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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Biofilm and *Helicobacter pylori*: From environment to human host

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Abstract

Helicobacter pylori (*H. pylori*) is a Gram negative pathogen that selectively colonizes the human gastric epithelium. Over 50% of the world population is infected with *H. pylori* reaching up to 90% of infected individuals in developing countries. Nonetheless the increased impact upon public health care, its reservoir and the transmission pathway of the species has not been clearly established yet. Molecular studies allowed the detection of *H. pylori* in various aquatic environments, even forming biofilm in tap water distribution systems in several countries, suggesting a role of water as a possible reservoir of the pathogen. The persistence of human infection with *H. pylori* and the resistance of clinical isolates to commonly used antibiotics in eradication therapy have been related to the genetic variability of the species and its ability to develop biofilm, demonstrated both *in vivo* and *in vitro* experiments. Thus, during the last years, experimental work with this pathogen has been focused in the search for biofilm inhibitors and biofilm destabilizing agents. However, only two anti- *H. pylori* biofilm disrupting agents have been successfully used: Curcumin - a natural dye - and N-acetyl cysteine - a mucolytic agent used in respiratory diseases. The main goal of this review was to discuss the evidences available in the literature supporting the ability of *H. pylori* to form biofilm upon various surfaces in aquatic environments, both *in vivo* and *in vitro*. The results published and our own observations suggest that the ability of *H. pylori* to form biofilm may be important for surviving under stress conditions or in the spread of the infection among humans, mainly through natural water sources and water distribution systems.

Key words: *Helicobacter pylori*; Biofilm, Water; Infection

Core tip: This review deals with the ability of *Helicobacter pylori* (*H. pylori*) to form biofilm, and the role of the biofilm as reservoir for *H. pylori* infection. The ability of *H. pylori* to grow and form biofilm *in vitro* and *in vivo* could be advantageous for the species to successfully avoid injuries due to chemical stressors - such as antimicrobial therapy *in vivo* - or stress induced by nutrient deprivation. Therefore, the ability of *H. pylori* to form biofilm should be kept in mind when epidemiological strategies are planned to prevent the spread of this ubiquitous pathogen and for treatment of human infection.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram negative pathogen

that selectively colonizes the human gastric epithelium. This species is defined as a urease, catalase and oxidase positive spiraled microaerophilic bacterium, mobile by means of 3-5 polar flagella. Although labile at pH 3.0, *H. pylori* is able to survive for decades in the highly acidic gastric pH by metabolizing urea though its urease enzyme, providing a protective neutral pH in the surrounding of the bacterial cell[1,2].

Over 50% of the world population is infected with *H. pylori* showing a prevalence of 90% in some developing countries where individuals are mainly asymptomatic. In these countries, the pathogen is acquired in the early infancy and its prevalence is higher among individuals belonging to low socioeconomic status groups[2]. The persistent infection with this pathogen is associated to the majority of the gastric pathologies, such as chronic gastritis, MALT Lymphoma, duodenal ulcer, gastric ulcer, and it shows a direct relationship with gastric adenocarcinoma development[3,4]. Nonetheless the wide variety of associated pathologies, only a reduced group of infected individuals develops a clinical severe disease which could be the consequence of environmental factors together with bacterial virulence factors and genetic susceptibility of the host.

H. pylori infected individuals remain colonized by this species during life due to the increased flexibility of the pathogen[5]. The human persistent infection with *H. pylori* and the antibiotic resistance showed by clinical isolates have been related to genetic variability of the species but also to its ability for developing biofilm as it has been demonstrated *in vivo*[6-8] and *in vitro*[9-13]. Thus, various studies have been done searching for biofilm inhibitors and biofilm destabilizing agents[14,15], to decrease recurrent *H. pylori* infections.

PATHOGENESIS AND EPIDEMIOLOGY OF *H. PYLORI* INFECTION

Gastric colonization by *H. pylori* induces superficial chronic gastritis in all infected individuals but few of them develop clinical symptoms[3]. Among the pathologies associated to the persistent infection with the pathogen, gastric adenocarcinoma was a matter of controversy for several years. However, large population studies clearly showed that infection with *H. pylori* significantly increases the risk for developing gastric cancer[16].

H. pylori needs at least four main cellular components to successfully colonize and establish an infection in the gastric mucosa: Urease enzyme, flagella, spiral morphology and adhesins. Additionally, although it is known that the species is able to grow under anaerobic condition[17], it is not clear whether its microaerophilic nature is also a requirement for mucosal colonization. Gastric acid has a pH < 4, which is deleterious for the majority of the bacterial species reaching the stomach by means of drinking water or foods. Thus, successful gastric mucosal colonization by *H. pylori* relies in its ability to convert urea into ammonium, by urease activity, rendering the pH surrounding the bacterial cell neutral, and the 3 to 5 polar flagella together with its spiral form that allows *H. pylori* to leave from the mucosal layer reaching the neutral environment of the gastric epithelial cells[17,18]. While reaching the target epithelial cells, *H. pylori* cells become bound to them through specific adhesion molecules.

Early infection by *H. pylori* and the persistence of this infection are related to an increased risk for developing peptic ulcer disease and gastric cancer[2]. In developing countries, a large number of adult asymptomatic population is usually persistently infected with the pathogen. This increased prevalence has been correlated with gastric cancer development[19]. This disease is considered the second cause of death among individuals died of cancer in the world.

WATER AS *H. PYLORI* RESERVOIR

In spite of the large number of researchs dealing with public health impact, the pathway of transmission and the reservoirs of this species have not been yet elucidated. *H. pylori* has been isolated from the gastrointestinal tract, including saliva and stools, suggesting that oral-oral and fecal-oral routes are the main transmission pathway[20]. However, molecular analyses show that *H. pylori* is also present in various aquatic environments suggesting that human fecal contaminated water sources could be a plausible reservoir of the pathogen[20]. In addition, zoonotic transmission by dogs, cats, sheep and flies as well as iatrogenic transmission by endoscopic procedures[20] have been proposed.

The species has been detected in several water sources, including lakes, rivers, tap water, well water, irrigation water and sea water (Table 1), but also has been detected in water distribution systems. Thus, drinking water could be the pathwayfor returning to humans, even though food and occasionally recreational waters may also participate in the *H. pylori* transmission cycle[21].

H. pylori remains viable after seven days in seawater, 16 d in saline and 11-14 d in distilled water, but this survival property is strongly affected by temperature[22,23]. Nonetheless, one of the main problems to accept that water could be a possible reservoir of *H. pylori* is the inability to routinely isolate the species from water samples in conventional microbiological culture techniques[24-29]. Nowadays, molecular methods, like polymerase chain reaction (PCR), are the most popular to search for *H. pylori* in water, although an intrinsic limitation of these techniques is to accurately differentiate live and dead cells[24,28-37]. However, new analytical methods such as *in situ* fluorescent hybridization (FISH) made it possible to successfully detect *H. pylori* in water distribution systems and various water sources[27,35,38,39] because the techniques detect rRNA, which is indicative not only of bacterial presence but also of viability due to the increased cellular rRNA content[40].

One of the difficulties for isolating *H. pylori* in culture is the ability of the species to change from a spiral

Table 1 Detection of *Helicobacter pylori* in acquatic environments

Methods	Water source	Ref.
Immunofluorescence	Surface and ground water	[60]
Bacteriological culture	Sea water and plankton	[28]
	Wastewater	[24,27]
	Tap water	[26,29]
	Contaminated wells water	[25]
Polymerase chain reaction	Tap water	[29-31]
	Sea water and plankton	[28,32,33]
	River and lake water	[34-36]
	Wastewater	[24,36,37]
Fluorescent hybridization	Tap water and ground water	[38]
	Wastewater	[27,35]
	Sea water	[39]
	Irrigation water	[39]
	River water	[35]

cultivable form to coccoid cells, which is described as a viable but non cultivable state (VBNC). This change is triggered by stressing conditions, like hyperosmolarity or nutritional deprivation[41], suggesting an adaptive mechanism of the species for remaining viable and infective for long periods[42,43]. This state also allows the cells to evade the chloride treatment of water used for human consumption, remaining infective but undetectable by conventional microbiological culture methods[44].

During the last decade, biofilm formation in water environments has been proposed as a strategy of *H. pylori* for surviving in the environment, and has called the attention of water as a reservoir of the pathogen. The first experimental evidence showing the ability of *H. pylori* to grow in biofilm arose in 1999, from the work of Mackay *et al*[45]. The authors found the species in the surface of tap water distribution systems. In 2001, Park *et al*[30] also detected *H. pylori* in the distribution system of tap water in Sweden by molecular analysis, even though the amplified DNA fragments were not sequenced to confirm the detection of the pathogen in the samples. A year later, another study informed the detection of 16S rRNA genes from *H. pylori* in biofilm samples obtained from water distribution system at western Africa[46]. In 2004, the species was detected by Watson *et al*[31] in biofilm and water samples from 11 England residential houses (kitchen, toilet and shower), seven educational establishments and hydrants. Among 151 samples analyzed, the genus was detected by DNA in 26% of the samples (39/151 samples) and in 15% of the samples (23/151 samples) the microorganism was identified at species level. Its presence was found mainly in biofilm samples from showers, suggesting a role of the tap water distribution systems as reservoir of *H. pylori*. Three years later, Braganra *et al*[38] detected, by FISH, spiral *H. pylori* cells (infectious form) in biofilm from water distribution systems in the United Kingdom suggesting that *H. pylori* would be resistant to chloride treatment.

Recently, the coexistence of *H. pylori* or *Legionella pneumophila* in biofilm together with other bacterial species including *V. paradoxus*, *M. chelonae*, *Acidovorax* sp., *Sphingomonas* sp. and *Brevundimonas* sp. has been analyzed[47]. *H. pylori* can be recovered in conventional microbiological cultures before 24 h if the biofilm is formed by only one species (*V. paradoxus*, *Acidovorax* sp. or *Brevundimonas* sp.), but remains cultivable at least 24 h if the biofilm is made by *M. chelonae*, a pathogen commonly found in tap water. This observation suggests that *M. chelonae* may play a role in supporting the incorporation of *H. pylori* to biofilm formed in tap water distribution systems.

BIOFILM: AN OVERVIEW

Bacterial species in natural environments usually live in communities of microorganisms on the surface of different materials, embedded by a self-produced matrix, usually of polysaccharide nature, that allows bacteria to adapt and survive under stress conditions. These structures are called biofilm[48]. The biofilm develops in several steps: (1) conditioning; (2) adhesion; (3) extracellular matrix synthesis; (4) maturation; and (5) dispersion. The processes render a uniform structure of cells deposit surrounded by a matrix that leaves open channels where water can diffuse freely. The matrix is composed by a polymeric substance, including polysaccharide molecules, DNA, proteins and lipids, which modify the bacterial surface and promote first adhesion of cells to surfaces[49].

It is currently accepted that biofilms are implicated in over 80% of the chronic infections caused by bacteria, including middle ear otitis, endocarditis, urinary tract infections and lung infections of patients with cystic fibrosis[50]. The importance of biofilm in medicine is due to its role in persistence of the infection because biofilm is not removed and bacteria in biofilms are 1000 more resistant to antibiotics and host defenses as compared to those free living bacteria[51]. The mechanisms involved in antibiotic resistance are: (1) delay in antibiotic diffusion, allowing the expression of resistance genes; (2) chemical charge of molecules like aminoglycoside antibiotics which show an altered diffusion throughout the negatively charged exopolysaccharide matrix; (3) presence of antibiotic hydrolyzing enzymes (such as β-lactamases); (4) lost of effectiveness of some antibiotics that need active growing cells to exert their function, because bacterial growth ratio is decreased in biofilm[51,52]; and (5) presence of reactive oxygen species as consequence of the oxidative burst by phagocytic cells that increase bacterial antibiotic resistance throughout the increase of mutagenicity in this microorganism by inducing its DNA-break repair systems[52]. In addition, recent experimental evidences suggest that biofilm is a virulence factor in a bacterial community, because the bacterial cells residing in the biofilm may acquire new virulence attributes that free living bacteria do not possess[53].

H. pylori biofilm formation *in vitro*

Like many of the bacteria investigated, both *in vitro* and *in vivo* experiments have shown that *H. pylori* may have living periods of biofilm forming cells. Roughly, 15 years

after *H. pylori* was successfully grown in culture[54], the first evidence of biofilm formation by this species arose. The biofilm obtained by growing the strain *H. pylori* ATCC 43504 in a chemically defined medium (Brucella broth supplemented with 0.1% β-cyclodextrin) was insoluble in water, and adhered to the glass in the interface glass-water[11]. Five years later, Cole *et al*[10] studied the ability of 19 *H. pylori* clinical isolates and reference strains in Brain Heart Infusion broth supplemented with 0.1% β-cyclodextrin. Both strains were able to form biofilm and the biofilm produced had a similar progression when compared to biofilm formed by other bacterial species[55]. The steps observed were initial binding, expansion to form microcolonies and growth in three dimensions with the presence of water channels for nutrients distribution.

On the other hand, Cole *et al*[10] also analyzed the effect of specific mutations of genes *luxS* and the type Ⅳ secretion gen *cagE* upon biofilm formation by *H. pylori*, detecting that both mutants were surprisingly twice more efficient in biofilm formation than the isogenic wild type parental strain.

The adherence of *H. pylori* to gastric mucosa is a key step in establishing positive interaction with host gastric epithelial cells. This adherence is mediated by BabA adhesin, which binds to Lewis b antigen and facilitates *H. pylori* colonization of human gastric epithelium[56]. On the other hand, gastric epithelial cells are protected by a mucous layer composed mainly by MUC5AC which contains a domain rich in glycans (including the Lewis b antigen), the target for *H. pylori* BabA adhesin. In opposite to MUC5AC, the MUC6 mucin synthesized in the deep mucosa and secreted by glandular mucosal cells works as a natural antibiotic by inhibiting *H. pylori* growth, avoiding the colonization of the deep gastric mucosa layer by the pathogen[56].

Because several papers indicate that mucin avoids adhesion of *H. pylori* to gastric epithelial cells[57], in order to understand what happens *in vivo*, Cole *et al*[10] studied the effect of mucin on the formation of biofilm by *H. pylori*,. It was observed that increasing concentrations of mucin favors significantly the planctonic growth of *H. pylori* over biofilm formation suggesting that this species lives primarily in biofilm but rapidly proliferates as free living bacteria after been in contact with the mucin in the human stomach. Nevertheless, it has been shown that biofilm formation *in vivo* also occurs[6-8].

A study carried out in 2009 with reference strains and clinical isolates showed that all the strains were able to form biofilm in the interface air-water of a cover slip[12]. The increased ability to form biofilm observed in one strain -*H. pylori* TK1402 - isolated from a Japanese patient with gastric and duodenal peptic ulcer disease called the attention of specialists. Scanning electron microscopy analysis showed the presence of outer membrane vesicles produced by this strain only in biofilm, suggesting that these vesicles might play a role in biofilm formation. One year later, the authors[13] search for virulence factors in this strain and showed that none of the traditional virulence factors described in the species were related to the ability of *H. pylori* TK1402 to form biofilm. Thus, new evidences are needed to better understand why this strain shows an increased ability to grow forming biofilm.

In vivo evidences of H. pylori biofilm formation

The first evidence of *in vivo* biofilm formation arose in United States during 2006[6]. The authors compared, by scanning electron microscopy, gastric biopsies from urease positive (presence of *H. pylori*) and urease negative (absence of *H. pylori*) patients, detecting the presence of dense mature biofilm in the pathogen positive biopsies while biofilm was absent in the urease negative biopsies, which is indicative that *H. pylori* is able to form biofilm in the human gastric mucosa.

In 2008, in Italy, a study was conducted to understand if the gastric diseases caused by this species are a consequence of its ability to form biofilm. The study was done with gastric biopsies obtained from patients receiving anti-*H. pylori* therapy three months prior to the analysis. The authors search for presence of the bacteria by culture and detection by reverse transcription polymerase chain reaction (RT-PCR) of the genes *glmM* and *luxS*. Only 30% of the samples were positive for *H. pylori* by culture whilst 90% of positivity was detected by RT-PCR suggesting that *H. pylori* could be present in these patients as VBNC coccid form. The analysis of the samples by scanning electron microscopy showed an S shape or spiral forms of the bacterial cells in all the samples that were also positive by conventional microbiological culture, with several coccid cells embedded in an extracellular matrix. On the other hand, the same study done with those positive samples only by RT-PCR showed predominance of coccid cells. In addition, positive samples for gene *glmM* were also positives for the Quorum Sensing related gene *luxS*, supporting its detection as a confidence marker of biofilm formation in the species. Interestingly, antibiotic susceptibility analysis done to the clinical isolates strains in this study showed that only one strain was resistant to Clarithromycin and none to Amoxicillin (the antibiotics used for eradication therapy) which was suggestive of a biofilm role in the eradication failure.

Anti-biofilm agents

Because antibacterial susceptibility of a particular strain is favored when the biofilm is destabilized[58], it is believed that a combination of antimicrobial agents and anti-biofilm molecules should be synergistic[59]. However, only two anti- biofilm compounds have been assayed against *H. pylori*: Curcumin, a natural dye extracted from *Curcuma longa*, and N-acetyl-cysteine, a mucolytic agent with anti-biofilm proved activity against other pathogens[60]. Curcumin acts upon biofilm formation in a doses dependent way when assayed at sub-inhibitory concentrations, suggesting its utility as coadjuvant to

standard first choice anti-*H. pylori* eradication therapy, especially in recurrent infections associated to biofilm formation.

One year later, N-acetyl-cysteine effect upon the formation of biofilm was analyzed both *in vitro* and *in vivo*[14]. N-acetyl-cysteine was able to avoid biofilm formation and to destabilize the already formed biofilm at concentrations over 10 mg/mL, similar to results observed with other pathogens[60]. On the other hand, *in vivo* studies with two groups of 20 individuals each, one group (treated) received N-acetyl-cysteine for one week before the anti-*H. pylori* first choice treatment while the other group (untreated controls) did not received the destabilizing agent, showed eradication of the infection in 65% of the cases for the N-acetyl-cysteine treated group as detected by the urea breath test. The control group showed only 20% of successful eradication, suggesting that N-acetyl-cysteine act as a biofilm destabilizing agent that favor *in vivo* the activity of antibiotic substances.

PERSPECTIVES

H. pylori biofilm formation and the role of coccid cells found in the environment or among clinical samples extend our frontiers in the understanding of the epidemiological cycle of this pathogen, but new challenges arises dealing with the identification of the molecular mechanisms allowing *H. pylori* to reactivate its metabolism acquiring the active divisionary form immediately after reaching the human stomach mucosa. The role of particular proteins in the regulation of the conversion from coccid to bacillary cells needs to be elucidated. Candidates of rod shape-promoting proteins - *i.e.*, the serendipity finding of YeaZ in *Escherichia coli*[61] - could give new insight evidences into the mechanism of infection of the species.

CONCLUSION

Our understanding of the role of water as reservoir of *H. pylori* infection and the pathway of transmission of the pathogen is still controversial although a fecal-oral route is accepted worldwide. New experimental evidence is needed to improve our knowledge on the survival strategies of *H. pylori* in the environment and how this reservoir contributes to the distribution of the infection among humans.

It has been suggested that biofilm formation is a critical step in bacterial survival in water and other environments. Thus, the ability of *H. pylori* to grow and form biofilm *in vitro* and *in vivo* could be an advantage for the species to successfully avoid injuries due to chemical stressors - such as antimicrobial therapy *in vivo* - or stress induced by nutrient deprivation in the environment. Under these stressing conditions, biofilm formation seems to play a key role for *H. pylori* survival, especially in water - included tap water and distribution systems- because the species may stay as spiral rod (active form) or acquire U form or coccid form (viable but non cultivable form). Thus, the ability of *H. pylori* to form biofilm should be kept in mind when epidemiological strategies are planned to prevent the spread of this ubiquitous human pathogen and treatment in human infection.

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