Monitoring levels of viable *Helicobacter pylori* in surface water by qPCR in Northeast Spain

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ABSTRACT

Helicobacter pylori infection is a risk factor for chronic active gastritis, peptic ulcers, gastric carcinoma and lymphoma. Although the infection may be acquired through different transmission routes, the presence and viability of *H. pylori* in water sources are not well known. Therefore, the aim of our study was to analyse the viability of *H. pylori* cells in urban surface waters collected at the Vallparadís public park in Terrassa, Barcelona, Spain. The water samples were analysed by viability quantitative polymerase chain reaction (qPCR) using propidium monoazide and specific primers for the *H. pylori* vacuolating cytotoxin (*vacA* gene). Viable *H. pylori* were found in 91.3% of the samples analysed, with an average concentration of 3.46 ± 1.06 log cell 100 mL^{-1}. Our work proves a quick and simple procedure for evaluating viable *H. pylori* cells in environmental samples by qPCR. Furthermore, the results provide evidence that urban surface waters may contain considerable levels of viable *H. pylori* cells, thus indicating they are a potential source of infection, which represents a public health concern.

Key words | cellular viability, *Helicobacter pylori*, propidium monoazide, qPCR, water

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INTRODUCTION

Helicobacter pylori is a microaerophilic Gram-negative bacterium that has been estimated to infect the gastric epithelium of half of the human population (Brown 2000). Furthermore, *H. pylori* is considered the causative agent of chronic active gastritis and an important risk factor in the multifactorial aetiology of peptic ulcers, gastric carcinoma and MALT (mucosa-associated lymphoid tissue) lymphoma (Gasparetto *et al.* 2012). Although transmission presumably occurs through faecal–oral and oral–oral routes, the main route of *H. pylori* transmission remains unknown. Evidence favours a faecal–oral route (Rolle-Kampczyk *et al.* 2004), where water plays an important role, acting as a natural reservoir and a source of infection (Hulten *et al.* 1998; Azevedo *et al.* 2008). Although some reports indicate the

presence of *H. pylori* DNA in water samples, the viability of the cells in the environment remains unknown.

In laboratory conditions, *H. pylori*'s ability to survive in an infectious state is hindered because its cultivability is rapidly lost (Baker *et al.* 2002; Moreno *et al.* 2007). In detrimental environmental circumstances, such as in bodies of water, some authors have suggested that *H. pylori* may transform from the bacillary to the coccoid form, remaining viable and virulent but non-cultivable (Giao *et al.* 2008; Dube *et al.* 2009). However, results have been reported to challenge such a hypothesis. Interestingly, some studies have reported that *H. pylori* is capable of spreading and remaining viable for several weeks in the presence of amoebae and biofilms (Winiecka-Krusnell *et al.* 2002; García *et al.* 2014). Therefore,

new methods should be developed to detect and study the viability of these bacteria in surface water.

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 (qPCR) and fluorescent *in situ* hybridization (FISH) (Bragança *et al.* 2007). Although qPCR is a fast and sensitive method to detect microbial DNA, it does not differentiate between viable and non-viable cells. Therefore, sample pretreatment with viability dyes, such as propidium monoazide (PMA), has been used in combination with qPCR to distinguish between viable and non-viable cells (Cawthorn & Witthuhn 2008; Slimani *et al.* 2012). PMA is a DNA-intercalating agent that selectively penetrates cells with compromised membranes, traditionally considered to be dead. 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 (Nocker *et al.* 2009; Fittipaldi *et al.* 2012). The effectiveness of this technique has been tested on different types of bacteria, including *H. pylori* in drinking water samples and by *in vitro* studies (Agustí *et al.* 2010; Santiago *et al.* 2015).

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

MATERIALS AND METHODS

Surface water samples

A total of 23 sampling points were randomly selected along a stream of water in the Terrassa's Vallparadís public park, in Northeast Spain. During the summer of 2014, four samples were taken each day, two in the morning and two in the afternoon, in different arbitrarily chosen parts of the stream. Each sample was collected aseptically in a 50 mL sterile vial and transported to the laboratory at 4 °C for subsequent analysis. In the laboratory the two samples collected in the morning were divided in half; two portions were treated with PMA and two served as controls; the same procedure was then performed for the afternoon samples, for a total of eight subsamples analysed each day. Subsamples were concentrated by centrifugation twice. A first step at $4,000 \times g$ for 30 min was performed to reach a 2 mL volume of concentrate which was placed into a microcentrifuge tube. Afterwards, concentrates were centrifuged at $10,000 \times g$ for 15 min (Minispin Plus-Eppendorf, Hamburg, Germany). The supernatant was discarded, and pellets were resuspended in 500 μL of phosphate buffered saline (PBS) at pH 7.4 for PMA treatment and DNA extraction.

PMA treatment

Four concentrates from the daily samples were treated with PMA using a modified method (Agustí *et al.* 2010). To produce a final dye concentration of 50 μM, 12.5 μL PMA (GenIUL, Terrassa, Spain) was added to each concentrate. Samples were incubated for 10 min in the Dark Box System (GenIUL) with occasional mixing and photoactivated for 15 min using the PhAST®-Blue system (GenIUL). The other four concentrates did not receive PMA treatment and were used as controls. Subsequently, both sets of concentrates were centrifuged at $10,000 \times g$ for 5 min. The supernatants were 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 DNA isolated was eluted in 200 μL buffer solution and stored at −20 °C until PCR amplification.

Quantitative PCR

qPCR was performed to simultaneously amplify s/m alleles of *vacA* gene *H. pylori* using previously reported specific primers according to Atherton *et al.* (1995). Additionally, qPCR specificity was tested using DNA of two laboratory *H. pylori* strains (Tx30a and 60190), which were donated by Dr Xavier Calvet (Department of Gastroenterology, Hospital de Sabadell, Barcelona); DNA from *Pseudomonas*

aeruginosa (CECT 110), *Escherichia coli* (CECT 101) and *Bifidobacterium longum* (CECT 4551) was also used as amplification controls. A conventional PCR protocol previously standardized by Dr Calvert's laboratory was run on a LightCycler 1.5 system (Roche, Mannheim, Germany). The PCR mixture contained 5 μL of genomic DNA, 0.25 μL of each primer (0.5 mM) and 4 μL of HOT FIRE-Pol® EvaGreen® qPCR Mix Plus (1×) dye solution (Solis BioDyne, Tartu, Estonia) prepared according to the manufacturer's recommendations. The mix was forced down the PCR tube by a short spin in a microcentrifuge. The protocol consisted of one step of 15 min at 95 °C for polymerase activation, and 45 cycles: 95 °C for 20 s, 50 °C for 20 s, and 72 °C for 20 s with slopes of 20 °C s^{-1}, 20 °C s^{-1} and 20 °C s^{-1}, respectively. Finally, melting point analysis was performed by raising the temperature slowly (0.1 °C s^{-1}) from 65 °C to 97 °C. A standard curve with a linear range across six logarithms of *H. pylori* DNA concentration was obtained from a series of 10-fold dilutions of a DNA Tx30a solution. Some of these dilutions were used as positive amplification controls, and PCR-grade water was used as negative control in all qPCR assays. Test detection limit was calculated according to the standard approach described in ISO-12869 (ISO 2012). Mean values of the cycles threshold (Ct) obtained ranged from 17 to 33.48, corresponding to concentrations from 1 to 10×10^7 colony-forming units (CFU) 100 mL^{-1}; thus, the detection limit was 150 CFU 100 mL^{-1}. Samples were considered positive when their melting temperature (Tm) values were similar to the Tm of the standard curve (89.33 °C); qPCR amplification of the non-*Helicobacter* bacteria showed negative signals in the melting curve analyses, demonstrating the assay's specificity. Bacteria concentration was expressed as logarithmic units of cells per 100 mL of sample.

Statistical analysis

The statistical analysis was conducted using SPSS for Windows version 20 (SPSS Inc., Chicago, IL, USA). Bacterial concentrations were expressed by the mean and the standard error of the mean. The coefficient of variation was used to compare the degree of variation across groups while the nonparametric, median-based Levene's test was used to compare sample variances between groups (Total

cell and Live). A probability (*P*) value of less than 0.05 was used as criterion of significance.

RESULTS

As shown in Figure 1, 21 (91%) 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^{-1}, while the total average concentration of viable cells was 3.46 ± 1.06 log cell 100 mL^{-1}. However, the results showed that when total concentrations were lower than 3 log cell 100 mL^{-1}, the concentration of viable cells coincided with the total cells detected. Additionally, it was observed that the samples collected on rainy days contained increased amounts of total *H. pylori* cells, but when compared with viable cells there was no significant statistical difference.

In order to estimate the degree of variance, the coefficient of variation was estimated for each sampling and is shown in Table 1.

Although some assays indicated variation, the analysis showed that these differences were not statistically significant ($P = 0.372$) (Table 2).

DISCUSSION

Evidence supporting the *H. pylori* water transmission hypothesis comes largely from two areas 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* in water sources (Aziz *et al.* 2015). The presence of *H. pylori* DNA in river waters has been previously reported by using PCR techniques. However, as previously mentioned, PCR is not suitable for determining the infective potential of a given sample. In this study, qPCR was combined with PMA to study *H. pylori* viability in environmental samples, and thus better understanding the potential role of surface water as a *H. pylori* transmission vehicle. A high frequency of viable *H. pylori* cells (91.3%) was found in the analysed samples. The presence of *H. pylori* in surface water can be explained by its ability to adapt to harsh conditions.
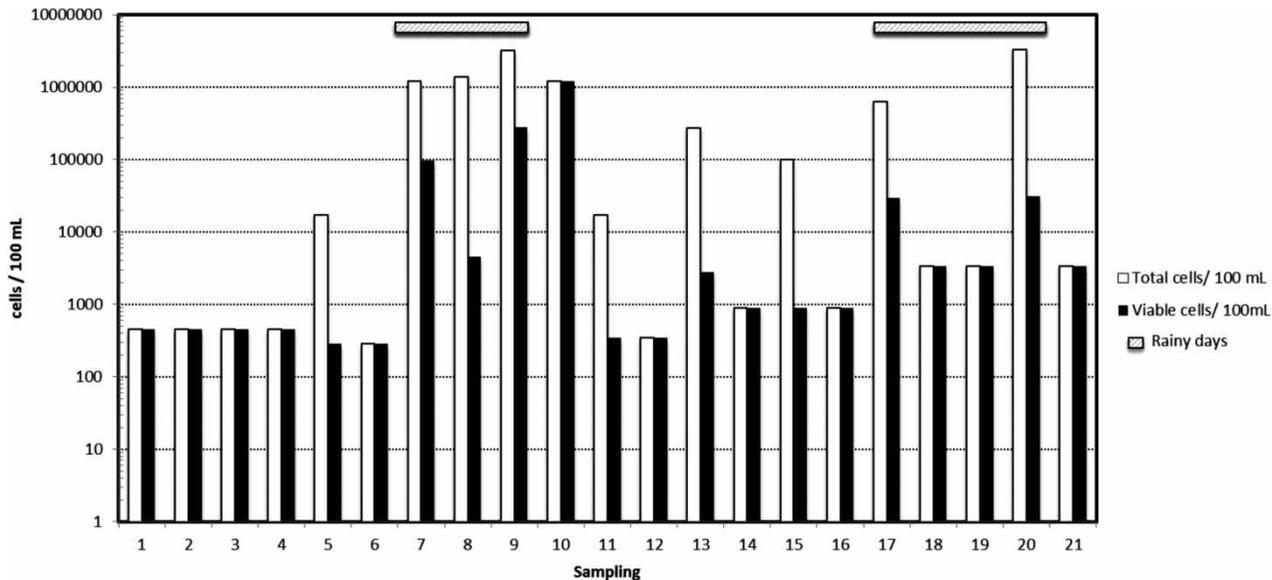
Figure 1 │ Concentration levels of *H. pylori* cells as determined by qPCR in surface water samples; total cells (white bars), living cells (black) and rainy days (shaded bar).

Therefore, the adherence of *H. pylori* to biofilms fosters a microaerophilic environment for the survival of the bacteria, which seems to be independent of temperature (Azevedo *et al.* 2008; Giao *et al.* 2008). In addition, it has been suggested that *H. pylori* can propagate and remain viable for several weeks in the presence of *Acanthamoeba* spp., which suggests that amoebae could be a natural reservoir for the bacterium (Winiecka-Krusnell *et al.* 2002; Greub & Raoult 2004). The PMA-PCR technique was used to demonstrate that *H. pylori* is able to survive chlorination treatment in association with *A. castellani* (Moreno *et al.* 2007).

In the present study, bacterial concentration levels ranged from 2.5 to 6 log units per 100 mL. It is important to note that higher bacterial levels were detected in samples collected on rainy days. This could be explained by surface water run-off, which can be caused by stormwater, a leading source of pollution to fresh incoming urban waters. Previous studies have indicated that surface water pollution by faecal bacteria and both inorganic and organic chemicals could be exacerbated following rainfall. Furthermore, precipitation causes a lowering in temperatures and a dilution effect that reduces salinity, and thus, favours the establishment of bacteria, including *H. pylori* (Gubler *et al.* 2001).

Although the minimum *H. pylori* infectious dose has not yet been established in humans, some studies suggest that 10^5 CFUs might be close to the minimum infectious dose

(Graham *et al.* 2004). In the present study, 21.7% (5/23) of the samples showed viability levels in concentrations over 4 log cell 100 mL^{-1}, while 8.7% (2/23) presented viable cell concentrations higher than 5 log cell 100 mL^{-1}. 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 (She *et al.* 2003).

In this study, *H. pylori* sample positivity was the same for both qPCR and PMA-qPCR methods alike. However, when bacterial concentrations were high (>4 log cell 100 mL^{-1}), mostly on rainy days, an average difference of 1.7 log cell 100 mL^{-1} was observed between methods, being higher for qPCR, which might mainly be due to the fraction of dead cells also accounted for by this technique. Although PMA has been proposed as a more appropriate DNA-intercalating dye with a substantially higher specificity for live cells than ethidium bromide monoazide (EMA) (Fittipaldi *et al.* 2012), the possibility of PMA uptake in bacterial cells with intact membranes should also be considered. It has been shown that membrane permeability undergoes changes in live cells dependent on the physiological status (Gedalanga & Olson 2009). However, a previous permeability study has shown that membranes of live *H. pylori* cells effectively prevent penetration of PMA

Table 1 │ Coefficient of variation (CV) of qPCR for PMA-treated (live cells) and non-treated (total cells) samples. The results are expressed as Log (cells 100 mL^{-1}) and cells 100^{-1}

	H. pylori					
Sampling	Log (PCR)	Log PMA-qPCR	PCR	qPCR-PMA	% live	CV (%)
1	2.660	2.660	457.6	457.6	100	0
2	2.660	2.660	457.6	457.6	100	0
3	2.660	2.660	457.6	457.6	100	0
4	2.660	2.660	457.6	457.6	100	0
5	4.238	2.453	17,280	284	2	38
6	2.453	2.453	284	284	100	0
7	6.078	4.986	1,198,000	96,800	8	14
8	6.137	3.656	1,372,000	4,528	0	36
9	6.511	5.451	3,244,000	282,400	9	13
10	6.088	6.084	1,224,000	1,212,000	99	0
11	4.229	2.543	16,934.4	348.8	2	35
12	2.543	2.543	348.8	348.8	100	0
13	5.439	3.448	274,840	2,804.8	1	32
14	2.952	2.952	896	896	100	0
15	5.000	2.952	100,000	896	1	36
16	2.952	2.952	896	896	100	0
17	5.801	4.468	632,400	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	

Table 2 │ Median-based Levene's test for comparing sample cell/100 ml group variance

	Sum of squares	Degrees of freedom	Mean squared	*F*	*P*
Between groups	28.850	1	28.850	0.817	0.372
Within groups	1,413.174	40	35.329		
Total	1,442.024	41			

(50 μM, exposure for 5 min) but allow the passage of EMA (Nam *et al.* 2011).

Sample pre-treatment with viability stains has been used mainly in combination with PCR. However, some practical limitations still remain when applied to environmental samples. In some cases, treatment with PMA can lead to false positive or negative results (Fittipaldi *et al.* 2011). The efficiency of the PMA-qPCR 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 microbial species. It has been suggested that the activity of the PMA can be saturated if high numbers of cells are present in the sample (Varma *et al.* 2009). Thus, the presence of high concentrations of other microorganisms different from the target microorganisms may lead to false positives. In order to achieve an improved estimation of the number of viable cells, the performance of three independent PCR amplifications for each sample – one regular qPCR reaction, one v-qPCR reaction and one v-qPCR on an aliquot subjected to lethal conditions inflicting membrane damage – was previously proposed (Fittipaldi *et al.* 2012). 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 overestimation of 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 (DVC-FISH) (Santiago *et al.* 2015).

CONCLUSION

Our study is the first to successfully identify viable *H. pylori* cells in small volumes of environmental water samples. It demonstrates the usefulness of PMA-based qPCR to detect *H. pylori* contamination and to estimate the concentration of live cells exposed to environmental stress conditions in a complex matrix such as urban surface water. In conclusion, our investigation provides a quick and simple procedure for evaluating *H. pylori* viable cells in environmental samples by quantitative viability PCR. Moreover, the results suggest that urban surface water can be contaminated with viable *H. pylori*, which strengthens the argument in favour of water as an environmental reservoir for *H. pylori* transmission. Nevertheless, and given the small number of samples analysed, it is not possible to state that

H. pylori is common in aquatic habitats. Consequently, we recommend additional larger studies using the PMA-qPCR technique to establish its presence and viability in other surface water sources.

ACKNOWLEDGEMENTS

FINANCING

DISCLOSURE

The authors declare no conflicts of interest.

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