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Molecular detection of *Helicobacter pylori* in a large Mediterranean river, by direct viable count fluorescent *in situ* hybridization (DVC-FISH)

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ABSTRACT

Although the precise route and mode of transmission of *Helicobacter pylori* are still unclear, molecular methods have been applied for the detection of *H. pylori* in environmental samples. In this study, we used the direct viable count fluorescent *in situ* hybridization (DVC-FISH) method to detect viable cells of *H. pylori* in the River Aliakmon, Greece. This is the longest river in Greece, and provides potable water in metropolitan areas. *H. pylori* showed positive detection for 23 out of 48 water samples (47.9%), while no seasonal variation was found and no correlation was observed between the presence of *H. pylori* and indicators of fecal contamination. Our findings strengthen the evidence that *H. pylori* is waterborne while its presence adds to the potential health hazards of the River Aliakmon.

Key words | Aliakmon river, DVC-FISH, *Helicobacter pylori*

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INTRODUCTION

Helicobacter pylori infection is widespread throughout the world, and is strongly associated with gastroduodenal disease, including chronic active gastritis, peptic and duodenal ulcer disease and gastric cancer (Suerbaum & Michetti 2002). The natural history and other aspects of the epidemiology of *H. pylori* infection are still unclear. The mode of transmission of *H. pylori* is also unknown, but fecal-oral transmission is highly probable (Go 2002). Several studies have suggested that use of fecal-contaminated potable or recreational water might aid the transmission of *H. pylori* (Brown 2000), while Baker and Hegarty reported a strong association between the presence of *H. pylori* in untreated well water and clinical infection (Baker & Hegarty 2001). Researchers also have detected *H. pylori* in foods like spinach (Buck & Oliver 2010) and milk (Angelidis *et al.* 2011). These results indicate the possible contribution of water and soil and that animals like sheep and bovines may constitute a natural host for *H. pylori*.

When *H. pylori* is exposed to variable environmental conditions, the cells enter a viable but non-culturable state (VBNC) and changes in metabolism and growth patterns are observed (Nilsson *et al.* 2002). This is the reason why *H. pylori* has not yet been isolated from environmental samples by traditional culture techniques and its presence in such samples has been shown using molecular techniques (Hegarty *et al.* 1999; Watson *et al.* 2004; Voytek *et al.* 2005). One of these techniques is the direct viable count (DVC) method combined with fluorescent *in situ* hybridization (FISH). The DVC procedure is based on incubation of samples in the presence of the antibiotic novobiocin which is an inhibitor of DNA gyrase, increasing thus, intracellular rRNA levels. Thus, incubation of environmental samples in the presence of this antibiotic, results in enlarged, swollen

doi: 10.2166/wh.2014.171

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viable cells. These elongated cells could be detected by epifluorescent microscopy and could be discriminated from non-viable cells by differences in size (Piqueres *et al.* 2006).

Water quality of rivers is one of the most important concerns facing communities that are dependent on the river for drinking water, agriculture, and watershed. Microbial contamination must be monitored continuously to determine the condition of the water in order to prevent the spread of diseases and to eliminate non-point source of contamination (Mendoza *et al.* 2004).

The current study describes the application of DVC-FISH for the detection of *H. pylori* in the River Aliakmon, Greece. The River Aliakmon is the longest and most important river in the region of Northern Greece; it originates from mountains inside Greek territory, it has a length of about 310 km and is used both for agricultural activities and for providing potable water to the city of Thessaloniki (1,000,000 inhabitants). Thessaloniki is the second biggest city of Greece, after the capital Athens, and is located in the Thermaikos Gulf, where the River Aliakmon discharges.

This work also constitutes one of the few reports of *H. pylori* molecular detection in river water worldwide. We also performed statistical analyses to examine the possible correlation between the occurrence of either total coliforms (TC) or *Escherichia coli* in the river water and the presence of *H. pylori*.

MATERIALS AND METHODS

Sampling and microbiological procedures

Two sampling sites (stations) were established in the River Aliakmon, while vehicular access to the river was the major factor determining sampling location: station P1 and P2, which were at a distance of 100 and 20 km respectively, from the city of Thessaloniki. Station P1 was located at the Veria dam and station P2 was located before the water treatment plant of Thessaloniki, where water was treated for drinking purposes.

Sampling was done twice a month over a period of one year: from September 2008 until August 2009. Thus, 24 fresh water samples were collected from the surface of each site, while the total number of samples from both sites was 48. Samples were taken about 25 cm below the surface of water, poured into sterile glass bottles, refrigerated immediately, transported to the laboratory and processed without further delay.

The indicator microorganisms were enumerated by membrane filtration using 0.45 μm pore size filters (Millipore, France) as specified in *Standards Methods* (American Public Health Association (APHA) 2005). The following microbiological parameters were examined: TC per 100 ml at 36 °C (m-Endo agar-LES-, Merck KGaA, Germany), fecal coliforms (FC) per 100 ml at 36 °C (m-FC agar, Merck KGaA, Germany), *E. coli* per 100 ml at 36 °C (m-Endo agar-LES-, Merck KGaA, Germany).

Direct viable count fluorescent *in situ* hybridization

As mentioned above, the DVC method in combination with fluorescent microscopy, discriminates among viable and non-viable cells in mixed microbial communities. Briefly, in the presence of the antibiotic novobiocin, which is a gyrase inhibitor, viable bacteria are able to elongate, having their division inhibited by the antibiotic. This results in large, swollen cells with increased intracellular rRNA levels. These cells, being a better 'rRNA target' for hybridization with the fluorescent probe, give a brighter and more intense signal than the signal of non-viable cells.

Samples were analyzed as described by Piqueres *et al.* (2006). Briefly, a volume of 200 ml of each water sample was centrifuged at 5,000 g for 20 min. The pellet was resuspended in 5 ml of PBS buffer (150 mM sodium phosphate, 150 mM NaCl, pH ± 0.1 (25 °C), Fluka Analytical/BioUltra, Sigma-Aldrich Switzerland), inoculated in 50 ml of *Brucella* broth supplemented with 5% newborn calf serum and 0.5 μg/ml novobiocin (Sigma-Aldrich) and incubated under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 37 °C. After 24 h of incubation, samples were taken from each flask, fixed for FISH with paraformaldehyde (PFA) 4% as described by Amann *et al.* (1995), and subsequently hybridized according to conditions described by Moreno *et al.* (2003).

Two fluoresceinated oligonucleotide probes were used for FISH. The 16SrRNA probe Hpy-1 (5′-CAC ACC TGA CTG ACT ATC CCG-3′), specific for *H. pylori*, has been

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described previously (Trebesius *et al.* 2000; Moreno *et al.* 2003), while the second probe was EUB 338 (5′-GCT GCC TCC CGT AGG AGT-3′), which is a universal probe complementary to a conserved region of the 16S rRNA of Eubacteria and was used as a positive control for visualizing the rest of the river water microflora. Probes were synthesized and labeled by Thermo Scientific (Germany). Probe EUB 338 was labeled at the 5′ end with fluorescein isothiocyanate (FITC, green fluorescence), whereas probe Hpy-1 was labeled at the 5′ end with carboxytetramethylrhodamine (TAMRA, orange fluorescence).

Probe specificity evaluation and bacterial strains

The probe Hpy-1 was initially developed by Trebesius *et al.* (2000). The specificity of the probe was confirmed by these researchers both by a gapped Basic Local Alignment Search Tool search and by *in vitro* experiments involving several bacterial species.

In our study the specificity of the Hpy-1 probe was assessed by *in vitro* experiments involving two sets of bacterial strains that occur commonly in river water. One set included strains that were obtained by Public Health England (Salisbury, UK). These strains, which are normally used in our laboratory microbial analysis testing, were *E. coli* NCTC 9528, *Enterococcus faecalis* NCTC 775, *Clostridium perfringens* NCTC 13170 and *Salmonella goldcoast* NCTC 13175. The second set consisted of bacteria that were isolated from three water samples from the River Aliakmon: 10 colonies of TC, *E. coli*, enterococci and *Salmonella* spp. were chosen randomly.

While the above strains served as negative control, a clinical isolate, the AHEPA Hospital (Thessaloniki) *H. pylori* strain J99 was used as a positive control for Hpy-1 hybridization (Angelidis *et al.* 2011). Each of the above strains was grown on the appropriate medium, colonies were suspended in PBS to an Optical Density$_{600}$ of 0.1 and fixed with paraformaldehyde (Gram-negative eubacteria).

Epifluorescence microscopy

For epifluorescence microscopy, slides were scanned under a Nikon Eclipse 80i microscope (Nikon Inc., Melville, NY, USA) at 40× (400× magnification); at least 50 microscopic fields of view (FOV) for each sample were examined. The Chroma HQ filter set ZyGreen was used for FITC (excitation wavelength 503 nm; emission wavelength 528 nm) and the ZyOrange filter was used for TAMRA (excitation wavelength 547 nm; emission wavelength 572 nm) (Chroma Technology Corp., Bellows Falls, VT, USA). Further examination and analysis of positive signals was done and color micrographs were taken under 100× (1,000× total magnification) and processed with the XCyto-Gen program (Alphelys, Plaisir, France).

Statistical analyses

Analyses were performed to quantify relationships between *H. pylori* and fecal contamination indicators such as fecal coliforms and *E. coli*. McNemar's test was used and all *p*-values were two-tailed with a significance level at 0.05 (statistical software package SPSS, version 12.0).

RESULTS AND DISCUSSION

As expected, the DVC treatment with novobiocin prior to hybridization resulted in elongation of viable cells compared to non-treated cells of control strain J99. FISH analysis showed elongated and non-elongated *Helicobacter* cells in the river water samples but we only considered as positive those samples which contained viable (twice elongated) *H. pylori* cells (Figure 1). We are aware also that viable coccoid forms of *Helicobacter* might be present in the sample, but because of formation of multiple (fluorescent) aggregates it was not easy to differentiate between coccoid and spiral forms just with epifluorescence microscopy. So we thought as viable the obviously long, swollen, isolated cells. For the semi-quantitative microscopic determination of viable *H. pylori* within river samples, the visible-stained rRNA of bacteria in at least 50 randomly chosen FOVs (1 FOV $= 0.24$ mm^2) were examined. We estimated that the *H. pylori* cell density in positive samples was 2.5×10^3 colony forming units (CFU)/ml.

Out of 12 months, Site 1 tested positive 12 times and Site 2, 11 times (overall percentage 47.9%, Table 1). The chi-square goodness of fit test ($\chi^2 = 14.75$, degrees of freedom $= 11$) showed that there was no seasonal variation

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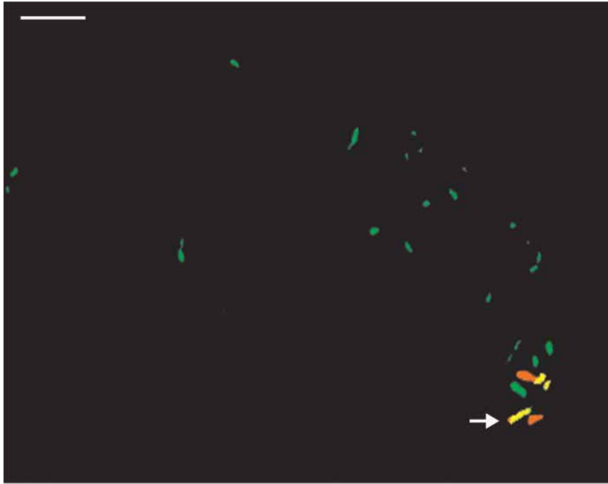

Figure 1 | FISH identification of *H. pylori* cells in river water samples after 24 h incubation with novobiocin (0.5 μg/ml). Arrow shows an elongated red fluorescent *H. pylori* cell, while the rest of water microflora were hybridized only with the EUB338 probe (green fluorescence). Bar: 10 μm. Please refer to the online version of this paper to see this figure in color: www.iwaponline.com/jwh/toc.htm.

($p > 0.05$). Furthermore, no significant relationships were observed between the presence of *H. pylori* and indicators of fecal contamination including *E. coli* (Figure 2, p for both sampling stations P1 and P2 was found to be >0.05). Our results are in accordance with those of other researchers, suggesting that differences in survival rates between *H. pylori* and fecal coliforms may explain the lack of correlation (Mendoza *et al.* 2004; Twing *et al.* 2011).

The presence of *H. pylori* in water samples has frequently been shown by polymerase chain reaction (PCR). However, the DVC-FISH combination is a tool to detect viable *H. pylori* cells in environmental samples and according to Moreno *et al.*, it has also shown to yield more positive results than PCR. To our knowledge, this is the first time that it has been possible to show the existence of VBNC *H. pylori* cells in Greek river water. The presence of *H. pylori* adds to the potential health hazards of the River Aliakmon. As no significant correlation was observed between the presence of *H. pylori* and indicators of fecal contamination, our results support previously published studies that fecal indicators cannot be used to detect the presence of *H. pylori* in surface water (Mendoza *et al.* 2004; Twing *et al.* 2011).

There is a growing body of data from other relevant investigations in different countries which provide evidence that *H. pylori* is present not only in surface waters, but in water distribution systems as well (Sasaki *et al.* 1999; Mazari-Hiriart *et al.* 2001; Watson *et al.* 2004). Moreno *et al.* showed that *H. pylori* cells are able to survive for short periods of time in chlorinated drinking water in the VBNC form (Moreno *et al.* 2007).

Advances in oligonucleotide probes, which result in new detection methods that depend on the recognition of specific gene sequences of emerging pathogens, would be of great help in elucidating the epidemiology of transmission of *H. pylori* and other VBNC waterborne pathogens. However, important practical limitations for the wide application of rRNA-targeted nucleic acid probes are: (i) low or heterogeneous ribosomal RNA content within the targeted bacterial cells results in lack of sufficient sensitivity to detect pathogens in environmental water samples (false-negative results); (ii) some water samples may be difficult to analyze because they carry a high load of bacteria, debris and particles, which result in a complex fluorescent background and false-positive signals; and finally (iii) data on the identification and the abundance of *in situ* stained microorganisms in the above environmental samples are mostly obtained by concentrating large water volumes,

Table 1 | Combined DVC-FISH results showing the presence/absence of H. pylori at each sampling site for a period of 1 year. P1: station located upstream and P2: station located before the water treatment plant of Thessaloniki

	Sep 08		Oct 08		Nov 08		Dec 08		Jan 09		Feb 09	
River Station P1	+	+		+	+				+		+	
River Station P2		+	+				+	+		+		
	Mar 09		**Apr 09**		**May 09**		**Jun 09**		**Jul 09**		**Aug 09**	
River Station P1		+		+		+	+	+			+	
River Station P2			+	+		+			+		+	+

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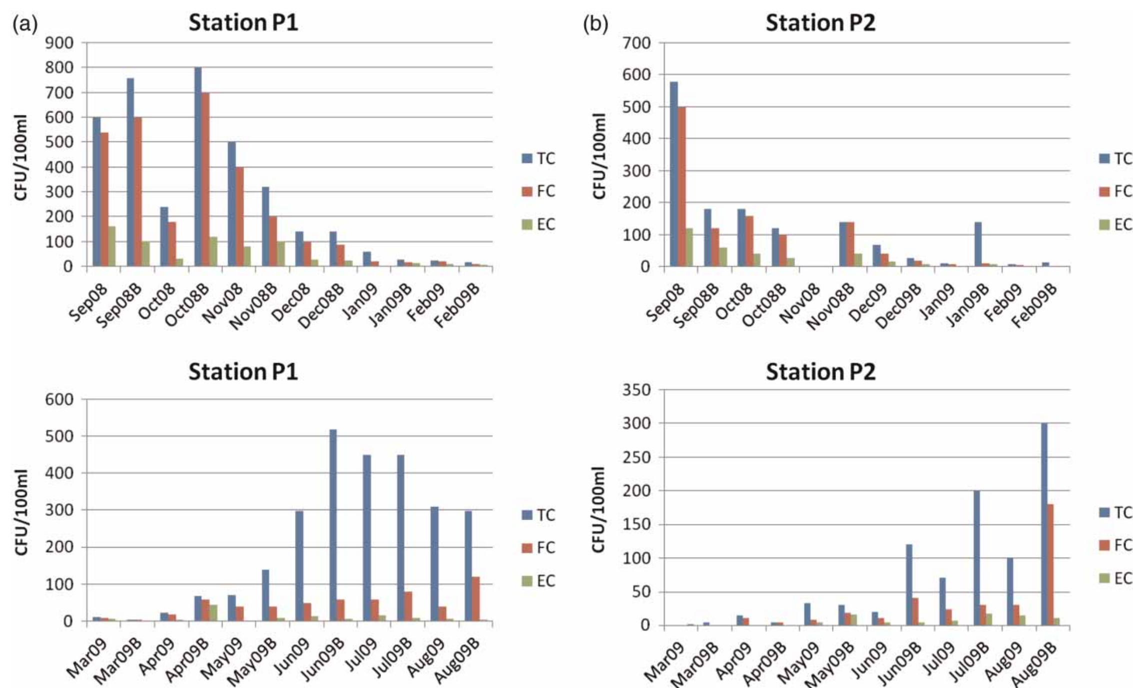

Figure 2 | (a) Water bacterial indicators for station P1. Since sampling was done twice a month, letter B in the horizontal axis stands for the second sampling during the same month. TC: Total coliforms, FC: Fecal coliforms, EC: *E. coli*. (b) Water bacterial indicators for station P2. Since sampling was done twice a month, letter B in the horizontal axis stands for the second sampling during the same month. TC: total coliforms, FC: fecal coliforms, EC: *E. coli.*

lengthy manual analysis and labor-intensive microscopic counting. These problems prevent wide application of molecular probes on a routine basis in laboratories dealing with water and environmental microbiology.

New approaches are needed in order to overcome the above problems and to increase sensitivity and specificity of nucleotide probes in environmental samples. These approaches may include molecular tools like a combination of FISH with flow cytometry and development of high density DNA microarrays (Manti *et al*. 2011; Gim & Rose 2012; Schauer *et al*. 2012).

CONCLUSIONS

In this study, we used DVC-FISH to detect the presence of *H. pylori* in the River Aliakmon, Greece, and provide evidence of the following:

- *H. pylori* live cells are present in the river water and there may be a possible waterborne route of transmission of this organism. According to a recent survey including a sample of 1990 patients from Northern Greece who underwent routine upper endoscopy, 21% of them were *H. pylori* carriers (Katsinelos *et al*. 2013). However, to date we have limited available scientific data to strongly correlate potable and recreational water with the transmission of *H. pylori* to humans. In the future we will need: (i) experimental studies in order to address the ability of *H. pylori* to survive in chlorinated water and to infect humans; and (ii) further prospective or retrospective epidemiological studies to verify the hypothesis whether water may serve as a potential vector for the transmission of *H. pylori*.
- Since no correlation was observed between the presence of *H. pylori* and fecal indicators, we can conclude that safety of water cannot be based just on fecal indicators and that new approaches and methods are needed for routine screening of water supplies and for providing potable water of adequate quality.
- The molecular method of fluorescent *in situ* hybridization is an efficient, although laborious, way to detect VBNC pathogens in water sources.

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ACKNOWLEDGEMENT

The authors wish to thank Stavros Papaioannou for collecting water samples from the River Aliakmon. The authors have declared no conflict of interest.

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First received 22 August 2013; accepted in revised form 11 May 2014. Available online 6 June 2014

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