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Additional Information

Use of fluorescent *in situ* hybridization to evidence the presence of *Helicobacter pylori* in water.

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Abstract-We have evaluated the use of a fluorescent *in situ* hybridization (FISH) technique for the detection of *Helicobacter pylori* in water (river and wastewater) samples. The assay was compared with PCR detection and isolation of cells on selective media. 16S rRNA and *UreA+B* sequence data were used as oligonucleotide probe and specific primers for FISH and PCR, respectively. Using FISH technique, *H. pylori* was detected in two river water and one wastewater samples, while PCR yielded only one positive result. *H. pylori* culture was not possible from any sample. According to these results, FISH technique has the potential to be used as a quick and sensitive method for detection of *Helicobacter pylori* in environmental samples.

Key words: *Helicobacter*, PCR, FISH, water, activated sludge, detection

INTRODUCTION

Helicobacter pylori is an etiological agent of gastritis, peptic and duodenal ulcer disease. In addition, infection with this organism is a recognized risk factor in the development of gastric mucosa-associated lymphoid tissue lymphoma and adenocarcinoma (Chaun, 2001). Although in most of cases infection does not result in clinical symptoms, the Public Health relevance of this infection is high. Chronic gastritis and peptic ulcer are very common diseases across populations. Moreover, gastric cancer remains second among causes of cancer deaths worldwide (Goodman and Cockburn, 2001). The prevalence of *H. pylori* infection in the world is assumed to be approximately 50%, with higher prevalence in developing than in developed countries (Dunn *et al.*, 1997). Despite of its major Public Health impact, the design of prevention measures is difficult due to our limited knowledge of transmission pathways (Goodman and Correa, 2000). The exact mode of transmission remains unclear, but faecal-oral and oral-oral routes have been suggested. In developed countries reports show a clustering of *H. pylori* infection within families, which supports an oral-oral transmission pathway. In these countries, no association between *H. pylori* infection and water is found (Hulten *et al.*, 1998). On the other hand, studies from developing countries with poor management of

their water supplies have shown an association between the prevalence of *H. pylori* and the source of drinking water (Klein *et al.*, 1991). Moreover, increased risk factors for the infection have been associated with the consumption of vegetables irrigated with untreated sewage (Hopkins *et al.*, 1993). These findings suggest that contaminated water may be a potential source of *H. pylori* infection.

So far, no reports have been published on successful culture of *H. pylori* from surface water. All bacillary *H. pylori* organisms change morphology on prolonged exposure to water, and enter a viable but non-culturable stage in which they appear as coccoid forms (Nilsson *et al.*, 2002). It has been suggested that this coccoid form is responsible for transmission in the environment, probably via contaminated water (Hulten *et al.*, 1998). However, as in this form *H. pylori* is non-culturable by ordinary techniques, its ability to survive, viability and virulence is still a matter of controversy (Engstrand, 2001).

Survival of *H. pylori* in water systems has been the subject of previous studies. By immunofluorescence techniques, the organism was found in a majority of the surface and shallow groundwater samples examined in Pennsylvania and Ohio (Hegarty *et al.*, 1999). Different PCR methods have been used too to detect *Helicobacter* in environmental samples (Sasaki *et al.*, 1999, Bunn *et al.*,

2002). Recently, *H. pylori* has been isolated from wastewater (Lu et al., 2002). Results from these studies suggest that *H. pylori* may survive in water for extended periods of time, which strongly supports a waterborne route of transmission.

During recent years, fluorescent *in situ* hybridization (FISH) with rRNA oligonucleotide probes has been used for detection and identification of microbial species in environmental samples (Amann et al., 1995). FISH method has the advantage of not being inactivated by sample inhibitors. Besides, a protocol to obtain the DNA from bacteria is not necessary, and positive results may be directly observed in the sample. This method has also been reported to allow for the detection of viable but non-culturable forms which could not be sometimes detected by PCR due to the decrease of DNA content (Amann et al., 1995). In clinical samples, FISH technique has shown to be more sensitive than cultural methods (Rüssmann et al., 2001).

In this work, we have evaluated the use of a fluorescent *in situ* hybridization (FISH) technique for the detection of *Helicobacter pylori* in water (river and wastewater) samples, and compared its effectiveness with PCR and cultural detection methods.

MATERIAL AND METHODS

For sensitivity assays, an overnight culture of *H. pylori* ATCC 43504 was serially diluted to give 10 to 10⁸ CFU/ml. The amount of cells of each dilution was calculated following plating on 5% sheep blood agar plates for 72 hours. 1 ml of each dilution was inoculated into a flask containing 100 mL of sterile river water. Samples were immediately filtered through 0.45 µm membrane filters (Whatman, Maidstone, England). The membranes were aseptically removed from the filtration unit, rolled, and transferred to 100 ml of Nutrient Broth supplemented with Dent Selective Supplement (Oxoid, SR 147E) at 37°C in microaerophilic atmosphere during 48 h. Aliquots were taken directly from the inoculated samples and after 1, 6, 17 and 24 h of enrichment for FISH detection.

For *Helicobacter* detection in non-inoculated samples, ten fresh water samples were obtained from Turia river (Valencia, Spain), with a periodicity of one sample per week. Fifteen wastewater samples were collected from a secondary wastewater treatment plant located in Valencia, Spain. Samples were obtained from the influent, effluent (water) and the aeration tank (activated sludge). All the samples were placed into sterile glass bottles, refrigerated and processed in less than six hours.

An amount of 200 ml of each sample was filtered as described above. The membranes were transferred to 100 ml of Nutrient Broth supplemented with Dent Selective Supplement and incubated in microaerobic conditions at 37°C for 48 h.

After enrichment, portions of 0.1 ml of enrichment broth were plated on Columbia Agar Base supplemented with 5% defibrinated horse blood and Dent Selective Supplement, incubated at 37°C and examined for the presence of characteristic colonies at 48 h, 3, 7 and 10 days.

For PCR analysis, an aliquot of 1 ml of each enrichment broth was obtained after 24 h of incubation, and DNA was extracted following the CTAB method (Wilson *et al.*, 1987), and purified by using a DNA Capture Column Kit (Genra Systems, USA). An amount of 5 µl from each extract was used to amplify an specific urease gene fragment of 2410 bp (Foxall *et al.*, 1992).

A final reaction volume of 50 µl was made by addition of 5 µl of each sample (100 µg), 200 ng of each primer (OW1: 5'-AGGAGAATGAGATGA-3' and OW2: 5'-ACTTTATTGGCTGGT-3'), 0.2 mM of each deoxynucleotide, 1.5 mM MgCl₂ and 2 U of *Taq* polymerase (New England Biolabs, U.K.). A negative control in which DNA was replaced with sterile distilled water was also included. The amplification consisted of an initial DNA denaturing step at 95°C for 5 min, followed by

30-cycle reaction (94°C for 1 min; 45°C, 1 min; 72°C, 2 min) and a final extension step at 72°C for 5 min.

PCR products were analysed by electrophoresis at 100 V for 1 h through 1% (w/v) agarose gels in TBE buffer pH 8.3 and visualized by staining with ethidium bromide under U.V. light. A 100 bp DNA ladder (FMC Bioproducts, Denmark) was used as a molecular weight marker.

For FISH analysis, 200 ml of each water sample and 1 ml of each enrichment broth were centrifuged (12000 rpm), and resuspended in 250 µl of PBS buffer. Sludge samples were treated first with tetrasodium pyrophosphate (Kepner and Pratt, 1994) to disperse sludge particles. Samples were fixed with three volumes of 4% paraformaldehyde for 2 h at 4°C as previously described by Aznar *et al.* (1994). Subsequently, fixed samples were centrifuged, washed with PBS buffer and finally resuspended in 1:1 PBS/ethanol (v/v) as previously described (Amman *et al.*, 1995).

FISH analysis was performed with a 16S rRNA oligonucleotide probe specific to *H. pylori* (HPY - CTGGAGAGACTAAGCCCTCC-) designed by us according to Sheng-Ang *et al.* (1991). The specificity of HPY probe for *Helicobacter pylori* detection was confirmed by a gapped BLAST search (Altschul *et al.*, 1997). HPY probe specificity was evaluated too by whole-cell hybridization with different *Helicobacter* and non-*Helicobacter* species

previous to its use (Table 1). Probe was synthesized and labelled by MGW Biotech (Mannheim, Germany) with 5 (6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) and tetramethylrhodamine-5-isothiocyanate (TRITC).

An aliquot of 20 μ l fixed sample was placed on a gelatine-coated slide, air dried, dehydrated (50, 80, 100% ethanol) and hybridized as described by Amann *et al.* (1995). Final concentration of formamide was established at 20 % in the hybridization buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl, pH 7.6) and NaCl concentration at 80 mM in the washing buffer (20 mM Tris-HCl, 0.01% SDS, 5 mM EDTA).

The EUB 338 universal probe, complementary to a region of 16S rRNA of the domain *Bacteria* was used as a positive control to simultaneously visualize the rest of water microflora (Buswel *et al.*, 1998). The use of this probe assures that hybridization procedure has been properly performed and oligonucleotides can really penetrate cells and attach to rRNA (Trebesius *et al.*, 2000).

Samples were checked too for autofluorescence before hybridization, and a fluorescent oligonucleotide sequence not complementary to *Eubacteria* rRNA (non-EUB probe) was used as a negative control, to check for non-specific binding of HPY probe to hydrophobic sample components (Amann *et al.*, 1992).

RESULTS AND DISCUSSION

Under stringent conditions, The HPY probe was able to detect all *H. pylori* strains tested, while other *Helicobacter* and non-*Helicobacter* species yielded negative results. In inoculated water samples, the detection limit of PCR assay for *Helicobacter* was 10^4 CFU/ml without enrichment, and 10^3 CFU/ml of initial inoculum after 24 h of enrichment. Longer enrichment periods did not improve the detection level. According to these results, a 24 h enrichment step was always performed when environmental samples were analyzed.

Coccoid forms could be easily detected in these inoculated samples. These results strongly supports other authors data, about the maintenance of viability and metabolic activity of these forms (Nilsson *et al.*, 2002).

FISH analysis allowed the detection of *Helicobacter pylori* cells in 2 river water samples (Fig. 1) and one wastewater sample (Fig.2). In all cases, rod-shaped forms were observed, while no coccoid form could be detected. All positive samples were obtained directly by centrifugation. After the enrichment step all samples were negative, confirming the inadequacy of liquid media for recovering stressed *H. pylori* cells (Roosendaal *et al.*, 1995).

When the seeded activated sludge samples were enriched, the sensitivity level strongly decreased due to

unspecific probe attachment of sludge flocs and interference of naturally occurring activated sludge microbiota in flocs. Höller and Schomakers-Revaka (1994) assumed that bacteria are not only attached to the surface of particles but are enclosed in the sludge particles. Microscopic examination of our positive sludge sample seemed to confirm this theory, as in some microscopic fields, HPY fluorescent signal could be observed to be included in sewage flocs (Fig. 3). This fact could explain the difficulties found for its detection in wastewater.

Only one out of the two river water FISH positive samples yielded the expected *Helicobacter* PCR 2410 bp band after 48 h of enrichment in selective broth (Fig 4). PCR assay was negative for the rest of river water and wastewater samples.

Isolation of *H. pylori* by cultural methods was not possible in any case. In fresh water, no growth was observed on selective medium. Detection of *H. pylori* from wastewater samples was impossible due to the massive growth of competitive biota in selective media used for isolation. For this reason, all the samples were considered "negative" as characteristic colonies could not be observed.

Our results demonstrate the presence of *H. pylori* in wastewater and surface water, and the inadequacy of available cultural methods for its detection. The findings

of this work seem to confirm too the ability of *H. pylori* to survive in sludge in the viable but non-culturable form. In our work, FISH technique has shown to yield more positive results than PCR. The *H. pylori* urease gene sequence primers have been applied extensively to a range of clinical samples and are established as sensitive and specific (Gzyl *et al.*, 1999; Kabir, 2001; Pacheco *et al.*, 2001). However, further studies should be necessary with water samples spiked with *H. pylori* strains to test its reliability when performed on environmental samples. Clearly, a number of important questions remain to be answered concerning the persistence of *H. pylori* strains in aquatic environments and the risk that this poses to human health. Although FISH technique needs more exhaustive evaluation, it can be a sensitive, specific and cost-effective tool to detect *H. pilory* in environmental samples. The detection of *H. pilory* by FISH will enable rapid analyses of water and sewage, improving its safety and quality, and contributing to elucidate the role of fecally contaminated water in the transmission of *H. pilory* infection.

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Table I. *Helicobacter* probe specificity test.

<u>Bacterium</u>	<u>Strain</u>	<u>Source and Origin</u>	<u>FISH</u>
<i>Helicobacter pylori</i>	43504	ATCC	+
<i>Helicobacter pylori</i>	00001	GEHO ¹	+
<i>Helicobacter pylori</i>	00002	GEHO	+
<i>Helicobacter pylori</i>	00003	GEHO	+
<i>Helicobacter pylori</i>	00004	GEHO	+
<i>Helicobacter pylori</i>	00005	GEHO	+
<i>Helicobacter felis</i>	49179	ATCC	+
<i>Helicobacter mustalae</i>	43772	ATCC	+
<i>Arcobacter butzleri</i>	12481	NCTC	-
<i>Arcobacter cryaerophilus</i>	11885	NCTC	-
<i>Arcobacter skirrowii</i>	12713	NCTC	-
<i>Arcobacter nitrofigilis</i>	11885	NCTC	-
<i>Campylobacter coli</i>	11366	NCTC	-
<i>Campylobacter fetus</i>	10842	NCTC	-
<i>Campylobacter helveticus</i>	12430	NCTC	-
<i>Campylobacter hyointestinalis</i>	11608	NCTC	-
<i>Campylobacter jejuni</i>	11168	NCTC	-
<i>Campylobacter jejuni</i>	12506	NCTC	-
<i>Campylobacter jejuni</i>	11828	NCTC	-
<i>Campylobacter lari</i>	11352	NCTC	-
<i>Campylobacter upsaliensis</i>	11845	NCTC	-
<i>Wollinella succinogenes</i>	11488	NCTC	-
<i>Clostridium perfringens</i>	13124	ATCC	-
<i>Enterobacter faecalis</i>	20478	DSM	-
<i>Enterobacter faecium</i>	20477	DSM	-
<i>Escherichia coli</i>	12900	NCTC	-
<i>Listeria monocytogenes</i>	19113	ATCC	-
<i>Pseudomonas aeruginosa</i>	10145	ATCC	-
<i>Salmonella typhimurium</i>	12117	NCTC	-

^aAbbreviations used for culture collection: ATCC, American Type Culture Collection; DSM, Deutsche Sammlung Von Mikroorganismen; GEHO, strains kindly provided by General Hospital, Valencia, Spain; NCTC, National Collection of Type Cultures.

Legend to the figures:

Fig. 1. Detection of *H. pylori* in water by *in situ* hybridization with a HPY probe.

Fig.2. Detection of *H. pylori* in wastewater by *in situ* hybridization with a HPY probe.

Fig. 3. Detection of specific HPY red fluorescence included in sludge flocks.

Fig.4. Detection of *H. pylori* in water samples by PCR. Lane 1: Positive control; Lane 2: Negative control; Lanes 2-8: water samples; M: 100 bp ladder

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