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Detection of viable *Helicobacter pylori* cells in urban surface water by quantitative PCR in northeastern Spain

Running Head: *H. pylori* detection in urban surface water by PMA-qPCR

Claudia P. Acosta-Astaiza,[*,†] Francesc Codony, [‡,§] Mariana Fittipaldi,[‡]
Carlos H. Sierra-Torres[*,†] and Jordi Morató[‡]

[*] Laboratorio de Genética Humana, Departamento de Ciencias Fisiológicas, Facultad de Ciencias de la Salud, Universidad del Cauca, Popayán, Colombia.

[†] Unidad de Salud Ambiental, Fundación InnovaGen, Popayán, Colombia.

[‡] Laborati de Microbiologia Sanitària i Mediambiental (MSMLab)-Aquasoft, UNESCO Chair in Sustainability, Universitat Politècnica de Catalunya, Terrassa, Spain.

[§] GenIUL R&D Laboratory, Terrassa, Spain

Correspondence: Claudia P. Acosta, Laboratorio de Genética Humana, Departamento de Ciencias Fisiológicas, Facultad de Ciencias de la Salud, Universidad del Cauca, Calle 5 No. 4-70, Popayán, Colombia. E-mail: cpacosta@unicauca.edu.co

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Abstract

Background: *Helicobacter pylori* is considered the causing agent of chronic active gastritis and has a role in the pathogenesis of peptic ulcers, gastric carcinoma and lymphoma. Although, it has been suggested that *H. pylori* can be acquired through different transmission routes, including water sources, the exact mechanisms are still unclear. In order to contribute to our understanding of infection pathways, the objective of this study was to analyze the viability of *H. pylori* cells in urban surface waters.

Material and Methods: Water samples, collected at the Vallparadís public park in Terrassa, Barcelona, Spain, were analyzed by viability quantitative PCR using propidium monoazide (PMA) and specific primers for the *H. pylori* vacuolating cytotoxin vacA gen.

Results: Among 23 urban surface water samplings collected, 21 (91.3%) were positive for the presence of *H. pylori* using PMA-qPCR, with an average concentration of viable cells of 3.46 ± 1.06 log cell/100 mL.

Conclusions: Our results are the first to demonstrate the usefulness of PMA-based qPCR to investigate the survival and infective potential of *H. pylori* cells under environmental stress conditions in a complex matrix such as urban surface water. Furthermore, these results strengthen the argument in favor of water as an environmental reservoir for *H. pylori* transmission.

Keywords: Surface water; *Helicobacter pylori*; Quantitative PCR; Cellular viability; Propidium monoazide.

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Helicobacter pylori is a microaerophilic Gram-negative bacterium that has been estimated to infect the gastric epithelium of half of the human population [1-3]. Furthermore, *H. pylori* is considered the causing agent of chronic active gastritis and an important risk factor in the multifactorial etiology of peptic ulcers, gastric carcinoma and MALT lymphoma [4-6]. Although transmission presumably occurs through fecal–oral and oral–oral routes, the main route of *H. pylori* transmission remains still unknown [7]. Evidence favors a fecal-oral route [8,9], where water plays an important role acting as a natural reservoir and a source of infection [10,11]. However, and in spite of some reports indicating the presence of *H. pylori* DNA in water samples, the viability of the cells in the environment remains unknown.

Under laboratory conditions, *H. pylori's* ability to survive in an infectious state is hindered because its cultivability is lost rapidly [11-13]. In detrimental environmental circumstances, such as in water bodies, some authors have suggested that *H. pylori* may transform from the bacillary to the coccoid form, remaining viable and virulent but non-culturable [14-16]. However, results have been reported to controvert such hypothesis [17]. Interestingly, some studies have reported that *H. pylori* is capable of propagating and remaining viable for several weeks in the presence of amoeba [18] and biofilms [19]. Therefore, methodologies should be developed to detect and study the viability of these bacteria in surface water [20].

Given the cultivability limitations under in vitro conditions, *H. pylori* detection in water samples has been conducted mainly using molecular techniques including polymerase chain reaction (PCR), quantitative PCR [21] and fluorescent *in situ*

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hybridization [22-25]. Although qPCR is a fast and sensitive method to detect microbial DNA, it does not differentiate between viable and non-viable cells [26]. Therefore, sample pretreatment with viability dyes, such as propidium monoazide [21] has been used in combination with qPCR to distinguish between viable and non-viable cells [21,27]. PMA is a DNA-intercalating agent that selectively penetrates cells with compromised membranes, traditionally considered dead cells [26]. Once inside the cell, PMA is covalently cross-linked to DNA through light photoactivation, resulting in irreversible DNA modification and subsequent inhibition of its amplification [28]. The effectiveness of this technique has been tested on different types of bacteria [28-30], including *H. pylori* both in drinking water samples and in vitro studies [31-33].

The aim of this study was to determine the presence of viable *H. pylori* in urban surface waters using viability qPCR to understand the role of surface water in the pathogen's transmission and improve our understanding of infection pathways.

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Material and Methods

Surface water samples

A total of 23 samplings were carried out during the summer of 2014 from a water stream in the Terrassa's Vallparadís public park, in Northeast Spain. During each daily sampling round, four samples were taken. Each sample was collected aseptically in 50-mL sterile vials, and transported to the laboratory under refrigeration (4°C) for subsequent analysis. Samples were concentrated by centrifugation twice. A first step at 4000 rpm for 30 minutes was performed to reach a 2 mL volume of concentrate which was placed into a microcentrifuge tube. Then, concentrates were centrifuged at 10000 rpm for 15 minutes (Minispin Plus-Eppendorf, Hamburg, Germany), supernatant was discarded, and pellets were resuspended in 500 µL of phosphate buffered saline, pH 7.4 (PBS) for PMA treatment and DNA extraction.

PMA treatment

Out of the four daily samples, two concentrates were treated with PMA using an adapted method from Agustí et al. [31]. Briefly, 12.5 µL PMA (GenIUL, Terrassa, Spain) were added to cell aliquots for a final dye concentration of 50 µM. Samples were incubated for 10 minutes in the Dark Box System (GenIUL, Terrassa, Spain) with occasional mixing and photo-activated for 15 minutes using the PhAST®-Blue system (GenIUL , Terrassa, Spain). The other two concentrates did not receive PMA treatment and were used as controls. Subsequently, the PMA-treated and PMA-non-treated concentrates were centrifuged at 14000 rpm for 5 minutes. The supernatants were

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discarded and the pellets were resuspended in 200 μL of sterile water.

DNA extraction

For DNA extraction, PMA-treated and -non-treated concentrates were processed using the E.Z.N.A.® DNA tissue kit (Omega Bio-Tek, Doraville, USA), following the manufacturer's recommendations. The isolated DNA was eluted in 200 μL buffer solution and stored at -20°C until use.

Quantitative PCR

qPCR was performed using previously reported *H. pylori* specific primers to simultaneously amplify either allele of the VacA gene [34]. Additionally, qPCR specificity was tested using DNA of two *H. pylori* strains (Tx30a and 60190), which were donated by Dr. Xavier Calvet (Department of Gastroenterology, Hospital de Sabadell, Barcelona), as positive controls; DNA from *Pseudomonas aeruginosa* (CECT110), *E. coli* (CECT 101), *Bifidobacterium longum* (CECT 4551), and PCR-grade water were also used as amplification controls in all qPCR assays. A conventional PCR protocol previously standardized by Dr. Calvert's laboratory was run on a LightCycler 1.5 system (Roche, Manheim, Germany). Briefly, the PCR mixture contained 0.25 μL of each primer (0.5 mM), 4 μL of HOT FIREPol® EvaGreen® qPCR Mix Plus (1x) dye solution (Solis BioDyne, Tartu, Estonia) prepared according to the manufacturers' recommendations, and 5 μL of genomic DNA. Capillary tubes were shortly spinned in a microcentrifuge at a low speed to bring samples to the tip. The protocol consisted of one step of 15 minutes at 95 ºC for polymerase activation, and 45 cycles: 95 °C for 20

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s, 50 °C for 20 s, and 72 °C for 20 s with slopes of 20 °C/s, 20 °C/s and 20 °C/s, respectively. Finally, melting point analysis was performed by raising the temperature slowly (0.1°C/s) from 65 to 97°C. Test detection limit was calculated according to the standard approach described in the ISO-12869 [35]. Bacteria concentration was expressed as logarithmic units of cells per 100 mL of sample.

Statistical analysis

The software package SPSS for Windows version 20 (SPSS Inc., Chicago, IL, USA) was used for conducting the statistical analysis. Bacteria concentrations were expressed with the mean and the standard error of the mean. The coefficient of variation was used for comparing the degree of variation across groups while the nonparametric, median-based Levene's test was used to compare sample variances between groups. A probability (*p*) value of less than 0.05 was used as criterion of significance.

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Results

From the serial 10-fold dilutions from a DNA solution of the Tx30a reference strain, a standard curve was obtained with a linear range across six logarithms of *H. pylori* DNA concentration for the threshold cycle (Cp) versus the genomic copy number of H. pylori cells (Fig. 1). The cycles to threshold (Ct) mean values ranged from 17 to 33.48, corresponding to concentrations from 1 to 10 x 10^7 genomic units (GU) per 100 mL. The detection limit was established in 150 GU/100 mL.

For qPCR, samples were considered positive when their melting temperature (Tm) values were similar to the Tm of the standard curve (89.33°C). As shown in Figure 2, 21 (91.3%) out of 23 surface water samples were positive for the presence of *H. pylori* using PMA-qPCR. The total average concentration of *H. pylori* cells was 4.2 ± 1.52 log cell/100 mL, while the total average concentration of viable cells was 3.46 ± 1.06 log cell/100 mL. However, the results showed that when total concentrations were lower than 3 log cell/100 mL, the concentration of viable cells coincide with the total cells detected. Additionally, it was observed that the samples collected during rainy days contained increased loads of *H. pylori*. qPCR specificity was confirmed as amplification of the non-*Helicobacter* bacteria showed negative signals in the melting curve analyses.

In order to estimate the degree of variance across groups, the variation coefficient was estimated for each sampling and is shown in Table 1. Although some assays indicated variation, the median-based Levene's test showed that these differences were not statistically significant ($p = 0.372$) (Table 2).

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Discussion

Evidence supporting the *H. pylori* water transmission hypothesis comes largely from two bodies of research: epidemiological studies showing an association between the prevalence of *H. pylori* and water-related sources and laboratory studies that have detected or isolated *H. pylori* from water sources [36]. The presence of *H. Pylori* DNA in river waters has been previously reported by using PCR techniques [37,38]. However, as previously mentioned, PCR is not suitable for determining the infective potential of a given sample. In this work, qPCR was combined with PMA to study the potential of surface water for acting as a *H. pylori* transmission vehicle, finding a high frequency of viable *H. pylori* cells in the analyzed samples (91.3%). The presence of *H. pylori* in surface water can be explained by its ability to adapt to harsh conditions. Thus, the adherence of *H. pylori* to biofilms fosters a microaerophillic environment for the survival of the bacteria, which seems to be independent of temperature [11,14]. In addition, it has been suggested that *H. pylori* can propagate and remain viable for several weeks in the presence of *Acathamoeba spp.*, which suggests that amoebas could be a natural reservoir for the bacterium [18,39]. Recently, the PMA-PCR technique has been used to demonstrate that *H. pylori* is able to survive chlorination treatment in occurrence with *A. castellani* [22].

In the present study, bacteria concentration levels ranged from 2.5 to 6 log units per 100 mL. It is important to note that higher bacteria levels where detected in samples collected during rainy days. This could be explained by the surface water runoff phenomenon, which can be caused by stormwater, a leading source of pollution to

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fresh receiving urban waters [40]. Previous studies have indicated that surface water pollution by fecal bacteria and both inorganic and organic chemicals could be exacerbated following rainfall [41]. Furthermore, raining causes a lowering in temperatures and a dilution effect that reduces salinity, and thus, favors the establishment of bacteria, including *H. pylori* [42].

Although the minimum *H. pylori* infectious dose has not yet been established in humans, some studies suggest that 10^5 colony-forming units might be close to the minimum infectious dose [43]. In the present study, 21.7% (5/23) of the samples showed viability levels in concentrations over 4 log cell/100 mL while 8.7% (2/23) presented viable cell concentrations higher than 5 log cell/100 mL. These levels might be considered an environmental hazard to human health given that viable coccoid forms are able to maintain their urease activity and preserve their ability to adhere to epithelial cells from gastric mucosa [44].

In this study, *H. pylori* sample positivity was the same for either qPCR or PMA-qPCR methods. However, when bacteria concentrations were high (> 4 log cell/100 mL), mostly on rainy days, an average difference of 1.7 log cell/100 mL was observed between methods, being higher for qPCR, which might be mainly due to the fraction of dead cells also accounted for this technique. Although PMA has been proposed as a more appropriate DNA intercalating dye with a substantially higher specificity for live cells compared to ethidium bromide monoazide (EMA) [26], the possibility of PMA uptaking in bacterial cells with intact membranes should also be considered. It has been shown that membrane permeability undergoes changes in live cells dependent

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on the physiological status [45]. However, a previous permeability study has shown that membranes of live *H. pylori* cells effectively prevent penetration of PMA (50 µM, exposure for 5 min) but allow the passage of EMA [32].

Sample pre-treatment with viability stains has been used mainly in combination with PCR (v-PCR). However, some practical limitations still remain when applied to environmental samples [46-48]. In some occasions, treatment with PMA can lead to false positive or negative results [46]. As it was reviewed by Fittipaldi et al. [26], the efficiency of the v-PCR technique depends on a complex set of parameters which include: dye concentration, cell concentration, live and dead cells ratio, PCR amplicon length and potentially targeted DNA sequence, turbidity, pH and salt sample concentration, incubation temperature, light source and even the type of microbial species. Varma et al. [48] has suggested that the activity of the PMA can be saturated if high numbers of cells are present in the sample. Thus, the presence of high concentrations of other microorganisms different from the “microorganisms in study” may lead to false positives results. In order to solve these issues, Fittipaldi et al. [26] has suggested the performance of three independent PCR reactions: one regular qPCR reaction, one v-qPCR reaction, and one v-qPCR on an aliquot subjected to lethal conditions inflicting membrane damage. These assays are intended to provide more reliable data regarding the number of live microbes by comparison and subtraction of results. The approach might help to reduce overestimating bacterial viability in complex matrices like environmental samples. Another alternative is the use of PMA-qPCR combined with other specific techniques, such as direct viable count combined with fluorescence in situ hybridization [22,33].

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Conclusions

The PMA-qPCR technique has been successfully used for detecting live *H. pylori* in water distribution systems [33] and it has also been suggested as a useful method for performing studies in environmental samples [22]. Our study is the first to successfully identify *H. pylori* cells in environmental samples of little volume. It demonstrates the usefulness of PMA-based qPCR to detect *H. pylori* contamination and to estimate concentration of live cells exposed to environmental stress conditions in a complex matrix such as urban surface water. In conclusion, our results suggest that urban surface water can be contaminated with viable *H. pylori*. Furthermore, our results strengthen the argument in favor of water as an environmental reservoir for *H. pylori* transmission. Nevertheless and given the small number of analyzed samples, it is not possible to state that *H. pylori* is common in all aquatic habitats. Consequently, we recommend additional larger studies to establish its presence and viability in other surface water sources.

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Conflict of interest

The authors declare no conflict of interest.

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Table 1 Variation coefficient of qPCR for PMA–treated (live cells) and non-treated (total cells) samples, expressed in Log cell/100 mL

	H. pylori					
Sampling	**Log Total**	**Log Live**	**Log Total / 100 mL**	**Log Live / 100 mL**	**% live**	**VC (%)**
1	2.660	2.660	458	458	100	0
2	2.660	2.660	458	458	100	0
3	2.660	2.660	458	458	100	0
4	2.660	2.660	458	458	100	0
5	4.238	2.453	17,28	284	2	38
6	2.453	2.453	284	284	100	0
7	6.078	4.986	1,198,000	96,8	8	14
8	6.137	3.656	1,372,000	4,528	0	36
9	6.511	5.451	3,244,000	282,4	9	13
10	6.088	6.084	1,224,000	1,212,000	99	0
11	4.229	2.543	16,934	349	2	35
12	2.543	2.543	349	349	100	0
13	5.439	3.448	274,84	2,805	1	32
14	2.952	2.952	896	896	100	0
15	5.000	2.952	100	896	1	36
16	2.952	2.952	896	896	100	0
17	5.801	4.468	632,4	29,348	5	18
18	3.523	3.523	3,336	3,336	100	0
19	3.523	3.523	3,336	3,336	100	0
20	6.517	4.496	3,288,000	31,308	1	26
21	3.523	3.523	3,336	3,336	100	0
Average	4.198	3.459	541,987	79,761	58	

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Table 2 Median-based Levene's test for comparing sample group variances

	Sum of squares	*Degrees of freedom*	*Mean squared*	*F*	*p*
Between groups	28.850	1	28.850	0.817	0.372
Within groups	1413.174	40	35.329		
Total	1442.024	41			

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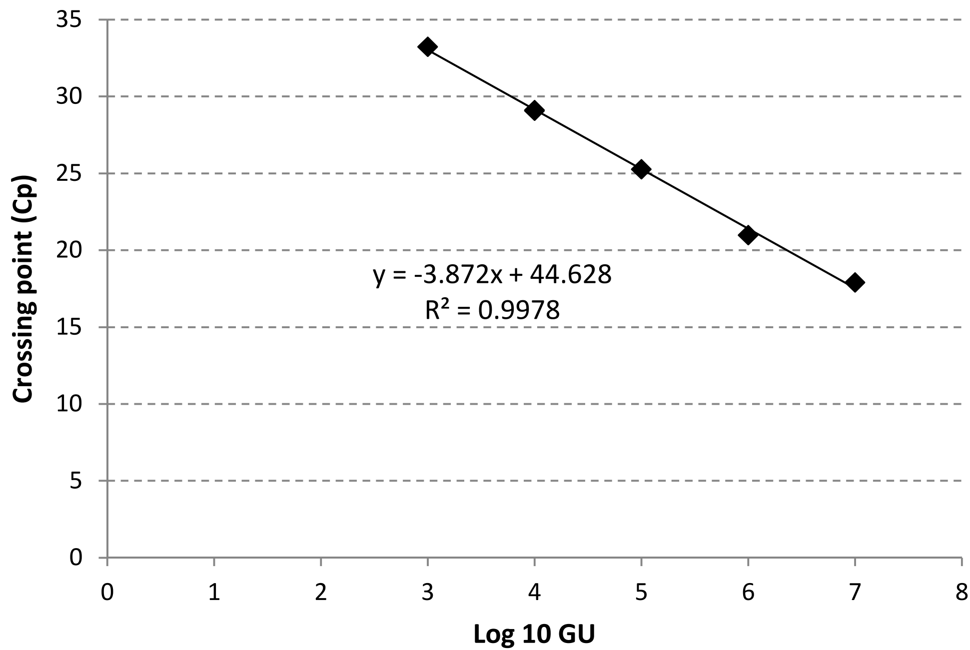

Figure 1 Linear regression of specific *H. pylori* VacA gene qPCR standard curve for serial 10-fold dilutions of DNA corresponding to concentrations from 1 to 10 x 10^7 genomic units (GU) per 100 mL.

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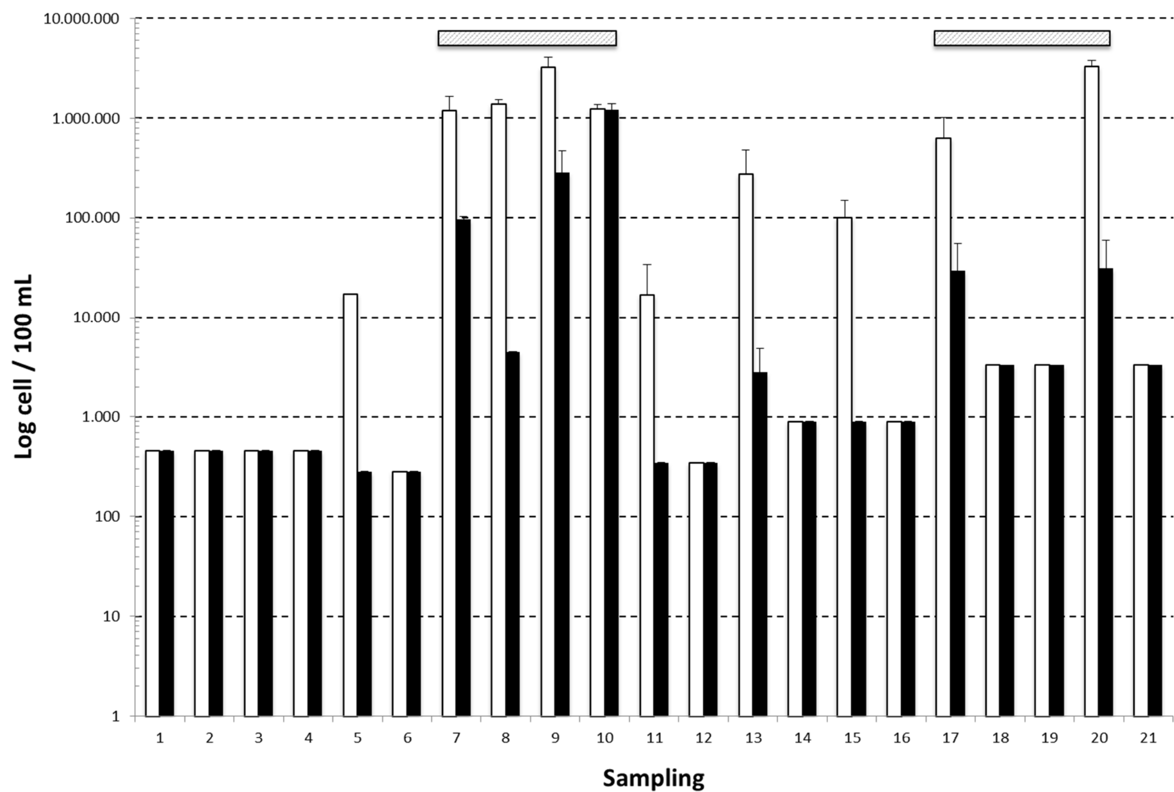

Figure 2 Concentration levels of *H. pylori* cells as determined by qPCR in surface water samples; total cells (white bars), live cells (black), and rainy days (gray); error bars represent the standard error of the mean.

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