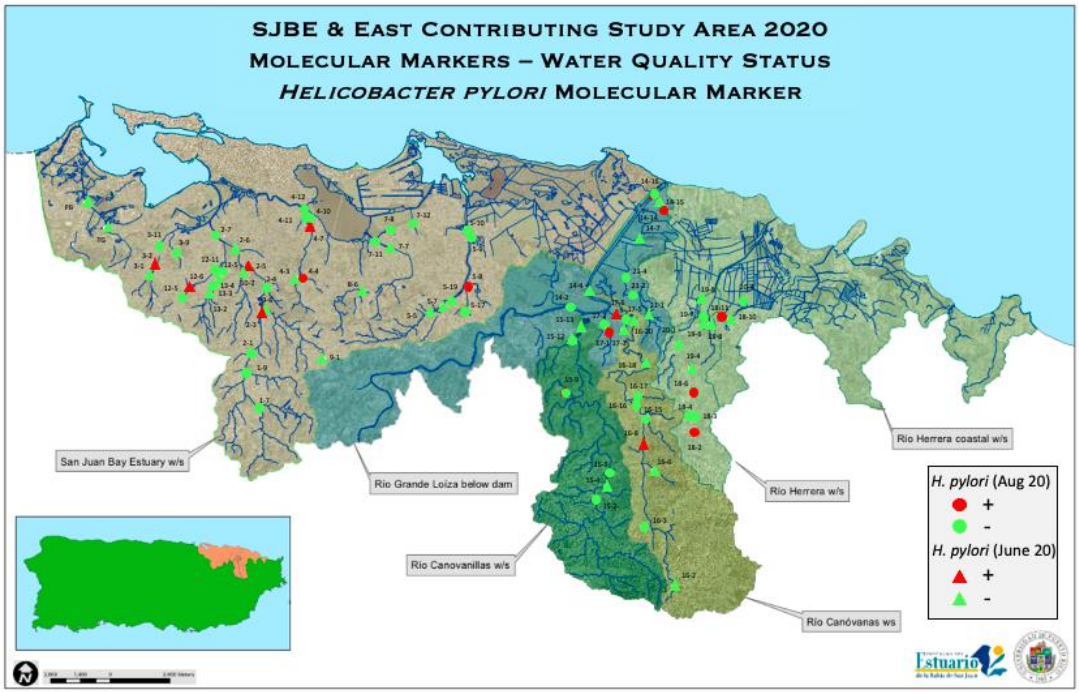

Figure 1. Presence of *Helicobacter pylori* in samples collected from the San Juan Bay Estuarian (SJBE) study area during both the dry (triangles) and wet (circles) seasons 2020. Red indicates a positive result, while green indicates a negative result.

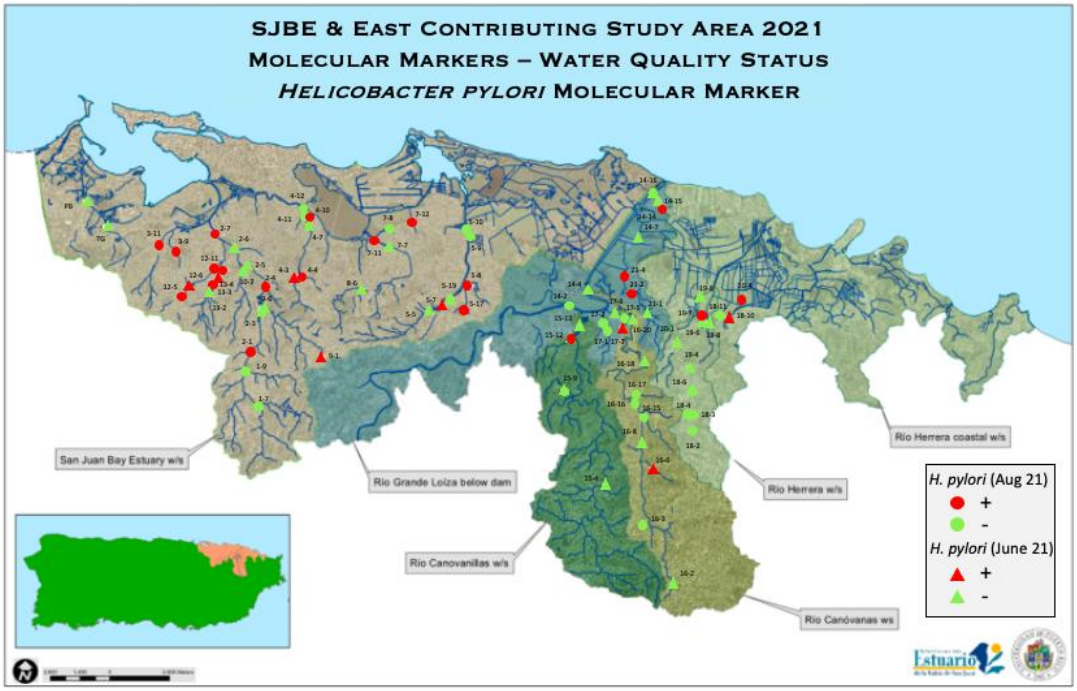

Figure 2. Presence of *Helicobacter pylori* in samples collected from the San Juan Bay Estuarian (SJBE) study area during both the dry (triangles) and wet (circles) seasons 2021. Red indicates a positive result, while green indicates a negative result.

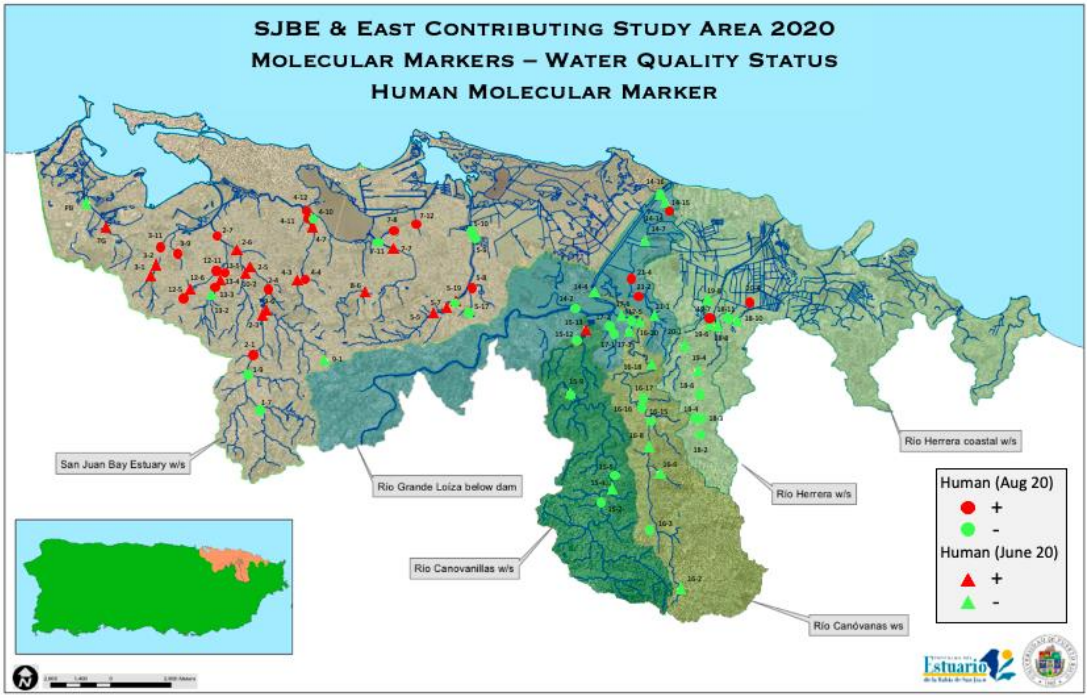

Figure 3. Presence of human fecal pollution in samples collected from the San Juan Bay Estuarian (SJBE) study area during both the dry (triangles) and wet (circles) seasons 2020. Red indicates a positive result, while green indicates a negative result.

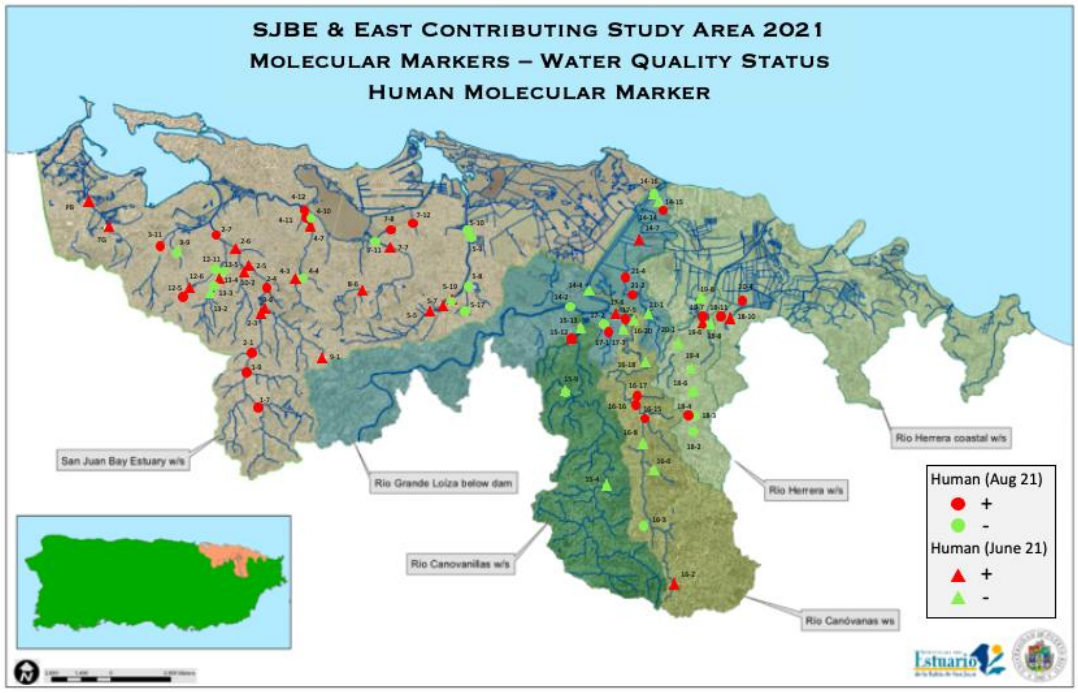

Figure 4. Presence of human fecal pollution in samples collected from the San Juan Bay Estuarian (SJBE) study area during both the dry (triangles) and wet (circles) seasons 2021. Red indicates a positive result, while green indicates a negative result.

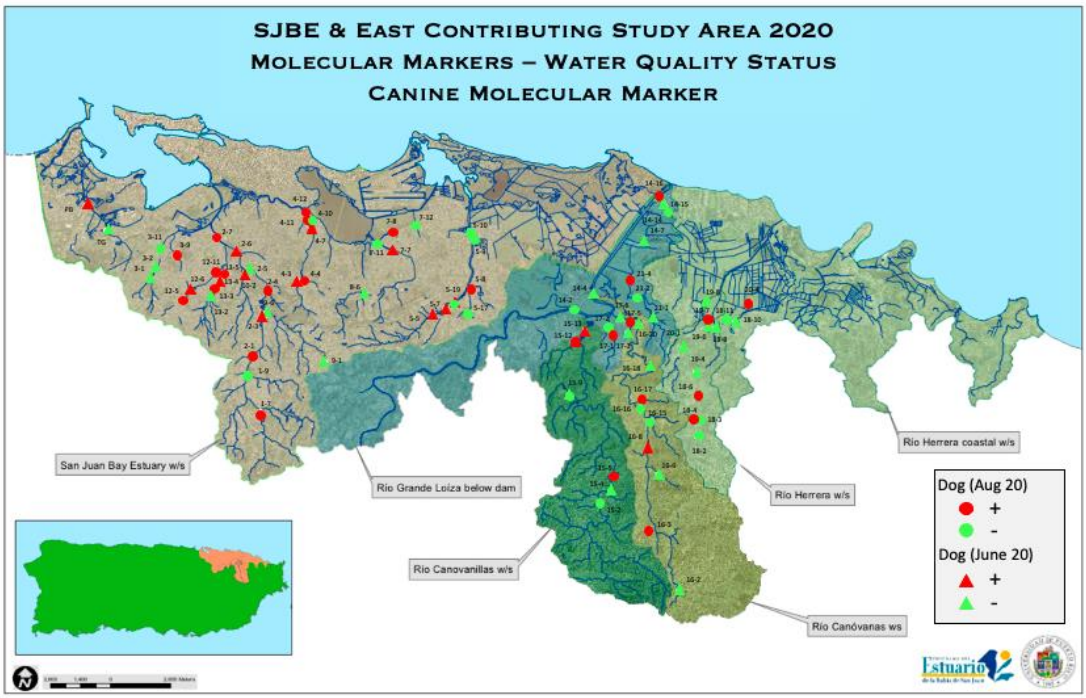

Figure 5. Presence of dog fecal pollution in samples collected from the San Juan Bay Estuarian (SJBE) study area during both the dry (triangles) and wet (circles) seasons 2020. Red indicates a positive result, while green indicates a negative result.

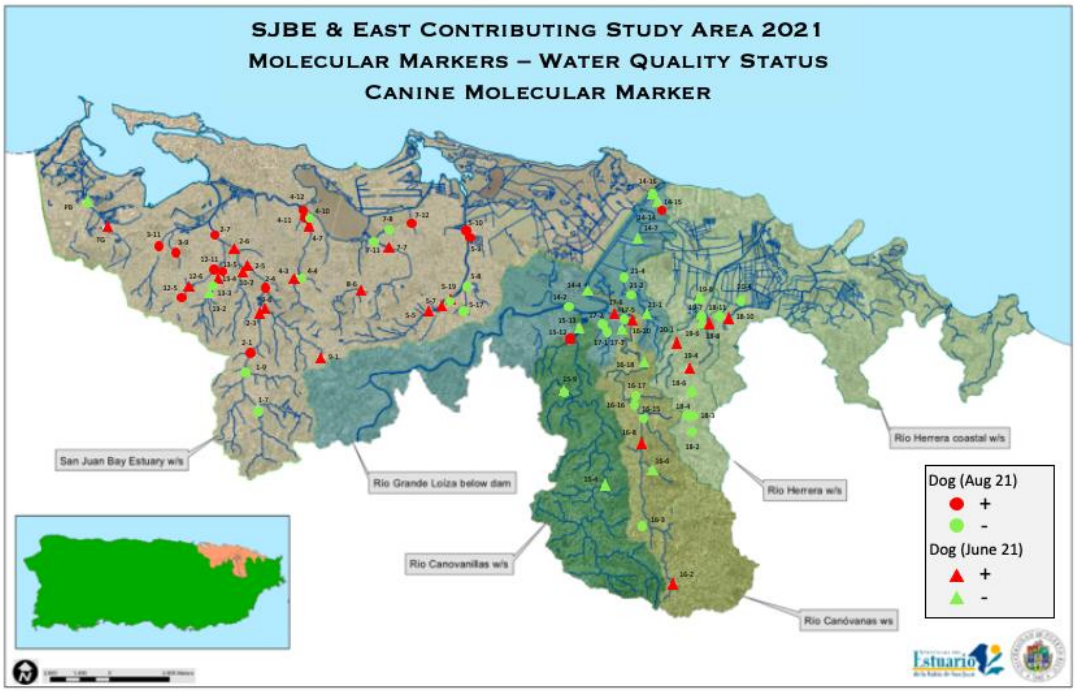

Figure 6. Presence of dog fecal pollution in samples collected from the San Juan Bay Estuarian (SJBE) study area during both the dry (triangles) and wet (circles) seasons 2021. Red indicates a positive result, while green indicates a negative result.

Discussion

Overall, *Helicobacter pylori* was detected in 16.86% of samples collected during the 2020 and 2021 wet and dry seasons (Table 3 and Figures 1-2). Previous studies of tropical regions have resulted in the detection of *H. pylori* being higher than the percentage detected in the current study (Sondos *et al.* 2018) With *H. pylori* infection rates as high as 33% in Puerto Rico, it was expected that *H. pylori* would be detected in the rivers and streams of the SBJE (González-Pons *et al.* 2017).

Helicobacter pylori presence in the SBJE system was higher during the dry seasons compared to the wet seasons (Table 3 and Figures 1-2). These results could be attributed to the dilution of water experienced in the study area during the dry seasons. The dilution of water in the study area can often result in larger numbers of pathogens per sample collected. A previous study of the coastal waters of Puerto Rico resulted in a greater detection of pathogenic *Leptospira* in samples collected during the wet season compared to the dry season (Truitt *et al.* 2020). While flooding or heavy rainfall on tropical islands has been shown to increase the enumeration of certain pathogens such as *Leptospira*, this phenomenon is not apparent in the current study (Truitt *et al.* 2020)

Human fecal contamination was detected at a consistent rate (~50%) throughout both the wet and dry seasons of the 2020 and 2021 sample collections (Table 3 and Figures 3-4). These findings are similar to the findings of Li *et al.* (2021) which resulted in the detection of human fecal contamination in approximately 50% of the samples collected during both the wet and dry seasons. The findings of the current study were as expected as a portion of the samples that were collected during this study were collected from an area of known human fecal contamination. The Rio Piedras watershed is the location in which the capital city of San Juan resides. This

watershed receives the discharge of two wastewater treatment plants (Puerto Nuevo Regional and Bayamon Regional WWTPs). It is common for people residing in areas of high elevation in San Juan to use septic tanks (Quiñones 2012, Garcia-Montiel 2014). Previous literature has described a possible fecal-oral route of *H. pylori* and the large amount of sewage and septic seepage in Puerto Rican rivers could be a possible source of *H. pylori* infections in this area (Khalifa *et al.* 2010, Goh *et al.* 2011). Previous research conducted by Holman *et al.* (2014) resulted in the detection of *H. pylori* in the coastal waters of the Rio Piedras watershed, which supports the findings of the current study.

The detection of human fecal pollution in the coastal waters of Puerto Rico could explain the presence of human fecal pollution in the rivers and streams of the SBJE system. This could also explain why more than half of the samples that were positive for *H. pylori* were also positive for human fecal pollution (Table 3 and Figures 3-4). Previous studies show that the coastal waters of the SBJE are highly contaminated with human fecal pollution (Bachoon *et al.* 2010). The findings of this study align with the findings of Holman *et al.* (2014), which implies that human fecal pollution may not be a reliable indicator of the presence of *H. pylori* in the SBJE.

In conclusion, *H. pylori* was detected in each of the four sampling events of this study and MST methods using host specific *Bacteroides* led to the identification of two sources of fecal contamination that may be positively related to the detection of *H. pylori* in the SBJE area. MST assays of samples containing *H. pylori* led to the identification of both human and dog specific *Bacteroides*. The eradication of *H. pylori* related diseases in this area begins with both the detection of *H. pylori* and the identification of fecal contamination hosts using MST. Future studies in this area should be conducted to determine the quantity and seasonal changes of *H. pylori* concentration in water sources.

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