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# Detection of *Helicobacter pylori* in biofilms by real-time PCR

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ABSTRACT

*Helicobacter pylori* is a cause of peptic ulcer disease and a causative agent of gastric cancer. Currently, a possible waterborne route of transmission or a possible survival in drinking water biofilms is discussed. *H. pylori*, like many other bacterial strains, has the ability to enter the viable but nonculturable state (vbnc) in case of unfavorable conditions. Therefore it is necessary to develop new analysis tools for vbnc bacteria.

We established a fast and reliable method to detect *H. pylori* in drinking water biofilms by quantitative real-time PCR which makes it redundant to use difficult cultivation methods for nonculturable bacteria. With this method it was possible to identify water biofilms as a niche for *H. pylori*.

The real-time PCR analysis targets the ureA subunit of the *Helicobacter pylori* urea gene which showed high specificity and sensitivity. The quantitative real-time PCR was used to detect *H. pylori* in biofilms of different age, unspiked and spiked with predetermined levels of cells. The drinking water biofilms were generated in a silicone-tube model. The DNA-sequences for probe and primers showed no cross-homologies to other related bacteria and it was possible to detect less than 10 genomic units of *H. pylori*.

This novel method is a useful tool for a fast screening of drinking water biofilms for *H. pylori*. The results suggest that drinking water biofilms may act as a reservoir for *H. pylori* which raises new concerns about the role of biofilms as vectors for pathogens like *Helicobacter pylori*.



## Introduction

*Helicobacter pylori*, a cause of the peptic ulcer disease and of gastric cancer (Ahmed, 2005; Blaser, 2005; Dunn et al., 1997; van Duynhoven and de Jonge, 2001), is being discussed as a possible waterborne organism (Brown, 2000; Bunn et al., 2002; Cellini et al., 2004; Engstrand, 2001; Exner, 2004). Investigations showed that the acquisition of *Helicobacter pylori* and the quality of drinking water are closely related (Ahmed et al., 2007; Nurgalieva et al., 2002; Reavis, 2005). Especially the consumption of untreated well water was found to be one main source for the acquisition of *H. pylori* (Baker and Hegarty, 2001; Horiuchi et al., 2001; Imanishi et al., 2003; Karita et al., 2003; Krumbiegel et al., 2004; Rolle-Kampczyk et al., 2004). Biofilms have been repeatedly reported as possible reservoirs for *H. pylori* in drinking water distribution systems to survive for a prolonged period of time (Azevedo et al., 2003, 2006; Giao et al., 2008; Percival and Thomas, 2009; Watson et al., 2004). The primary mode of transmission remains undetermined although faecal–oral and oral–oral transmission routes are widely accepted (Adams et al., 2003; Blaser, 2005; Brown, 2000; Bunn et al., 2002; Castillo-Rojas et al., 2004; Cellini et al., 2004; Dunn et al., 1997; van Duynhoven and de Jonge, 2001; Engstrand, 2001; Herbarth et al., 2001; Malfertheiner et al., 2004). *H. pylori* enters the viable but nonculturable state (vbnc) at unfavorable conditions (Adams et al., 2003; Cole et al., 1997) and has therefore only rarely been cultured from water-samples and biofilms (Bunn et al., 2002; Castillo-Rojas et al., 2004).

The hygienic aspects of biofilms have gained importance since it had become widely accepted that they are natural habitats for the majority of microorganisms (Donlan and Costerton, 2002; Flemming and Wingender, 2001). Especially in health-care facilities the control of watersupplies and the surveillance and treatment of plumbing systems posed a new challenge for the prevention of nosocomial infections (Anaissie et al., 2002; Kelley et al., 2004; Exner et al., 2005, 2007).

*H. pylori* forms biofilms and like many other (opportunistic) pathogens and biofilm-associated bacteria they cannot be detected by conventional drinking water analysis methods like cultivation on culture medium for several reasons (Cole et al., 1997; Owen et al., 2006). Additionally, the main focus of the conventional drinking water analysis lies on coliform bacteria but it is supposed that there exists no correlation between the presence of coliform bacteria and *H. pylori* (Exner et al., 2005; Exner and Kistemann, 2000). As a consequence of the vbnc state, *H. pylori* cannot be easily cultivated and it gives the organism the ability to survive in water distribution sys-

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**Table 1**
Excerpt from the water quality analysis for Bonn (SWB, 2008).

| Parameter | Unit | Threshold | Measured value |
|---|---|---|---|
| Temperature | °C | – | 8.4 ± 1.1 |
| Elec. conductivity | mS/m | 250 | 25 ± 5 |
| pH value (at 10 °C) | | ≥6.5 to ≥9.5 | 8.3 ± 0.2 |
| Total organic carbon | mg/l | No anormal changes | 0.9 ± 0.2 |
| UV-extinction | m-1 | No anormal changes | 1.6 ± 0.4 |
| Anion | | | |
| Borat | mg/l | 1 | 0.03 ± 0.01 |
| Bromate | mg/l | 0.01 | <0.0005 |
| Bromide | mg/l | – | <0.2 |
| Chloride | mg/l | 250 | 19 ± 2 |
| Fluoride | mg/l | 1.5 | <0.l |
| Nitrate | mg/l | 50 | 15 ± 3 |
| Nitrite | mg/l | 0.5 | <0.01 |
| Phosphate | mg/l | – | <0.01 |
| Sulfate | mg/l | 240 | 27 ± 2 |
| Silicate | mg/l | – | 3.0 ± 0.8 |
| Acid capacity | mmol/l | – | 1.0 ± 0.3 |
| Cation | | | |
| Ammonium | mg/l | 0.5 | <0.02 |
| Sodium | mg/l | 200 | 103 ± 3.0 |
| Potassium | mg/l | – | 2.5 ± 0.5 |
| Calcium | mg/l | – | 26.9 ± 5.0 |
| Magnesium | mg/l | – | 5.5 ± 0.7 |
| Carbonate hardness | °dH | – | 2.7 ± 0.1 |
| Total hardness | mmol/1 | – | 0.89 ± 0.16 |
| Organic trace-elements | | | |
| Tri-haloaenmethane | mg/l | 0.05 | <0.001 |
| Tri- and tetraclilorme-ethene | mg/l | 0.01 | <0.0002 |
| Benzopyrene | mg/l | 0.000 | <0.000005 |
| Polycyclic aromatics | mg/l | 0.000 | <0.000025 |
| Cyanide | mg/l | 0.05 | <0.005 |
| Bacterial parameter | | | |
| Colony count 20 °C | /1 ml | 100 | <1 to < 10 |
| Colony count 36 °C | /1 ml | 100 | <1 to < 10 |
| Colifonne bacteria | /100 ml | n.u. | n.n. |
| *Escherichia coli* | /100 ml | n.n. | n.n. |
| Clostridium | /100 ml | n.n. | n.n. |
| Euterococcus | /100 ml | n.n. | n.n. |
| Faecal-streptococcus | /100 ml | – | n.n. |
| Leaionella | /100 ml | – | n.n. |

tems for longer periods of time (Moreno et al., 2007) Therefore it seems necessary to develop new strategies for the prevention and control of pathogens like *Helicobacter pylori* in drinking water.

The aim of this study was to establish an exact and reliable detection- and quantification-method for *H. pylori* by real-time PCR especially for biofilms. The method was established for untreated water and freshwater-samples and adjusted for the detection of *H. pylori* within biofilms. Additionally the influence of the biofilm on the sensitivity of the real-time PCR and the resulting loss of detectable cells was investigated.

The investigations were carried out to corroborate the presumption that drinking water biofilms may serve as a reservoir for *H. pylori*. The results may then be used as a basis for a risk assessment concerning the possible acquisition of *H. pylori* via drinking water.

## Materials and methods

### *Biofilm growth (autochthonous water flora)*

The biofilms were generated in a silicone-tube model at the Institute for Hygiene and Public Health, University of Bonn (Gebel et al., 2009). The model simulates a drinking water distribution system in which drinking water from a reservoir of surface water (a barrage near Bonn) is flowing continuously through silicone tubes with a diameter of 4 mm und a wall thickness of 1 mm. In order to avoid a growth of algae the system is kept in the dark. In less than 50 days a stable biofilm with more than $10^6$ CFU/cm$^2$ is formed by natural water bacteria. The biofilms used in this assay had an age of 10 and 30 months, respectively. The drinking water analysis was done by the "Wahnbachtalsperrenverband" and the Institute for Hygiene and Public Health Bonn (Table 1).

### *Bacterial strains, growth conditions and cell preparation*

The bacterial strain of *H. pylori* (DSM 4867) used in this work was received from the DSMZ Braunschweig, Germany and several clinical strains were received from a multicentre trial (Reischl et al., 2005), that means that only the species of these strains was determined but without further specifications except for the cell concentration. *H. pylori* was grown on Columbia Agar with 5% sheep blood and 1.5 ml brain–heart infusion broth under microaerophilic conditions by using a CampyGen™-Pak (Oxoid). The cultures were grown at a temperature of 37 °C for 3–4 days.

The colonies were harvested from the plates and suspended in Trypton–NaCl (0.9%). After washing the suspension 3 times by centrifuging (12,000 rpm for 2 min) and re-suspending with WSH (A. dest; solution A: 19.84 g $MgCl_2$/l, 46.24 g $CaCl_2$/l; solution B: 35.02 g $NaHCO_3$/l) the optical density was adjusted to 0.130 at 620 nm in a Lambda2 UV/VIS Spectrophotometer (Perkin-Elmer, Massachusetts, USA).

### *Cultivation and biofilm formation in a closed circuit*

In order to visualize the ability of *H. pylori* to generate monoculture biofilms a closed circulation system was developed. The