

# Detection of free and plankton-associated *Helicobacter pylori* in seawater

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## ABSTRACT

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**Aims:** To detect both free and plankton-associated *Helicobacter pylori* in seawater samples collected on the Italian coast of the Adriatic Sea using a nested-PCR.

**Methods and Results:** Dissolved oxygen, pH, salinity and chlorophyll 'a' were the parameters recorded together with the characterization of zooplanktonic organisms. Plankton-associated *H. pylori* DNA was searched for in water samples filtered through 200 and 64 µm nylon nets whereas free bacteria were retained with the subsequent filtration through 0.22 µm pore-size membranes. Nested-PCR using primers for the *glmM* (*ureC*) gene was performed to reveal the presence of *H. pylori*. The DNA sequencing of amplified products confirmed the specificity of the assay. The sensitivity of the nested-PCR assay for *H. pylori* detection was 62 CFU per 100 ml in spiked water samples. *Helicobacter pylori* either free or bound to planktonic organisms was found in seven of 12 monthly samples. In particular, free bacteria were detected during the summer sampling and in November, December and March associated to planktonic cells.

**Conclusions:** The presence of free and plankton-associated *H. pylori* in seawater suggests that it can be a significant reservoir and a potential route of transmission for the microorganism.

**Significance and Impact of the Study:** Our study seems to provide a promising background to define new and effective strategies for surveillance of this human pathogen.

**Keywords:** *Helicobacter pylori*, nested-PCR, planktonic organisms, route of transmission, seawater.

## INTRODUCTION

*Helicobacter pylori* is the causative agent of gastritis and duodenal ulcer and its presence on the human gastric mucosa has been related to several other diseases including gastric cancer, gastric lymphoma (mucosa-associated lymphoid tissue, MALT) and coronary heart disease (Mendall *et al.* 1994; Go 2002).

How the microorganism is transmitted remains unclear and the currently suggested route is person-to-person transfer by faecal-oral and oral-oral mode (Parsonnet *et al.* 1999; Cellini *et al.* 2001).

It has been hypothesized that water supplies contaminated by sewage are a potential route of *H. pylori* transmission (Hulten *et al.* 1996; Hegarty *et al.* 1999; Baker and Hegarty 2001; Horiuchi *et al.* 2001; Mazari-Hiriart *et al.* 2001a; Park *et al.* 2001; Lu *et al.* 2002). Recreational bathing in sewage contaminated water and consumption of contaminated seafood have also been suggested as possible transmission routes (Brasher *et al.* 1998). These hypotheses emphasize the need to investigate the presence of free *H. pylori* in the seawater, but even attached to planktonic organisms, such as Copepods and Cladocerans as a new possible transmission route of the microorganism to humans.

In this paper, a rapid and simple multi-step DNA preparation method and a nested-PCR assay for detection of *H. pylori* in seawater are described. The nested-PCR specifically amplifies a highly conserved region of the

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phosphoglucosamine mutase *glmM* (*ureC*) gene which is unique and essential for *H. pylori* growth (Labigne *et al.* 1991; Bickley *et al.* 1993; De Reuse *et al.* 1997) and it has been previously reported (Bamford *et al.* 1998) to improve sensitivity in *H. pylori* detection in samples containing both prokaryotic and eukaryotic cells as well as many organic impurities. The sensitivity of the assay in our hands was assessed by using seawater samples artificially seeded with serial logarithmic dilutions of *H. pylori*.

Water samples were collected monthly over 1 year on the Italian coast of the Adriatic Sea in a sampling station at a depth of 5 m. Together with the search of *H. pylori*, routine parameters (total and faecal coliforms and enterococci) for the assessment of the microbiological quality of coastal recreational waters were also recorded.

## MATERIALS AND METHODS

### Water sampling

Water samples were collected once a month from May 2001 to April 2002 in a sampling station at latitude 42°29'41" North and longitude 14°12'19" East (5 m depth) at about 500 m far from the coast. The recorded standard water pollution parameters showed very few variations during the routine sampling because the station is far from the mouth of rivers and hardly influenced by marine currents. Each water sample was collected at the beginning of every month at the same hour (about 9 AM) on the first day with favourable climate conditions for sampling.

Dissolved oxygen, salinity, pH and chlorophyll 'a' were recorded by using a multiparameter probe (CTD Ocean Seven 316; Idronaut s.r.l., Brugherio, Milan, Italy), starting at 30 cm from the bottom up to the surface of the sampling point, and data were elaborated by a standard REDAS software (RS 232; Idronaut). For zooplankton detection, subsurface water samples (20 cm below the water surface) were taken using a trawl net (WP-2; IDROMAR s.r.l., Genova, Italy) 0.57 m diameter, 2.6 m in length and 200 µm mesh size, by horizontal towing for *ca* 5 min at a speed of two knots. A digital flowmeter (Hydro-bios; Apparate bow GmbH, Kiel-Holtenau, Germany) was used to determine the water volume passing through the plankton net.

The collected plankton was poured into a sterile plastic container up to a final volume of a 500 ml in sterile seawater and 4% of formalin.

For *H. pylori* detection, a Niskin bottle was used to collect 3 l of water, refrigerated at 4°C, and processed within 4 h.

Water samples were concentrated through two steps of filtration using 200 µm and 64 µm nylon net filters (IDROMAR) to detect *H. pylori* associated with planktonic cells of different sizes. In particular, water samples were first passed through the 200 µm mesh plankton net and the collected

sediment together with the filter were gathered in sterile plastic containers with 3 ml of sterile seawater and sterile glass-beads. The presence of glass-beads facilitated, through gentle agitation, the detachment of sediments from the filter. The filtered water was successively passed through the 64 µm mesh plankton net and the filter was treated as above. Finally, the filtered water was passed through 0.22 µm pore-size standard membrane filters (Pall Gelman Laboratory, Milan, Italy) to detect *H. pylori* free cells and the filter was treated as above indicated. Each of these preparations resuspended with 3 ml of sterile water was divided in three 1 ml aliquots and subsequently used for experiments. The aliquots were centrifuged at 12 000 × *g* for 20 min. Pellets were stored at -20°C until DNA extraction. All experiments were conducted in triplicate. One litre of water was also collected by Niskin bottle, immediately refrigerated at 4°C and analysed within 4 h for enumeration of total-faecal coliforms and enterococci by membrane filters methods according to the Italian National Standard Methods for Analytical Procedures for Recreational Waters (1982) conformed to European Microbiological Parameters (EEA UNEP 1999). Briefly, aliquots of 100 ml were filtered through 0.45 µm pore-size standard membrane filters (Pall Gelman Laboratory) and the washed filters were transferred on a solid selective media; in particular, LES Endo agar (Oxoid Ltd, Basingstoke, London, UK) was used to enumerate total coliforms, MFC medium agar (Oxoid) was used for faecal coliforms and M-Enterococcus agar (Oxoid) was used in detecting enterococci.

### Zooplankton examination

The amount of zooplankton per m<sup>3</sup> was evaluated through the total water volume filtered during the sampling with trawl net and the zooplankton analysis was performed according to standard methods (EEA UNEP 1999; Skjoldal *et al.* 2000).

Briefly, the zooplankton analysis was carried out on four samples of 5 ml each, taken from total sample after gentle agitation. Cladocerans and Copepods which are the major components of zooplankton, were identified by a Leica standard stereomicroscope (Leica, DMIL, Germany) using a Petri disc with a net on the bottom to count the number of microorganisms in the plate. Examinations were performed blinded by three biologists. All experimental data are shown as mean ± SD of quadruplicate determinations.

### Artificial seeding of seawater with serial logarithmic dilutions of *H. pylori* prior to PCR analysis

*Helicobacter pylori* ATCC 43504 was used to spike seawater to determine the sensitivity of the nested-PCR assay. The microorganism was grown on chocolate agar (CA) plus 1%

IsoVitaleX (Becton Dickinson and Co., Cockeysville, MD, USA) for 5 days in microaerophilic atmosphere (Campy Pak Jar, Oxoid Ltd) at 37°C. Bacteria were harvested in Brucella broth (BB) (Biolife Italiana, Milan, Italy) and diluted to a concentration of *ca* 0.1 optical density at 600 nm. Serial tenfold dilutions were prepared in BB up to 10<sup>8</sup> CFU ml<sup>-1</sup> times the original broth concentrations.

To enumerate the bacteria, 100 µl aliquots were spread onto four CA plates and incubated in microaerophilic environment for 5 days at 37°C. The bacterial concentration was estimated by calculating the average colony count for the four agar plates. Aliquots of serial dilutions were added to 100 ml of seawater.

Seawater samples were obtained collecting 2 l of seawater in the sampling station by Niskin bottle from which aliquots of 100 ml were prepared. Before seeding with serial *H. pylori* dilutions, within 4 h from collection, one seawater sample of 100 ml was analysed to quantify the background flora (Italian National Standard Methods for Analytical Procedures for Recreational Waters 1982 DPR 470/82; EEA UNEP 1999). In the same time of sampling, the absence of *H. pylori* DNA was detected in 1 l of seawater by using the method described before.

Each contaminated sample was filtered through 0.47 µm pore-size standard membrane filters (Pall). Filters were put in sterile plastic containers with 1 ml of sterile seawater and sterile glass-beads. The suspensions were centrifuged at 12 000 × *g* for 20 min. Pellets were stored at -20°C until DNA extraction. One aliquot of concentrated bacteria was also centrifuged at 12 000 × *g* for 20 min and bacterial genomic DNA was extracted by using a QIAGEN kit (Qiagen, Milan, Italy). The extracted sample was stored at 4°C until tested.

### DNA extraction

Bacterial DNA was extracted by the method of McKeown *et al.* (1999). Briefly, the pellets stored at -20°C were suspended in 500 ml TE buffer (pH 8). A 50 µl aliquot was boiled for 5 min [boiled preparation (b.p.)]; the remaining aliquot was centrifuged, the supernatant removed and the pellet was suspended with 300 µl of extraction buffer (20 mM l<sup>-1</sup>, Tris-HCl pH 8, 0.5% Tween 20). Proteinase K was added to a final concentration of 0.5 mg ml<sup>-1</sup>. After incubation for 1 h at 55°C, the proteinase K was inactivated by heating at 98°C for 10 min and 50 µl were stored (preparation a). A 300 µl of phenol-chloroform-isoamyl alcohol was added to the remainder which was vortexed then centrifuged at 12 000 × *g* for 10 min, and 30 µl was removed from the aqueous layer (preparation b). The remainder of the aqueous layer was transferred to another tube; 750 µl of cold 100% ethanol and 30 µl 3 M sodium acetate were added (pH 5.2), mixed and kept at -80°C for 30 min, then centrifuged

at 12 000 × *g* for 15 min. The pellet was air-dried and resuspended in 30 µl of distilled water (preparation c). Six microlitre of the preps were used for PCR.

### PCR primers

Oligonucleotide primers 1 and 2 (5'-AAGCTTTT TAGGGTGT TAGGGGTTT-3', 5'-AAGCTTACTTTCTA AACTAAACGC-3') were used to obtain a 294 bp PCR product of the *H. pylori glmM (ureC)* gene. Primers 3 and 4 (5'-CTTTCTTCTCAAGCGGTTGTC-3', 5'-CAAGCCATCGCCGGTTTTAGC-3') were used to amplify a 252 bp region located 21 base pairs internal to primers 1 and 2 (Bamford *et al.* 1998). The primers were synthesized by EuroBio (Labtek s.r.l EURO BIO, Corsico, Milan, Italy).

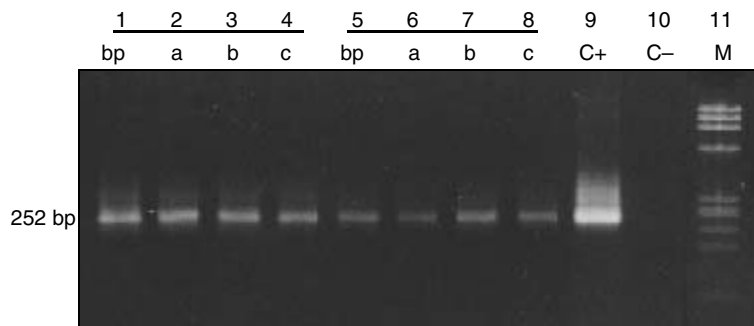
### Nested-PCR amplification and DNA sequencing

Six µl of the four DNA lysate preparations were added to a final volume of 50 µl of reaction mixture containing 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3) 1.5 mM MgCl<sub>2</sub>, 200 µM of desossinucleotide triphosphate, 50 pmol of each primers (1 and 2) and 1 U of Amplitaq DNA polymerase (Applied Biosystems, Monza, Italy). PCR amplification was performed according to the following profile: 95°C for 5 min and 33 cycles at 95°C for 2 min, 57°C for 2 min, 75°C for 1 min, followed by 75°C for 5 min in an Applied Biosystem Thermocycler 2400. After PCR, 1 µl of the final product was transferred in a second step reaction mixture and reamplified for 30 cycles with the primers 3 and 4 under the followed conditions: 95°C for 5 min and 30 cycles at 95°C for 2 min, 54°C for 2 min, 75°C for 1 min, followed by 75°C for 5 min. Positive control reaction consisted of *H. pylori* genomic DNA from type strain ATCC 43504, while as negative control three microorganisms (*E. coli* ATCC 25922, *Saphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853) were chosen and tested with the same primers. Ten microlitres samples of PCR products were analysed by electrophoresis in a 1.5% (w/v) agarose gel at 100 V for 45 min. Gels were stained with ethidium bromide and photographed.

When more accurate determinations of the size of PCR products were desired, a VISIGEL separation matrix (Stratagene; Eppendorf s.r.l., Milano, Italy) was used and electrophoresis was performed for 45 min at 100 V.

The PCR product was purified by spin column QIAQuick (Qiagen) and cycle-sequenced (on both strands) by using the ABI PRISM Big Dye Terminator Cycle Sequencing kit (Applied Biosystems).

In the sequencing primers UreC 3 (5'-CTTTCTTCTCAAGCAATTGTC-3') and primer UreC 4 (5'-CAAGCCATCGCCGGTTTTAGC-3') were used. DNA sequences were carried out by using an automated



**Fig. 1** Results of Visigel electrophoresis patterns of nested-PCR products obtained by running *Helicobacter pylori* spiked seawater samples. Lanes 1–4: samples spiked with 435 CFU 100 ml<sup>-1</sup> and extracted by four different methods of DNA purification (b.p., boiled preparation; a, b, c, preparations); lanes 5–8: samples spiked with 62 CFU 100 ml<sup>-1</sup>; lane 9: positive sample in BB; lane 10: unspiked aliquot; lane 11:  $\Phi \times 174/HaeIII$  Marker

sequencer, ABI PRISM 310, version 3.4.1 (Applied Biosystems). The resulting nucleotide sequence of the 252 bp region of the *glmM* (*ureC*) gene was aligned using the Sequence Navigator software package (Applied Biosystems). Sequence comparison was subsequently carried out using BLAST Search in National Center of Biotechnology Information (NCBI).

### Statistical analysis

All experiments were carried out in triplicate. Student's *t*-test was used to detect a possible correlation among the presence of *H. pylori* and other microorganisms, chlorophyll 'a' and zooplankton. A *P*-value of <0.001 was considered significant.

### RESULTS

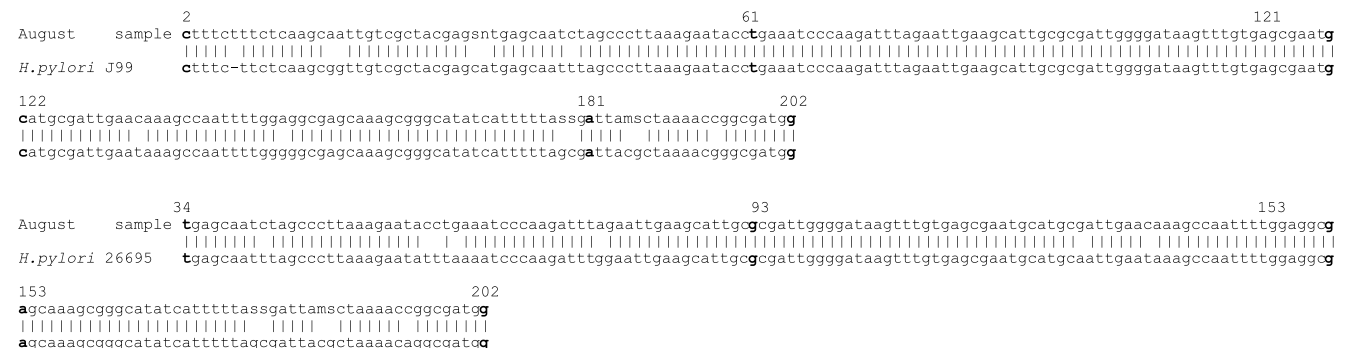
During 1 year of monitoring, *H. pylori* DNA was detected in samples collected monthly from the Adriatic Sea indicating the presence of this bacterium in two different forms: free and attached to planktonic organisms.

The coastal site, elected for monthly water collection was characterized by 97.32%  $\pm$  8.4 of dissolved oxygen (range 10.4–85.2%, with major values during summer months), 34.47 g l<sup>-1</sup>  $\pm$  4.8 of salinity (range 37.6–19.8) and

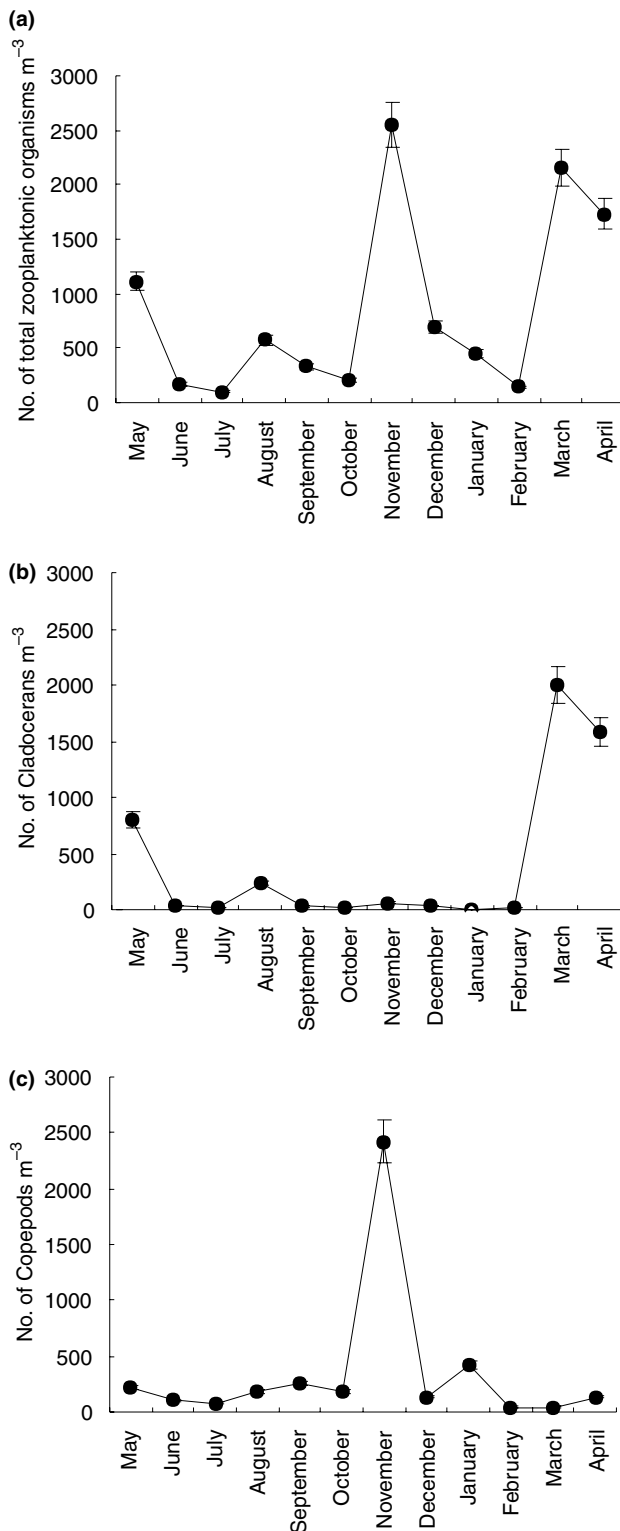
7.73  $\pm$  0.35 of pH (range 7.7–8.9). Variation in these parameters did not influence the presence of *H. pylori*. Chlorophyll 'a', considered as an indicator of seaweed biomass, expressed values ranging from 0.2 to 2.1  $\mu$ g l<sup>-1</sup> with the highest levels during the spring (2.1, 1.7, 1.3  $\mu$ g l<sup>-1</sup> in March, April and May, respectively).

To determine the minimum number of *H. pylori* cells that could be amplified by nested-PCR, the sensitivity of the assay was evaluated by spiking 100 ml seawater samples with serial dilutions of *H. pylori* ATCC 43504. Sixty-two CFU was the minimum number of cells that were required to give positive amplification products from each DNA preparation. Figure 1 shows the results obtained from different steps of DNA extraction from water samples spiked with 435 and 62 CFU of *H. pylori* respectively in presence of a microbial background of 4.5  $\times$  10<sup>2</sup> total coliforms, 0.2  $\times$  10<sup>2</sup> faecal coliforms and 0.6  $\times$  10<sup>2</sup> enterococci.

The oligonucleotide primers UreC 1, UreC 2 and UreC 3 and UreC 4, were highly specific in detecting *H. pylori* DNA and the sequences of UreC 3 and UreC 4 amplified fragments confirmed that the amplicons originated from *H. pylori glmM* gene sequence with a percentage of alignment over 90%. Figure 2 shows an example of the obtained alignment using BLAST search of the *glmM* (*ureC*) ORFs of a *H. pylori* positive seawater sample to *H. pylori* J99 and 26695, respectively.



**Fig. 2** Alignment of the *glmM* (*ureC*) ORFs of a positive seawater sample to *H. pylori* J99 and 26695, respectively. The comparison shows an identity of 93% in the first alignment and 92% in the second one



**Fig. 3** Number of zooplanktonic organisms (a), Cladocerans (b) and Copepods (c) per m<sup>3</sup> of seawater monthly detected during 1 year of sampling

The total zooplankton, from which Cladocerans and Copepods were identified, was evaluated in each sampling during the study. Figure 3a shows the mean  $\pm$  SD from each month; in particular, Fig. 3b shows a peak of Cladocerans in March and Fig. 3c of Copepods in November. The highest values corresponding to a blooming of zooplanktonic organisms were paralleled by the presence of *H. pylori* DNA. According to these data, the bacterium detected amplifying a highly conserved region of the *glmM* (*ureC*) gene by a nested-PCR assay, was found attached to planktonic organisms in November, December and March (Table 1 and Fig. 4). In fact, *H. pylori* DNA was detected bound to planktonic cells on nylon nets of 200 and 64  $\mu$ m in November and December, and on a 64  $\mu$ m filter in March but not all of the four DNA lysate preparations resulted positive. Positive results were also recorded from June to September. In the summer time, together with *H. pylori* positive samples bound to planktonic cells, amplified products obtained from free samples were also detected. In this condition, the *H. pylori* free DNA was revealed through the more purified DNA procedure extraction (preparation c). These results could be related to the significantly ( $P < 0.001$ ) increased summer concentrations of total coliforms (Fig. 5), one of the three major groups of indicator bacteria (total coliforms, faecal coliforms and enterococci) normally utilized for determining the state of pollution of the sea by sewage.

## DISCUSSION

The results of this study provide evidence of the presence in seawater of *H. pylori* either free or attached to planktonic organisms.

Mazari-Hiriart *et al.* (2001b), recently reported that an enhanced presence in freshwaters of faecal indicator bacteria is paralleled by detection of free *H. pylori*.

These findings can be explained with the release of the microorganism from sewage containing the faeces of diseased individuals or carriers.

In this study, *H. pylori* was detected in free state during the summer months, when a greater bacterial pollution of coastal marine waters occurs.

In addition, our data show a significant presence of *H. pylori* linked to Copepods and Cladocerans. In fact, *H. pylori* cells bound to plankton were detected either in summer months or in November/December during the blooming of Copepods and in March when Cladocerans are present in a greater number. Therefore, we can suppose that zooplanktonic organisms represent a sort of protected niche for survival of *H. pylori*.

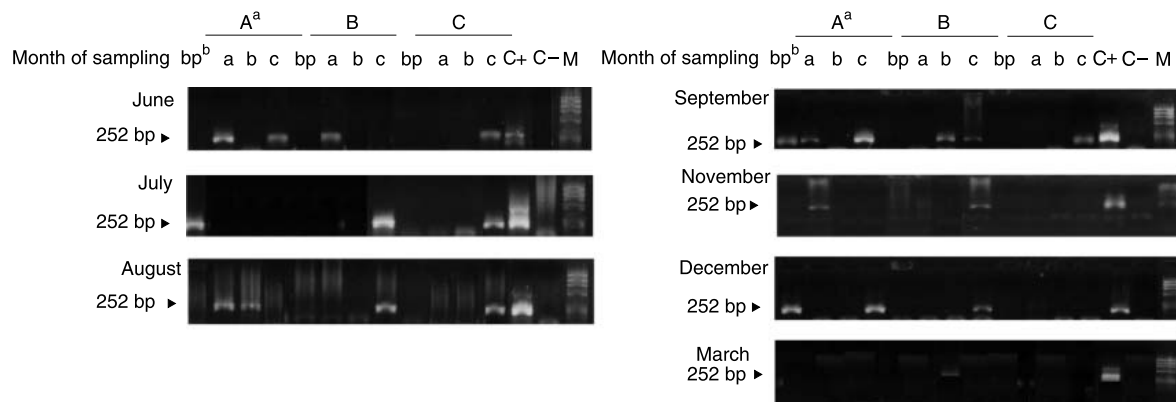
On the contrary, it has been documented (Dumontet *et al.* 1996; Montanari *et al.* 1999; Tarsi and Pruzzo 1999) that the

**Table 1** Results of *Helicobacter pylori* detection by nested-PCR from seawater samples

Month of sampling	<i>Helicobacter pylori</i> detection											
	A*				B				C			
	bp†	a	b	c	bp	a	b	c	bp	a	b	c
May	-	-	-	-	-	-	-	-	-	-	-	-
June	-	+	-	+	-	+	-	-	-	-	-	+
July	+	-	-	-	-	-	-	+	-	-	-	+
August	-	+	+	-	-	-	-	+	-	-	-	+
September	+	+	-	+	-	-	+	+	-	-	-	+
October	-	-	-	-	-	-	-	-	-	-	-	-
November	-	+	-	-	-	-	-	+	-	-	-	-
December	+	-	-	+	-	-	-	+	-	-	-	-
January	-	-	-	-	-	-	-	-	-	-	-	-
February	-	-	-	-	-	-	-	-	-	-	-	-
March	-	-	-	-	-	-	+	-	-	-	-	-
April	-	-	-	-	-	-	-	-	-	-	-	-

\**H. pylori* bound to planktonic cells was searched by filtering seawater through (A) 200  $\mu\text{m}$  and (B) 64  $\mu\text{m}$  nylon for nets. *H. pylori* free cells were searched for by filtering through (C) 0.22  $\mu\text{m}$  diameter pore-size membrane.

†DNA was extracted through four levels of purification (bp, boiled preparation; a, b, c preparations).



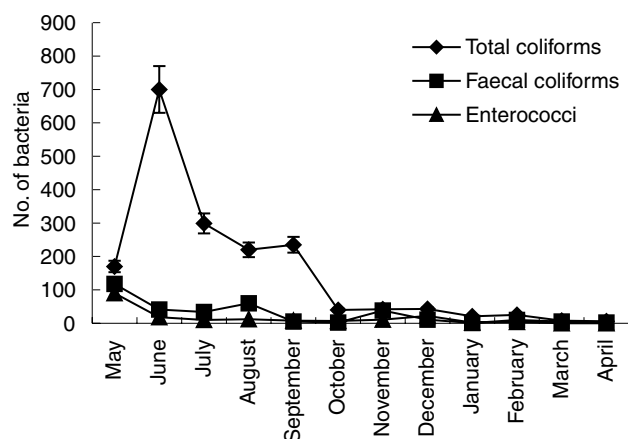
**Fig. 4** Agarose gel electrophoresis of nested-PCR products of *H. pylori* detected in positive seawater samples. <sup>a</sup>*H. pylori* bound to planktonic cells was searched for by filtering seawater through (A) 200  $\mu\text{m}$  and (B) 64  $\mu\text{m}$  nylon nets. *H. pylori* free cells were searched for by filtering through (C) 0.22  $\mu\text{m}$  diameter pore-size membrane. <sup>b</sup>DNA was extracted through four levels of purification (bp, boiled preparation; a, b, c, preparations).

C+ = positive control; C- = negative control; M =  $\Phi \times 174$ / *Hae*III

adhesion of other bacterial species on the surface of live planktonic cells significantly contributes to their survival and diffusion in aquatic environment with epidemiological and ecological implications. The finding of *H. pylori* bound to planktonic organisms is especially interesting also for the role of the latter in the seafood chain and its subsequent potential role in the spread of *H. pylori* infection.

We hypothesize that the presence of *H. pylori* DNA in seawater can be attributed to the presence of viable whole bacteria, although this study provides little direct evidence for this. It has been demonstrated that *H. pylori* is able to enter a VBNC state (Cellini *et al.* 1998) in which coccid,

nonculturable cells can react against environmental stressing stimuli (Cellini *et al.* 1994b; Donelli *et al.* 1998; Mizoguchi *et al.* 1998; Nilsson *et al.* 2002). The VBNC state is a survival strategy of bacteria to overcome environmental stress (Barer *et al.* 1998; Barer and Harwood 1999; Lleo *et al.* 2000). In this state, bacteria are able to maintain their metabolic activity, their pathogenicity (Colwell and Huq 1994; Mizoguchi *et al.* 1998; Lleo *et al.* 2001) and in some cases, to revert from dormant to active re-growing condition (Nilsson *et al.* 1991; Kaprelyants and Kell 1993; Cellini *et al.* 1994a, 1998; Colwell and Huq 1994; Lleo *et al.* 1998, 2000, 2001); moreover, the existence in aquatic environment of VBNC



**Fig. 5** Values of total and faecal coliforms and enterococci during 1 year of sampling

bacteria, is well documented (Colwell and Huq 1994; Eguchi *et al.* 2000; Lleo *et al.* 2001; Moore *et al.* 2001). Then, our positive samples are supposed to contain 'resting' forms of *H. pylori* which are able to survive in marine environments in which a massive presence of prokaryotic and eukaryotic cells is associated with an intensive activity of degradative enzymes against dead/damaged bacteria and free DNA (Hoppe 1991; Huston *et al.* 2000).

The nested-PCR used for *H. pylori glmM* gene detection, provided sensitive and specific results. The *glmM* gene was selected as a suitable target site for nested-PCR as it is unique to *H. pylori*; despite a very high level of expression, the DNA is present as a single chromosomal copy per bacterial cell (Labigne *et al.* 1991).

Moreover, as also demonstrated by other authors (Bickley *et al.* 1993; Bamford *et al.* 1998), the presence of other microorganisms in the sample, does not interfere with the detection of *H. pylori*. The use of four different steps of DNA preparation, was crucial to achieve an increased PCR sensitivity. In fact, by the two first steps of DNA extraction (b.p. and a preparations) a higher amount of DNA was obtained from each sample even in the presence of PCR inhibitors. Instead, by the further two steps (b and c preparations), more purified DNA was obtained, probably with a partial loss in the amount, but with the removal of PCR inhibitors.

In this study, variable PCR results were obtained using the four DNA different preparations suggesting a combined use of extraction methods to obtain the most effective results. For the free *H. pylori* DNA detection, the more purified sample obtained using ethanol, sodium acetate together with phenol-chloroform-isoamyl alcohol (preparation c) showed the best sensitivity.

In conclusion, our study provides evidence of the presence of *H. pylori* either free or bound to planktonic cells, indicating that polluted coastal marine environments may be

a significant reservoir of *H. pylori* and hypothesizing that faecal-oral transmission via contaminated marine waters can occur. These findings, obtained with the experimental procedure here described for a fast and reliable *H. pylori* monitoring in seawater samples, seem to provide a promising background to define new and effective strategies for surveillance of this human pathogen.

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