Development of an optimized technique for the recovery of H. pylori from water and drinking water biofilms

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At present, the route of transmission is perhaps one of the most controversial areas of **Helicobacter pylori** research. Drinking water and associated biofilms have been suggested as possible environmental reservoirs for the bacterium, however, successful and consistent detection of the bacterium in real systems has not yet been accomplished. A new protocol that offers improved recovery ratings of **H. pylori** from water and associated-biofilms has been developed and evaluated. This protocol includes optimized incubation atmospheres and a new recovery culture medium, as well as an assessment of existing techniques for the detachment of H. **pylori** from surfaces. Even though obvious advantages in terms of cell counts are obtained using this method, certain problems, such as contamination by faster-growing species of the culture medium in heavily contaminated waters may still hinder a successful recovery of the pathogen. Future research will involve further improvement of the protocol and monitoring of real drinking water distribution systems.

Introduction

Helicobacter pylori is a Gram-negative bacterium closely related to Campylobacter spp., with the ability to survive and replicate in the human gastrointestinal (GI) tract After colonization of the GI tract, a precarious balance is established between the bacterium and the host which can lead to the development of peptic and duodenal ulcer disease and gastric MALT lymphoma, among other detrimental conditions (**Blaser** and Atherton, 2004). Most of the human population, however, carries the microorganism asymptomatically, which implies that even when H. pylori presence is detected, the individual may be left untreated.

Because isolation of the bacterium outside the human GI tract remains elusive, there has been increasing research in order to identify the route or routes of transmission from one host to another. One of such routes, implicated by epide-

miological and microbiological (namely by molecular biology techniques) evidence, is the ingestion of contaminated water supplies (e. g. Bunn *et al.*, 2002, Klein *et al.*, 1991). Therefore, drinking water and associated biofilms have been suggested as possible environmental reservoirs for the survival of the bacterium. However, viability is intimately associated with cultivability, and H. *pylori* cultivability in water usually ends after a short time when compared to other known waterborne pathogens (Table 1). Furthermore, demonstration of the existence of viable H. *pylori* either in the planktonic phase or in biofilms associated with drinking water using plate-spreading techniques has yet to be accomplished. Excluding the fact that only very recently these surveys have started to be performed on a large scale (Degnan *et al.*, 2003), only two other distinct explanations can be advanced for this apparent failure: either H. *pylori* is in fact not able to survive in potable water systems, or some of the recovery technique parameters, such as the medium composition and the incubation atmosphere, are not adequate for replication and colony formation when the pathogen is water-stressed.

Table 1. Comparison of the cultivability times in water, described for different temperatures, between different waterborne pathogens and *H. pylori*

Microorganism	Culturability	T (°C)	References
H. pylori	≈ 6 hours	23	(Adams *et al.*, 2003)
	24 days	4	(Shahamat *et al.*, 1993)
Yersinia enterocolitica	10 days	30	(Chao et *al.*, 1988)
Legionella pneumophila	36 to 42 days	42	(Ohno *et al.*, 2003)
	>60 days	25	
Escherichia coli O157	49 to 84 days	25	(Wang and Doyle, 1998)
	91 days	8	
Salmonella typhimurium	>45 days	20–30	(Santo Dorningo *et al.*, 2000)
Campylobacter jejuni	43 hours	22	(Buswell *et al.*, 1998)
	29 to 36 days	4	(Cools *et al.*, 2003)

While cultivability using traditional plating techniques has been for a long time the gold-standard to assess the survival of a microorganism in a given environment, the development of new methods to evaluate the same parameter (Breeuwer and Abee, 2000) has questioned the ability of a single cell to divide in artificial nutrient media as a suitable indication of the cell's life and death. The notion of viable but noncultivable (VBNC) has then arisen to describe organisms that are not cultivable at a given time or condition, but may revert to a state of cultivability later or under different circumstances (Nystrom, 2001). Resuscitation techniques have therefore been developed for several microorganisms to alleviate this state, again enabling cultivability (e. g. Shleeva *et al.*, 2004, Wong *et al.*, 2004).

In this work, the development of an optimized technique for the recovery of H. *pylori* from water and drinking water biofilms is reported. The protocol includes optimized incubation atmospheres and a new recovery culture medium, as well as an assessment of existing techniques for the detachment of H. *pylori* from

surfaces.

Methods

Culture Maintenance

H. pylori NCTC 11637 was maintained on Columbia Agar (CA) (Oxoid, UK) supplemented with 5% (v/v) defribinated horse blood (Biomerieux, France) in the presence and absence of 1% (v/v) fetal calf serum (Merck, Germany). Plates were incubated at 37 °C in 2.5 L jars (BioMerieux) under microaerophilic conditions created using a Microaer gas pack (BioMerieux), and streaked onto fresh plates every 2 or 3 days.

For the new medium evaluation 7 additional clinical isolates (three obtained from adults and four obtained from children) were tested. The clinical isolates belong to the strain collection of the National Institute of Health in Lisbon, Portugal. Clinical isolates were stored at -80 °C upon arrival and subcultured only once before the medium evaluation experiment, in the same conditions as the reference strain.

Development of a New Medium Based on Helicobacter pylori Special *Peptone* Agar (HPSPA)

The use of nutrient rich media for the assessment of water-stressed H. pylori has been proven to have a deleterious effect in the recovery of the pathogen when compared to low nutrient media (Azevedo et *al.*, 2004). However, that same study reported that diluting the media too much would prevent the pathogen from growing. Based on these results it was attempted to develop a medium that could support H. pylori growth, but contain as low concentration of nutrients as possible. The first step was to identify the component, or components, which halted the growth of H. pylori when medium was diluted by more than a factor of two. Using HPSPA as the reference medium and H. pylori NCTC 11637 as the test strain, several sets of experiments were conducted. In each set, media were prepared by varying the concentration of one of the individual components in half and quarter-strength HPSPA. An initial assessment of the media performance was obtained by plating an appropriate quantity (between 20 and 200 CFU per plate) of water-stressed H. pylori onto the different media and comparing the results with the number of colonies obtained in a standard HPSPA medium. Mean colony she was also obtained by measuring the sizes of 10 or more colonies of one plate, in plates with similar number of colonies. Based on the results obtained and summarized on Table 2, a new low nutrient medium was designed (wHPA) and subsequently compared with HPSPA. The concentration of the different components in the final version of wHPA was: 2.5g of special peptone, 1g of meat extract, 4g of yeast extract, 5g of sodium chloride, 0.2g of pyruvic acid and 10g of agar per liter of distilled water, supplemented with 2.5% (vol/vol) of defibrinated horse blood.

Media Evaluation and Incorporation of Antibiotics

Cells from 2 days old cultures from the different strains were harvested from CBA plates and suspended in 10 mL of autoclaved tap water until a concentration of ca. 10^8 CFU per ml was achieved. This inoculum was then transferred to a sterile 1000 mL bioreactor, also containing autoclaved tap water, to achieve a final concentration of ca. 10^6 CFU/ml. The bioreactor was maintained at room temperature (approx. 24±2°C) and continuously stirred (120 rpm) using a magnetic bar. After 2 hours, sampling was performed. Before serial dilution (1:10) in sterile tap water, samples were vortexed for 10s for homogenization. *H. pylori* was enumerated by surface plating 100 ml of the different dilutions onto the appropriate agar media (pH 7±0.2). Plates were incubated at 37 °C for 6 days under the same microaerophilic conditions used for culture maintenance. For NCTC 11637, one experiment was also carried out following a time course (instead of sampling only after 2 hours, samples were also taken from the same bioreactor after 4,6 and 10 hours).

Table 2. Range of the concentrations of components from the HPSPA media tested for *H. pylori* growth.

Component	Range of concentrations tested[b]	Minimal concentration to support acceptable growth on half-strength HPSPA[ab]	Minimal concentration to support acceptable growth on quarter-strength HPSPA[a,b]
Special Peptone	1-10	3	n.p.[d]
Yeast extract	1.5-5	1.5	5
Meat extract	1-10	1	10
Sodium pyruvate	0.1-1	0.1	n.p.
Sodium chloride	0-5	2.5	5
Granulated agar	10-15	10[c]	n.p.
Hone blood	1-5	2.5%	n.p.

[a]The medium was considered to support acceptable growth if colony counts recovered in that medium were in the same order of magnitude as the reference media, HKPA.
[b]In %(vol/vol) for defribinated horse blood and g/L for every other component
'Concentrations of agar lower than 10 g/L prevented the medium from solidifying properly.
[d]Growth is not supported at acceptable levels even when the highest concentration was tested.

For the evaluation of described antibiotics in the new medium, both HPSPA and wHPA were prepared by the standard method, with 3,500 U of polymyxin B, 7.5 mg of amphotericin B, 10 mg of vancomycin, 5 and mg of trimethoprim; 5 mg of cefsulodin per liter (all from Sigma) was added aseptically after heating to 50°C.

These media were compared with the antibiotic-free media using the procedure described above. with NCTC 11637 as the test strain.

Techniques for the Removal of Adhered *H. pylori*

After allowing *H. pylori* to attach to stainless steel coupons (grade 304: SS304) for diierent periods of time (from 2h to 192h) by static adhesion, three methods were applied to detach the pathogen: vortexing with glass beads, scraping and sonication. In all cases coupons were immersed in 10 mL of sterile distilled water. Vortexing was carried out with 1 mm diameter glass beads (Sigma) for 30s in a Heidolph Reax 2000 (Heidolph Instruments GmbH and Co. KG, Schwabach, Germany). Scraping was performed systematically, 10 times in each direction, and sonication was used for 3×1min bursts with 10s interval and 10% amplitude (GEX 400 Ultrasonic Processor; Sigma). Then, 100 mL of the distilled water containing detached cells were dispensed on HPSPA plates to determine the numbers of attached *H. pylori*.

Statistical Analysis

Media evaluation results were statistically analyzed employing a one-way ANOVA for the experiment with time and a t-test for the others. Computations were performed using SPSS (SPSS Inc., USA). Results were considered statistically relevant if $P=0.05$.

Results

Media Development

Sodium chloride was found to be important not only to support growth but also in terms of colony size, and a strong positive correlation was observed between this parameter and sodium chloride concentration. In fact, colony size was larger in the diluted medium with high sodium chloride concentration than in the standard medium. Of the other components, only yeast and meat extract at high concentrations appeared to support the growth of *H. pylori*, when all other components were reduced to a quarter of their normal concentration. However, the colony size obtained for wHPA in this case was smaller than the colony size obtained for HPSPA. Because the content of defribinated horse blood was also reduced, wHPA is a more transparent medium, which allows easier colony identification when compared to HPSPA.

Media Evaluation.

The new medium wHPA provided better recoveries for 5 of the eight strains (Table 3). For 3 of the strains the recovery improved more than 30%, whereas all other strains stayed in the range of a -10% to 10% improvement It is also important to note that, of the strains where higher counts were obtained for HPSPA, 2 out of three presented P values higher than 0.9, meaning that statistically the two media are probably similar in respect to *H. pylori* recovery from water.

Table 3. Percentage of improvement of HPSPA and wHPA media (n=3-12)

Strain	Provenience	Pathology	Percentage of improvement
1320	adult	Peptic ulcer	40%
968	child	Gastritis	-7%
1330	adult	Peptic ulcer	10%
957	child	Peptic ulcer	-3%
1198	child	Peptic ulcer	45%*
1152	child	Peptic ulcer	1%
1342	adult	Gastritis	-5%
NCTC 11637	type strain		34%*

* Statistically significant (P<0.05)

For NCTC 11637, the comparison of the cultivable counts was also performed with time (data not shown). ANOVA showed that, for this strain, there was a significant difference (P<0.01) between wHPA and HPSPA. This result was already expected because a statistically significant result has already been obtained between HPSPA and half-strength HPSPA (Azevedo *et al.*, 2004).

The effect of addition of antibiotics has already been described in the literature (Degnan *et al.*, 2003). Using the new medium, the percentage of improvement for the type strain was 31.5% between wHPA and HPSPA with no antibiotics, and 16.3% between wHPA and HPSPA with antibiotics.

Techniques for the Removal of Adhered H. pylori

The methods tested to remove the cells (sonication, scraping and vortexing with glass beads) were only able to detect the bacteria after two hours attachment and even then only at very low levels (up to 500 CFU/cm^2). However, the efficiency of removal, as assessed by DAPI total counts before and after the detachment method, ranged between 60-70% for the sonication and vortexing methods.

Discussion

This work describes a reformulated medium with the specific purpose of recovering *H. pylori* from water and water associated biofilms. Perhaps the most striking result is the fact that not all strains appear to recover better in low nutrient medium, which may indirectly imply that while optimizing the medium for *H. pylori* NCTC 11637 one or more essential nutrients for some of the other strains has been reduced too much, preventing these strains from growing properly. An al-

ternative interpretation is that adaptation to water does not occur for all of H. *pylori* strains. This may, however, not come as a surprise, since it has been demonstrated that this pathogen presents extensive strain and interstrain variation at both the genomic and the protein level (Enroth and Engstrand, 2001). Differences in adaptation could actually suggest that H. *pylori* uses multiple routes to be transmitted from person to person, with non water-adapted species preferring other transmission routes, such as person-to-person. The existence of multiple transmission routes to infect human individuals has also been indicated by other authors (Graham and Malaty, 2002, Megraud, 2003), and has also been observed for *Campylobacter* spp., a closely related species (Stelzer *et al.*, 1991). Because it appear obvious that H. *pylori* strains that are able to adapt to environmental conditions will possess a better chance of being transmitted through the water, wHPA is likely to be very useful, even though it brings no obvious advantages for other strains tested in this study, apart from being more transparent.
The antibiotics at the concentrations described in Degnan *et al.* (2003) were tested for suitability in this new medium, and were found to have a small deleterious effect on the growth of H. *pylori*. Even though wHPA with antibiotics is still superior to HPSPA with antibiotics, a reassessment of the concentrations of each of the compounds in the selective supplement might be necessary to further enhance the medium performance.

The efficiency of wHPA was due, in a large part, to maintaining the sodium chloride concentration from HPSPA. In other studies, restriction of salt and salted food intake was suggested as a practical strategy to prevent gastric cancer in areas with high *H. pylori* prevalence (Tsugane *et al.*, 2004), and suspicions of seawater as a transmission vehicle have recently arisen (Cellini *et al.*, 2004). Sodium chloride has also been implicated in pathogenesis as it potentiates the vacuolation toxin activity (Ma *et al.*, 2002). Sodium chloride appears therefore to be a key element for H. *pylori* growth and metabolism.

This work also shows that new microbial cultivation media need to be not only species-specific, but also adjusted to the environment the microorganism is recovered from. For the enumeration of heterotrophic microorganisms present in water or other low nutrient environments, it has already been shown that the low-nutrient medium R2A obtains much better results than other heterotrophic high-nutrient media (Massa *et al.*, 1998, Reasoner and Geldreich, 1985). Low nutrient medium efficiency is probably related to osmotic effects, which were considered to be the most important parameter for killing *H. pylori* in regionally different types of honey (Osato *et al.*, 1999). Also, Enroth and co-workers have shown that coccoid forms of H. *pylori* have lower density than the spiral counterparts (Enroth *et al.*, 1999). As it has been shown that transformation from spiral to coccoid is a common process when the microorganism is exposed to water, it is possible that this is in fact a response mechanism of the bacterium to withstand the osmotic effects caused by exposure to water.

Because recovery of attached *H. pylori* was only possible after the 2 hours attachment time, there appears to be no protection conveyed to the pathogen provided by attachment on stainless steel. It would, however, be expected that cultivability would remain for at least 6 hours, which is the time the bacteria becomes noncultivable in the planktonic state, but this difference could be due to the lack of a more suitable and sensitive method for the recovery of attached bacteria. Studies to recover viable *H. pylori* from water have been unsuccessful so far. Timing to perform the sampling is one of the issues, as infection does not immediately cause disease, but only one exposure appears enough to cause infection. The next step of this work will therefore include screening of the pathogen during indicator bacteria failure in drinking water systems and associated biofilms, where increased chances of detection exist, using these new methods and the incubation technique described in Azevedo et *al.* (2004).

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References

Adams, B. L., Bates, T. C. and Oliver, J. D. (2003) Survival of *Helicobacter pylori* in a natural freshwater environment. *Applied* and *Environmental* Microbiology **69**, 7462-7466.

Azevedo, N. F., Pacheco, A. P., Vieira, M. J. and Keevil, C. W. (2004) Nutrient shock and incubation atmosphere influence recovery of culturable *Helicobacter* pylori from water. *Applied* and Environmental Microbiology **70**, 490-493.

Blaser, M. J. and Atherton, J. C. (2004) Helicobacter pylori persistence: biology and disease. *Journal of Clinical Investigation* **113**, 321-333.

Breeuwer, P. and Abee, T. (2000) Assessment of viability of microorganisms employing fluorescence techniques. International *Journal* of Food *Microbiology* **55**, 193-200.

Bunn, J. E. G., MacKay, W. G., Thomas, J. E., Reid, D. C. and Weaver, L. T. (2002) Detection of *Helicobacter pylori* D N A in drinking water biofilms: implications for transmission in early life. *Letters* in Applied Microbiology **34**, 450-454.

Buswell, C. M., Herlihy, Y. M., Lawrence, L. M., McGuiggan, J. T., Marsh, P. D., Keevil, C. W. and Leach, S. A. (1998) Extended survival and persistence of *Campylobacter* spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and -rRNA staining. Applied and *Environmental* Microbiology **64**, 733-741.

Cellini, L., Del Vecchio, A., Di Candia, M., Di Campli, E., Favaro, M. and Donelli, G. (2004) Detection of free and plankton-associated *Helicobacter* pylori in seawater. *Journal of Applied* Microbiology **97**, 285-292.

Chao, W. L., Ding, R. J. and Chen, R. S. (1988) Survival of *Yersinia Enterocolitica* in the Environment. *Canadian Journal* of Microbiology **34**, 753-756.

Cools, I., Uyttendaele, M., Caro, C., D'Haese, E., Nelis, H. J. and Debevere, J. (2003) Survival of *Campylobacter* jejuni strains of different origin in drinking water. *Journal of Applied* Microbiology **94**.

886-892.

Degnan, A. J., Sonzogni, W. C. and **Standridge, J. H. (2003)** Development of a plating medium for selection of Helicobacter pylori from water samples. *Applied* and *Environmental Microbiology* 69.29 14-2918.

Enroth, H. and Engstrand, L. (200 1) An update on Helicobaaer *pylori* microbiology and infection for the new millennium. *Scandinavian Journal* of Infectious Diseases 33,163- 174.

Enroth, **H., Wreiber,** K., Rigo, R, **Risberg,** D., **Uribe, A. and** Engstrand, **L. (1999)** In vitro aging of *Helicobacter* pylori: Changes in morphology, intracellular composition and surface properties. *Helicobacter* **4,** 7-16.

Graham, D.Y. and Malaty, H. M. (2002) Commentary: What remains to be done regarding transmission of Helicobacter *pylori*. *International Journal* of Epidemiology 3 1,646-647.

Klein, P. D, Graham, D.Y., **Gaillour, A., Opekun, A. R. and** Smith, E. **O. (1991)** Water Source as Risk Factor for *Helicobacter Pylori* Infection in Peruvian Children. *Lancet* 337, 1503-1506.

Ma, F. **J.,** Zhao, W.Y., Kudo, M, Aoki, K. and Misumi, **J.** (2002) Inhibition of vacuolation toxin activity of Helicobaaer pylori by iodine, nitrite and potentiation by sodium chloride, sterigmatocystin and fluoride. Toxicology *in Vitro* **16,** 53 1-537.

Massa, S., **Caruso, M., Trovatelli, F. and Tosques,** M. **(1998)** Comparison of plate count agar and R2A medium for enumeration of heterotrophic bacteria in natural mineral water. *World Journal* of Microbiology and Biotechnology **14, 727-730.**

Megraud, **F. (2003)** When and how does Helicobacter pylori infection occur? Gastroenterologie *Clinique* Et *Biologique* 27,374-379.

Nystrom, T. (2001) Not quite dead enough: on bacterial life, culturability, senescence, and death. Archives of *Microbiology* **176,** 159-164.

Ohno, A., Kato, **N., Yamada,** K. **and Yamaguchi,** K. (2003) Factors influencing survival of *Legionella pneumophila* serotype 1 in hot spring water and tap water. *Applied* and Environmental Microbiology 69, 2540-2547.

Osato, M. S, Reddy, S. G. and Graham, D.Y. (1999) Osmotic effect of honey on growth and viability of *Helicobacter* pylori. Digestive *Diseases* and Sciences **44, 462-464.**

Reasoner, D. **J.** and **Geldreich,** E. **E. (1985)** A new medium for the enumeration and subculture of bacteria from potable water. *Applied* and Environmental Microbiology 49, 1-7.

Santo **Domingo,** J.W., Harmon, S. and Bennett, **J.** (2000) Survival of Salmonella species in river water. Current Microbiology **40, 409-17.**

Shahamat, M., Mai, U., Paszkokolva, C., **Kessel,** M. and **Colwell,** R. R. (1993) Use of Autoradiography to Assess Viability of *Helicobacter Pylori* in Water. *Applied* and *Environmental Microbiology* 59, 1231-1235.

Shleeva, M., **Mukamolova, G. V., Young,** M., Williams, H. D. and **Kaprelyants, A.** S. (2004) Formation of 'non-culturable' cells of Mycobacterium *smegmatis* in stationary phase in response to growth under suboptimal conditions and their Rpf-mediated resuscitation. *Microbiology-Sgm* 150, 1687-1697.

Steizer, W., Jacob, **J.** and **Schulze,** E. **(1991)** Environmental Aspects of Campylobacter Infections. *Zentralblatt* Fur *Mikrobiologie* **146,** 3-15.

Tsugane, S., **Sasazuki, S.**, Kobayashi, M, **Sasaki,** S. and Grp, **J. S.** (2004) Salt and salted food intake and subsequent risk of gastric cancer among middle-aged Japanese men and women. *British Journal* of Cancer 90, 128-134.

Wang, **G. and** Doyle, M. P. **(1998)** Survival of enterohemorrhagic *Escherichia coli* O157:H7 in water. *Journal* of Food Protection 61,662-667.

Wong, H. C., Wang. P. L., Chen, S.Y. and Chiu, S.W. (2004) Resuscitation of viable but non-culturable *Vibrio parahaemolyticus* in a minimum salt medium. *Fems* Microbiology *Letters* **233, 269-275.**