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Detection of H. pylori in biofilms formed in a real drinking water distribution system using peptide nucleic acid fluorescence in *situ* hybridization

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Helicobacter pylori has previously been detected in real drinking water distribution systems (DWDS) using PCR based techniques, but this approach will not discriminate between live and dead cells and their spatial relationship *within* the biofilm. On the other hand, in situ detection using fluorescence in situ hybridization (FISH) has been used successfully *for* spatial resolution but only in lab-grown experiments. In the present work, therefore, two flow cells were placed in a by-pass of the DWDS, *and* coupons were regularly removed for the detection of ***H.* pylori** by *peptide* nucleic *acid (PNA)* FISH, as well as for the assessment of CFU and total bacteria and visualization under scanning *electron* and *epifluorescence* microscopy. Chemical and physical parameters of the water *feeding* the flow cells were also monitored. Cells exhibiting similar morphology to ***H. pylori*** that were PNA FISH positive could be sparsely detected in the coupons. The technique showed promise despite the large morphological heterogeneity of microorganisms present in biofilms and associated autofluorescence.

Introduction

Helicobacter pylori is a Gram-negative bacterium closely related to *Campylobacter* spp. Although most people harbouring H. pylori are asymptomatic, the bacterium has been implicated in the development of certain diseases, such as gastric ulcers and stomach cancers. Despite the fact that most of the world population is infected, the route (or routes) of transmission among the human population is yet to be identified. Epidemiological studies have implicated drinking water as one of the possible vehicles of infection (e.g. Constanza et al., 2004, Herbarth et *al.*, 2001, Klein et *al.*, 1991), but culture techniques have so far failed to identify H. pylori in drinking water distribution systems (DWDS) or associated biofilms.

By 2002, two research groups had already reported the in situ detection of *Helicobacter* spp. in biofilms associated with DWDS using a nested PCR technique (Bunn et al., 2002, Park et al., 2001). The same technique had previously allowed Mackay et *al.* to demonstrate that H. pylori could persist for up to 192 h in a mixed species drinking water biofilm after inoculation in a modified Robbins device (1999). This approach will not, however, discriminate between live and dead cells and provide information about spatial relationships with the biofilm. *In* situ detection using fluorescence in situ hybridization (FISH) has been successfully used by our group for the spatial resolution in lab-grown experiments (Azevedo et *al.*, 2003a, Azevedo et *al.*, 2003b). These studies have demonstrated that H. pylori could incorporate into drinkingwater heterotrophic biofilms, and that it could be located both directly attached to the surface and in the basal layer of frond or stacks.

In the present work, a peptide nucleic acid (PNA) probe that has been shown to specifically detect H. pylori in lab-grown biofilms, was tested in coupons of two flow cells placed in a by-pass of a DWDS. PNA probes have been shown to offer several advantages in relation to the standard DNA probes, namely in terms of specificity and sensitivity (Stender et al., 2002). Chemical and biological parameters of the water and associated biofilms have been also monitored so that a correlation between them and an eventual H. pylori presence could be established.

Methods

Description of the DWDS and *Installation* of the Flow Cells

The drinking water distribution system under study was a small network that supplied treated river water to a population of 1,500 habitants in the North region of Portugal. In short, water was collected from the river bedrock after filtration through sand, filtered again through activated carbon filters and then treated with chlorine before pH adjustment with a solution of calcium hydroxide. Water was then pumped to a storage reservoir. The flow cells were connected in parallel to the distribution system using a ½" WC pipe at approx. 6 km downstream of the reservoir (Figure 1). This location corresponded to the end of the network, where chlorine concentrations were expected to be low. The biofilm forming devices consisted of a semicircular Perspex duct with ten spaces on its flat surface where coupons of different materials can be inserted. In this case, both cells were equipped with PVC coupons with 2.4×1.4cm area. A more detailed description of flow cells potential for different biofilm-related studies can be found in Pereira et al. (Pereira et al., 2002).

Water and biofilm sampling

Biofilm and water samples were collected from October 2004 to January 2005. The water samples were taken just after the passing of the water through the flow cells and were routinely analyzed for pH, temperature and total and free

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chlorine. For the determination of chlorine, the N,N-diethyl-p-phenylenediamine (DPD) colorimetric method was used (Lovibond, Salisbury, UK). On two other occasions, a more complete analysis of the water parameters was also performed (Table I).

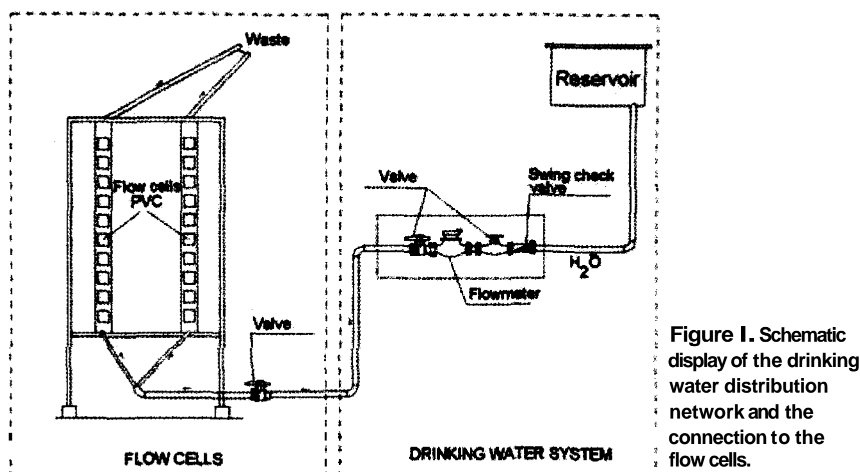

Figure I. Schematic display of the drinking water distribution network and the connection to the flow cells.

Table I. Analysis of water parameters after the passing through the flow cells. Results are the average of two samples.

Parameter	Units	Value
Turbidity	NTU	0.53
Alkalinity	$CaCO_3$	16.85
Ca_2^+	mg/L	7.65
Mg_2^+	mg/L	0.8
Na^+	mg/L	6.3
K^+	mg/L	1.1
NH_4^+	mg/L	<0,05
HCO_3^-	mg/L	20.5
SO_4^{2-}	mg/L	3.3
Cl^-	mg/L	9.35
Mn, total	mg/L	<25
NO_3^-	mg/L	5.8
NO_2^-	mg/L	<0,01
TOC	mg/L	1.8

For the biofilm sampling, one coupon from each flow cell was removed and replaced by a new one. The new coupons had been previously sterilized by washing

in a solution of sodium hypochlorite (15% v/v) for 10 min, and then cleaning with sterile water for 5 min. The removed coupons were transported under refrigeration to the laboratory in sterile boxes filled with cotton soaked in a solution of 20% glycerol. After arrival, coupons were washed in 5 mL of sterile water to remove any loosely attached microorganisms. The processing time of the coupons up to this step was kept at just under 2 hours.

Total and Cultivable Cell Counts in *Biofilms*

Biofilm material from the coupons was scraped into 10 mL of sterile water and vortexed for 2 min to promote the dispersion of cell clusters. For the cultivable cell counts, 100 mL of the suspension and associated 1:10 dilutions were plated in triplicate on R2A agar and incubated at room temperature for 14 days. For the total cell counts, 3 to 8.7 ml of the suspension was filtered through a 25 mm black Nuclepore® polycarbonate membrane with a pore size of 0.2 µm (Whatman, Kent, UK). After filtration, cells on the membrane were stained with 100 mg/mL of 4',6-diamino-2-phenylindole (DAPI) (Sigma-Aldrich Quimica, Sintra, Portugal) for 5 min and preparations were stored for up to one week in the dark before visualization. No significant decay of fluorescence was noticed during this time span. Cells were visualized under an epifluorescence microscope (Carl Zeiss, Germany) equipped with a filter block sensitive to DAPI fluorescence. A total of at least 10 fields (with approx. 30 cells each) were counted using an ocular grid and the average was used to calculate total cells (TB) per cm^2.

Scanning Electron Microscopy *(SEM)* Analysis

Coupons from the flow cells were immersed for 15 minutes in solutions with increasing concentrations of ethanol up to 100% (v/v), and placed in a sealed desiccator. The coupons were mounted on aluminium stubs with carbon tape, sputter coated with gold and observed with a Leica Cambridge S-360 SEM (Leo, Cambridge, UK).

Detection of *H. pylori* in *Biofilms* of DWDS using PNA *FISH*

The PNA probe and the hybridization procedure have been previously tested and described elsewhere (Azevedo et al., 2003a). In short, the PVC coupons with biofilm attached were immersed in 90% ethanol for 10 minutes for fixation. They were then covered with a hybridization solution containing 200 nM of a fluorophore-labelled *H. pylori*-specific PNA probe for 90 minutes at 63°C. Subsequently, coupons were washed at the same temperature for 30 min and visualized under epifluorescence microscopy. As a control experiment, each time the hybridization procedure was performed one PVC coupon was submitted to the same process, but with no PNA probe added during the hybridization step.

Results

In Figure 2, the cultivable and total cell counts obtained from biofilms for each of the flow cells is shown. Cultivable cell numbers were nearly undetectable until 12

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days of biofilm growth, but then increased until 50 days of operation reaching 6×10^2 CFU/cm^2. However, due to a perturbation in the chlorine of the DWDS that caused the free chlorine in the system to reach 0.8 mg/L, cultivable cell numbers decreased again to approx. 1×10^2 CFU/cm^2, which was similar to the values obtained after 12-25 days of operation. It is not clear whether a plateau had been reached for CFU's after 50 days or if counts were still increasing. Similarly to the cultivable bacteria, total counts only reached high numbers (of 10^5 TB/cm^2) after 12 days. In this case, a peak in the counts was obtained after 32 days, and a pseudo steady-state, where small changes in the total counts were derived from a balance between the sloughing off and attachment of bacteria, appeared to be reached. The addition of chlorine also affected this parameter, and TB decreased to 5×10^5 TB/cm^2 after 50 days. When comparing the results obtained from the two flow cells, significant differences can be easily observed. These differences are more noticeable when comparing CFU values, where for some cases, more than double the number of the cells could be recovered from one flow cell when compared to the other. However, no flow cell presents constantly higher values, implying that these variations are due to the natural heterogeneity of biofilm formation on surfaces. During the whole experiment, water pH values remained in the range of 7-7.6 whereas temperature decreased steadily from 17 °C to 9 °C as the winter arrived.

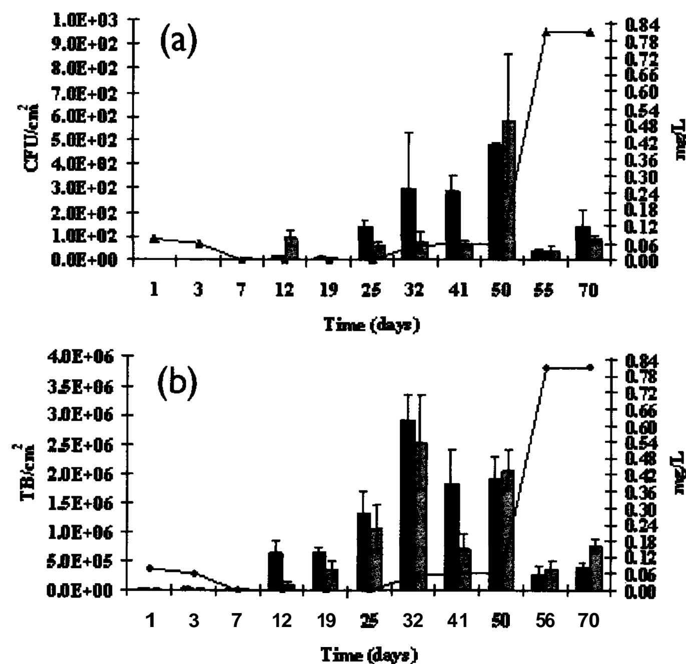

Figure 2. CFU (a) and TB counts (b) of biofilms formed in coupons of the two flow cells (each bar correspond to values from one flow cell) obtained along time. Error bars represent standard deviation. The line corresponds to free chlorine concentration in the water at the time of the sampling.

Because free-living amoebae, which are typical predators found in biofilm ecosystems, were already found to provide conditions favouring the survival of H. *pylori* (Winiecka-Krusnell *et al.*, 2002), it was attempted to detect the presence of protozoa in biofilm coupons using SEM (Figure 3). Even though several different types of eukaryotic microorganisms have been consistently found in samples after more than 25 days exposure, only a small fraction exhibited a similar morphology to the microorganisms of interest Eukaryotes detected appeared to be mostly diatoms (single celled photosynthesizingalgae).

Figure 3. SEM images of mature biofilrn sections showing the presence of different kinds of eukaryotic microorganisms. In the left picture the elongated forms resemble *Nitzschia* spp., a diatom that can be commonly found in freshwater environments. In the right picture, the structure from the bottom left resembles *Cyclotella* spp., also a diatom typically found in freshwater environments, whereas the top right structure is possibly a type of protozoon. These structures were nearly always observed associated with biofilm structures. Bars represent 10 mm.

As reported in a previous paper that detected H. *pylori* in a spiked lab-model biofilm formation system, autofluorescence from both biofilm microorganisms and stacks was observed (Azevedo *et al.*, 2003b). The autofluorescence of biofilm material could obviously be a drawback for the PNA FISH detection, because of an increase in the risk of H. *pylori* misidentification and the possibility of overlooking the fluorescent signal of the probe against a brighter background. Therefore, strategies to eliminate the risk of a misidentification were included. The first invoked the observation of all filter sets available in the epifuorestence microscope: a cell was only considered to be *H. pylori* if it emitted fluorescence in the filters sensitive to the probe and if no signal was detected in the non-sensitive filters. Secondly, it was considered that an unequivocal detection would depend on finding individual cells directly attached to the surface exhibiting typical *H. pylori* morphology. These considerations implied that cells embedded in stacks or fronds, where individual cells are not easily distinguished, were not considered to be H. *pylori* even if the fluorescence emission in the different filter sets was similar to that provided by the probe. Nevertheless, H. *pylori* has been consistently detected directly adhered to the surface in nearly all of the coupons that have been used to perform the hybridization experiment (Figure 4).

Notably, all morphological forms of the pathogen could be observed (spiral, rod and coccal). In the control coupons, however, it was not possible to detect any *H.*

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pylori-positive sample demonstrating that the method provides sensitive and meaningful results.

Originally, it was also intended to correlate the presence of *H. pylori* with the values obtained for the different water parameters. However, the presence of autofluorescing material in the samples prevented an accurate quantification of the pathogen attached to the coupons implying that such correlations could not be confidently established.

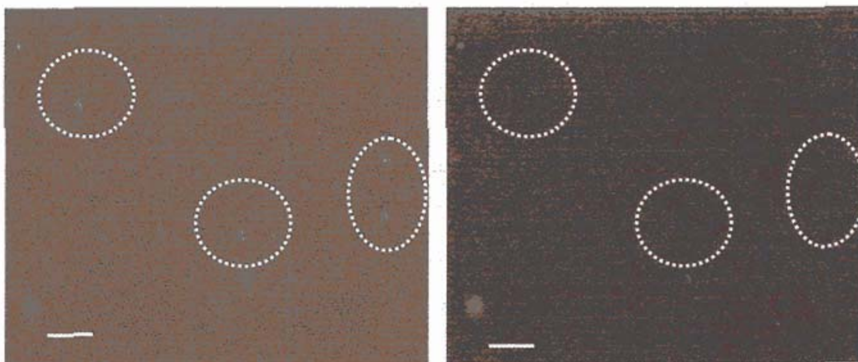

Figure 4. Episcopic fluorescence images of a mature biofilm formed in a PVC coupon that has been exposed to drinking water for 72 days. After hybridization of the coupon with the specific PNA probe, *H. pylori* cells could be identified: Inside the white circles, spiral, rod and cocci-like *H pylori* cells are detected by the Live/Dead filter sensitive to the probe (a); In the same area, the Cy3 filter not sensitive to the probe is not able to pick those same cells (b). ?Bars represent 10 mm.

Discussion

Installing biofilm-forming devices as a by-pass or directly connected to a DWDS has been a commonly used strategy to allow a more efficient monitoring of biofilm formation in real systems (e. g. Hallam et *al.*, 2001, Lehtola et *al.*, 2004). In our case, the flow cell not only allowed easy sampling of the coupons for standard microbiological procedures (such as DAPI and heterotrophic plate count), but also provided an important advantage when performing the hybridization procedure: the fact that the surface where the analysis is made is flat. In fact, our group has previously attempted to detect H. *pylori* in pipes that were part of a DWDS, but the curvature as well as the corrosion exhibited by the pipes prevented a successful detection (unpublished data).

Because of heavy autofluorescence found in certain areas of the biofilm, a correlation between water parameters and *H. pylori* numbers could not be established. However, it has been surprising that detection has been accomplished even after the increase in chlorine in the DWDS. A possible answer is given in a study by Baker and Hegarty (2002). When analyzing the effect of oxidizing disinfectants on H. *pylori*, they concluded that the pathogen would be more resistant than *Escherichia coli* to chlorine.

Besides this work, only Moreno et al. (2003) was capable of detecting H. pylori in non-inoculated water samples using FISH technology. Because sampling was performed in river and wastewater samples, one of the possible reasons this screening might have been successful was the lower amount of autofluorescence-emitting substances when compared to biofilm samples. This study, together with other studies using the PCR technique (Krumbiegel et al., 2004, Park et al., 2001, Rolle-Kampczyk et al., 2004, Watson et al., 2004), appear to indicate that H. pylori can indeed be commonly found in real water systems. It will now be important to determine the preferred conditions for H. pylori attachment to biofilms, so that possible risk zones can be identified.

This work reports the in situ detection of H. pylori in biofilms formed in DWDS. The success of this detection is mainly due to two different factors: the installation of flow cells in a DWDS and the application of PNA probes instead of the standard DNA probes. Further improvement of the method is however needed before coupling it with viability dyes that will give an indication of the pathogen physiology in these systems. Nevertheless, spiral cells that are usually associated with the active form of the bacteria have been found, leading to renewed concerns about the potential for transmission of H. pylori through water.

Acknowledgments

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