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Can Drinking Water Serve as a Potential Reservoir of *Helicobacter pylori*? Evidence for Water Contamination by *Helicobacter pylori*

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Additional information is available at the end of the chapter

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1. Introduction

This review was designed to highlight the available evidence that suggests drinking water and possible survival in water as a probable transmission mode for *H. pylori*.

Although the natural niche for *H. pylori* is the human stomach, for widespread infection to occur the organism may need to survive in the external environment [1]. Documented evidence relating to the survival of *H. pylori* outside the gastric niche is extremely limited. However, there are no established culture methods for the detection of viable *H. pylori* in the environment, in particular drinking water supplies, preventing the development of true epidemiological and risk assessments [2]. A number of drinking water studies have identified *H. pylori* in water pre- and post-chlorination [3]. Baker and colleague [4] found that *H. pylori* were more resistant to low levels of free chlorine than *E. coli* or *C. jejuni*. Moreno and colleagues [5] have shown that *H. pylori* could survive disinfection procedures that are normally used in drinking water treatment when bacterium *H. pylori* was found in the viable but not-culturable (VBNC) state. However, they did find that culture of *H. pylori* was lost after 5 min in water despite free chlorine levels of 0.96 mg/2l of water. Children born into high-income families supplied with municipal water are considered 12 times more likely to become colonized with *H. pylori* than those supplied from community wells. This suggests that municipal water is a possible risk factor in the transmission and acquisition of *H. pylori*. It is plausible to suggest that breaks in municipal pipes allow for infiltration of contaminated surface water [6].

2. *Helicobacter pylori* — sources and pathways of transmission

2.1. A Brief history

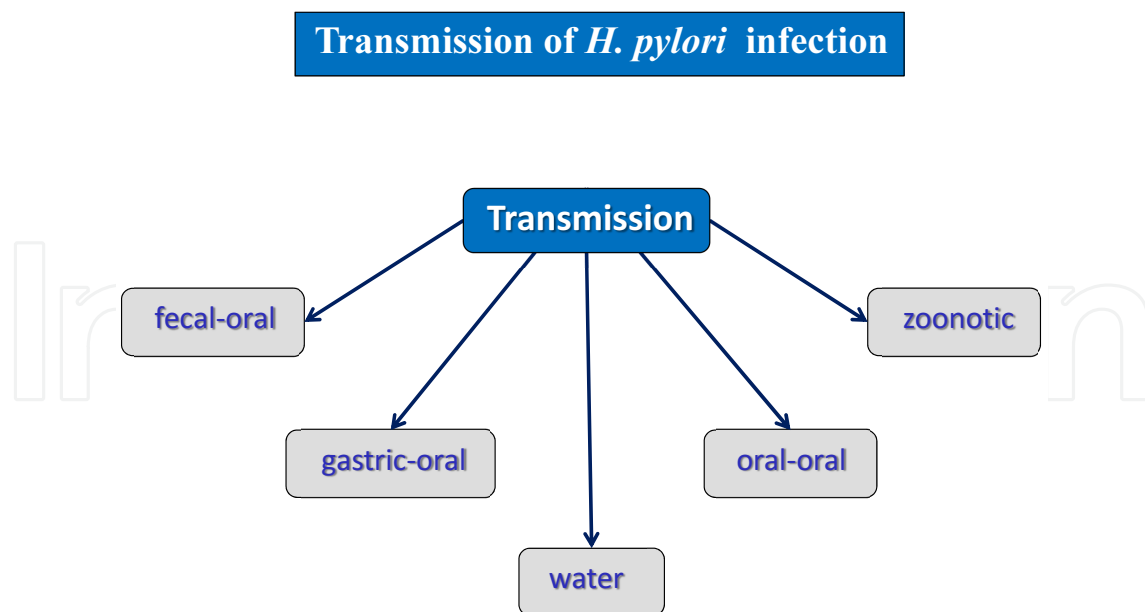
It has been over a century since Walery Jaworski at Cracow University detected a spiral bacteria named *Vibrio rugula*, in the sediment after gastric washing from patients with gastric cancer and over a quarter of century since Marshall and Warren drew attention to the spiral bacteria, *H. pylori* as a pathogen in various gastric diseases. The Nobel Prize was awarded to Marshall and Warren in 2005 for the discovery of *H. pylori* within the gastric mucosa and its role in gastritis and peptic ulcer disease [7].

H. pylori infecting almost half of the world's population, has become the cause of one of the most common infections. It is a pathogen of constant interest among researchers still looking for the answers to many questions. One of them is the question: "How are *H. pylori* transmitted and what is its source?"

The answer to this question hangs within the realm of conjecture, because - so far - no dominant route of infection by *H. pylori* has been defined. The following routes of transmission are currently taken into consideration: oral - oral transmission, fecal - oral transmission, iatrogenic transmission and vector-borne transmission (Fig. 1). The most likely way in which the *H. pylori* infection is spread has been found to be by passing from person to person by oral- oral or fecal - oral transmission [8, 9]. All the scientists involved in research on the etiology of *H. pylori* infection are unanimous about the fact that this bacterium has to get invaded into the stomach through the mouth - this is a non-invasive micro-organism, and therefore cannot colonize the gastric mucosa in any other way.

2.2. Oral-oral transmission

Data for the confirmation of oral - oral transmission is based on various observations that are related to, among others, research on infection within the family. Research showed that if one person in the family was found to be infected with *H. pylori*, the probability of infecting the other family members was significantly higher [10, 11]. By studying the frequency of infected couples, Italian and German epidemiologists have shown the relationship between the prevalence of infection among uninfected spouses and the length of time in which they live with an infected partner [12, 13]. German studies [14, 15] have also demonstrated particularly strong dependence of the cross-infection between mother and child, with emphasis on the key role of the mother in this form of transmission. Evidence for the dominance of the oral- oral and fecal - oral transmissions was also provided by research conducted in closed institutions such as care facilities for the mentally and physically handicapped, nursing homes, preschools/ kindergartens and orphanages. Research conducted by Lambert and others [16], carried out on adult residents of a home for mentally and physically handicapped persons in Australia, reported a higher prevalence rate of *H. pylori* infection in the population of the institution, than in the general population, as well as the relationship between the prevalence of infection and the length of stay in such a care facility. Similar conclusions were reached by Dutch and Japanese researchers [17, 18]. Indeed, a higher degree of *H. pylori* infection was also detected



Lee A et al., 1999, Brown LM et al., 2000 Konturek PC et al. 2009

Figure 1. The different mode of transmission of *H.pylori* in humans.

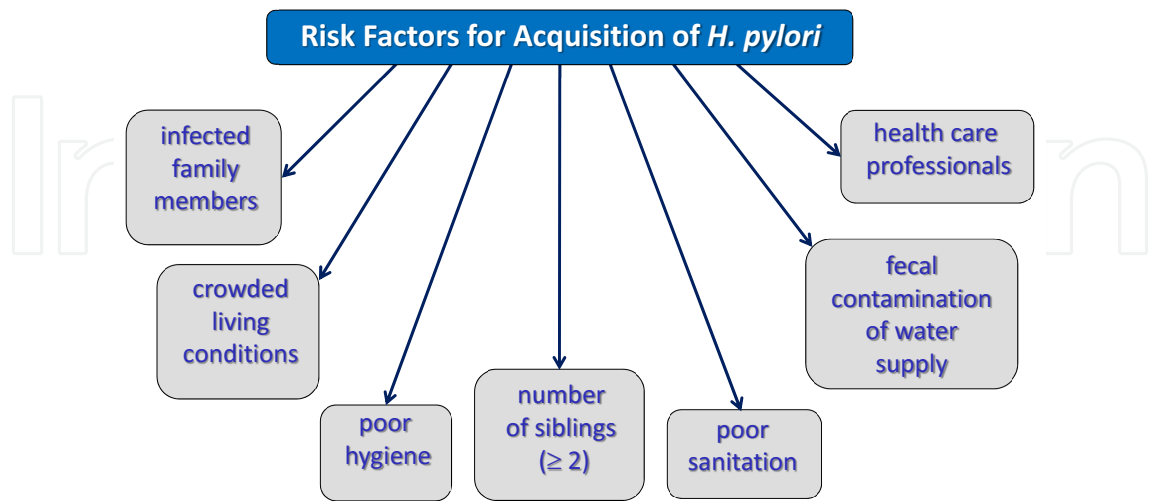
in closed institutions: in England [19, 20], Russia [21], and France [22]. The oral - oral route of transmission is confirmed by the presence of bacteria in the saliva, the dental plate and dental pockets. Evidence that the bacterium *H. pylori* can be transmitted by saliva come from studies conducted by Mègraud's [23], Pytko - Polonczyk et al [24] and Parsonnet et al [25]. Mègraud in the cited work [23] also reported an increased risk of transmission associated with the chewing of food by mothers in West Africa before the feeding it to their children. The transmission of bacteria through vomiting up the stomach contents into the mouth was confirmed in clinical trials by Parsonnet et al [25] who bred *H. pylori* from all samples collected from 16 vomiting seropositive asymptomatic patients.

2.3. Fecal -oral transmission

An important argument for transmission of *H. pylori* by the fecal - oral route was the isolation and growth of the bacteria from human faeces by Thomas et al [26]. Another proponent of the spreading of the *H. pylori* infection by the fecal - oral route is a David Graham, who demonstrated the transmission's similarity to other bacterial gastrointestinal infections transmitted in this way, in particular hepatitis A (HAV) [27].

An important factor in the acquisition of *H. pylori* infection by the fecal - oral route may be the contamination of water with faeces (Fig. 2). The active, spiral form of *H. pylori* can survive in river water for at least a week, and coccoid of the bacteria can survive for a year or more. Klein et al [28] also demonstrated that *H. pylori* can survive in water, and then penetrate into particular parts and organs of the gastrointestinal tract through the contaminated water. They

found that the water resources in Lima (Peru) may be responsible for the bacterium *H. pylori* infection, especially if it is stored in tanks or extracted from urban scenes.



Lambert JR. et al., 1995, Tytgat GN., 1995, Renner H et al., 1998, Plonka M et al., 2006

Figure 2. Major epidemiologic risk factors for acquisition of *H.pylori* in humans.

2.4. Iatrogenic and vector-borne transmission

In view of the widespread use of the gastroscopy as a means for diagnosing the upper gastro-intestinal tract disorders, the iatrogenic transmission of *H. pylori* could be a potential risk factor for infecting patients undergoing colonoscopies. Tytgat [29] proposed that the major cause of this phenomenon is the complex structure of gastroscope and the difficulties involved in its disinfection. It should be noted, however, that the risk of bug infection in this way is negligible. Lin et al [30, 31] believe that the medical staff performing endoscopic examinations are at greater risk of becoming infected with *H. pylori*, as opposed to dentists and dental nurses whose level of risk is negligible. This suggests that infected gastric mucosa may play a more important role in the spread of infection than infected saliva. Although the principal reservoir for *H. pylori* infection seems to be a human being, there may be other reservoirs of *H. pylori*, such as livestock. Handt et al [32] isolated *H. pylori* from cats and implied that this so called “pet transmission” may have public health implications. However, there is no convincing epidemiological data of *H. pylori* infection in cats, which is why the current risk of infection from cats is considered to be rather small. Other animal organisms that were found to be colonized with *H. pylori* are monkeys [33]. The monkey to human mode of transmission of *H. pylori* could be, however, unlikely due to limited human contact with monkeys. However, a much more likely vector in the transmission of *H. pylori* is a housefly. It was Grubel et al [34, 35] who found that houseflies infected with *H.pylori* in the laboratory may be a reservoir of living bacteria, found both in the gut and on the hairs covering the fly's body. The probability that flies can carry *H. pylori* from infected faeces to food or human mucosal surfaces is indirectly confirmed by numerous epidemiological studies conducted in countries with particularly difficult sanitary conditions such as Colombia, Peru and Japan (Fig. 3). However, there is no direct evidence that

the flies which have been in contact, under natural conditions, with faeces infected by *H. pylori* are the source of infection or that this microorganism can be transferred from contaminated flies to food in an amount sufficient enough to infect humans [36]. Goodman et al [37] have found interesting results on the risk of *H. pylori* infection in children from the Colombian Andes, who played with sheep (Fig. 3). A study by Dore et al [38] revealed a significantly higher prevalence of *H. pylori* infection among Sardinian sheep herders, but not among members of their families. Papiez et al [39] and Plonka et al [40] detected an increased risk of *H. pylori* infection in both Polish sheepherders from the Tatra Mountains, as well as entire families in that region. Despite the significance of issue at hand, the epidemiology of the *H. pylori* infection still remains obscure. Studies often present conflicting results, especially when it comes to sources and modes of transmission of infection and associated risk factors. In world literature, authors of various studies emphasize the high volatility of risk, depending on environmental and geographical factors. They also emphasize the lack of a sufficient number of epidemiological studies in the countries of Central and Eastern Europe, including Poland.

Evidence for waterborne *H. pylori* transmission

- ✓ Bacterial survival in laboratory aquatic environments
- ✓ Risk of *H. pylori* transmission from drinking well water in Japan
- ✓ *H. pylori* detection by polymerase chain reaction in water samples from Colombia and Peru
- ✓ Association with drinking water source in Andean countries
- ✓ Association with raw vegetable consumption in Andean countries
- ✓ Detection with swimming in rivers and swimming pools

Hopkins RJ et al., 1994, Goodman KJ et al., 1996, Karita M et al., 2003, Plonka M et al., 2011, Twing KI et al., 2011

Figure 3. Literature evidence that water may serve as a potential source and reservoir of *H. pylori*.

2.5. Water serves as a potential reservoir of *H. pylori*

The breakthrough discovery that water can be a source and route of the *H. pylori* infection resulted in a flurry of research. The fact that water can be a source of *H. pylori* infection was reported in a study, conducted in the Colombian Andes, by Goodman and others [37]. They found that drinking water from streams, bathing and swimming in streams and pools significantly increases the risk of infection in children in Colombia (Fig. 3). Similar conclusions were reached by Klein et al [28] on the basis of the abovementioned studies conducted in Lima. However, Teh et al [41] of Taiwan and Hopkins et al [42] in Chile found no increased risk in

subjects who acquired their drinking water from rivers, and even in those that swam near the polluted beaches in local rivers, irrigation canals or lakes. The problem of *H. pylori* contamination of water resources requires further studies with a proper way of assessment of bacteria in water environment.

3. Methods for the detection of waterborne *H. pylori*

The following methods are used in order to detect the presence of *H. pylori* in water samples taken from a variety of intakes:

1. Culturable methods – that utilize the specialized media bacterial growth, and
2. The methods of molecular assessment of *H. pylori* using the polymerase chain reaction (PCR).

3.1. Culturable methods in the culturing of *H. pylori*

H. pylori is a Gram-negative bacterium, measuring 2 to 4 μm in length and 0.5 to 1 μm in width. Culturing *H. pylori* from areas outside the human stomach has been difficult because of a morphological change in the bacterium and overgrowth by competing microorganisms. *H. pylori* rapidly transforms into a coccoid form which is in a viable but nonculturable (VNBC) state. The VNBC state could be responsible for the difficulty in isolating *H. pylori* from water samples [43]. Coccoid forms are repeatedly observed in several environments, but since it is not known if they represent cell death or a resistant state, their role in the transmission pathway of *H. pylori*, especially by animals and food, is still controversial [44, 45].

We found only a few studies that report successful isolation of *H. pylori* from environmental water samples [46]. In one case, the bacterium was isolated from a municipal wastewater canal on the US-Mexico border, which is compatible with a fecal – oral route of contamination [47]. This canal was found to be heavily contaminated with untreated raw sewage in an area known to have a high *H. pylori* prevalence. The authors used a combination of non standardized immunomagnetically separation (IMS) and culturing techniques. Following separation the bead-*H. pylori* conjugates were streaked onto Columbia blood agar plates and incubated for 3 to 5 days in microaerophilic condition at 37°C, the small, gray colonies were selected and stained with Gram stain to verify morphology. Colonies with Gram-negative rods or coccoid forms were tested by three diagnostic techniques: a rapid urea test, of cytochrome oxidase test and catalase test. In order to fully confirm detection of *H. pylori* one should remember that besides to be positive in all the three mentioned tests, *H. pylori* is a Gram-negative bacterium. A total of 37 out of 132 isolates from culture were selected as putative *H. pylori* and then 23 out of 37 isolates were confirmed to be *H. pylori* by 16S rRNA PCR (Fig. 4).

In the second case, *H. pylori* was isolated from a seawater sample [48]. Colonies were only obtained in residues on 200 μm filters but not in residues on 0.64 and 0.22 μm filters. *H. pylori* could only be isolated from fractionated seawater samples, containing large zooplankton organisms; without zooplankton, *H. pylori* cells could not be recovered from any other fractions

by growth-dependent detection protocols [48, 49]. This suggests that bacterium *H. pylori* requires an organic or proteins matrix to remain culturable. This should be further investigated and other studies attempting to isolate *H. pylori* from water sources using similar approaches are needed.

In another study [50] drinking water samples (n= 600) were collected from ground-drilled water supplied by water and sanitation agencies in different localities within the Lahore metropolitan area, Pakistan, and were used within six hours for culturing of *H. pylori*. Water Samples with isolated bacteria was cultured on modified *H. pylori* medium and detected by biochemical tests. The culture-based method sometimes may lead to false negative results due to contamination of microbial species present in the environment that contain the combination of antibiotic resistant genes. In order to remove this discrepancy the grown bacterial cultures were rechecked on another *H. pylori* agar plate followed by urease and catalase tests and was also checked under a microscope to minimize the probability of mixed microbial growth. It was found that 225 out of 600 drinking water samples (37.5%) were positive for *H. pylori* on the *H. pylori* agar medium. The samples that were positive for *H. pylori* were selected on the basis of the formation of a discrete red color zone around the bacterial colony during incubation in 5% CO₂ at 37°C for 3-5 days. And then PCR analysis showed that 90 out of 225 *H. pylori* positive drinking water samples were confirmed 139 bp gene segment of 16S rRNA *H. pylori* strains from water in the Lahore metropolitan area were studied to find the presence of virulence genes and also to correlate with the global genetic structure and evolution of *H. pylori*. The presence of *H. pylori* in drinking water samples of Lahore metropolitan city was approximately 40%, which is an alarming situation. The presence of a high percentage of *H. pylori* contamination in different water sources may be due to low socioeconomic factors such as a lack of public health education, poverty, overcrowding, poor sanitation and unsafe water supplies (Fig. 2). The results for the presence of *H. pylori* in the test samples are in agreement with the results of other studies [51]. The epidemiological survey also suggests that water is the potential source for the transmission of *H. pylori* infection [52] suggesting that water is one of the vectors required for prevalence and transmission of *H. pylori*.

Survival studies in water samples demonstrate that *H. pylori* can be cultured for a limited period of time in a temperature dependent manner [53, 54]. Elevated temperature results in loss of cultivability [55]. The presence of *H. pylori* associated with biofilm from wells, rivers and water distribution systems has been reported recently [56, 57]. Biofilms are slimy films of bacteria, other microbes and organic materials that cover underwater surfaces, particularly inside plumbing. This makes them rather inaccessible and provides a matrix difficult to be reached by disinfectants. The detachment of biofilms is the principal form of contamination of treated water [58, 59]. Aside from these studies, positive culture of *H. pylori* from drinking water has not been successful, despite efforts to produce a culture-specific media sensitive and selective enough to isolate and grow this organism. A simple plating medium for the detection of *H. pylori* in the environment was investigated independently by Stoodley et al [59], however, the culturable methods employed were unsuccessful in the culturing of *H. pylori*.

3.2. The methods of molecular assessment of *H. pylori*

3.2.1. Fluorescent *in situ* hybridization (FISH)

Fluorescent *in situ* hybridization (FISH) with rRNA oligonucleotide probes has been used for detection and identification of VBNC forms of bacteria [62]. In addition to PCR, FISH was validated as a quick and sensitive method for detection of *H. pylori* in environmental samples [63].

In the United States, actively respiring *H. pylori* from surface and well water has been detected using fluorescent antibody - tetrazolium reduction (FACTC) microscopy [64] and confirmed using species-specific PCR [65]. Sen et al. [66] investigated the development of internal controls for PCR assays by spiking drinking water with 100 cells of *H. pylori* and demonstrated similar cycle thresholds to those of recombinant *Escherichia coli* during chlorine disinfection. In addition to PCR, FISH was validated as a quick and sensitive method for detection of *H. pylori* in environmental samples [63]. These findings suggest the presence of *H. pylori* in the natural environment and a possible waterborne route of transmission.

3.2.2. The determination of gene expression by PCR method in the biological material collected from humans and from water sources

This method allows the identification of DNA which is specific for *H. pylori* in biological material: gastric juice, saliva, feces and as shown, this method could be useful to detect the presence of *H. pylori* in water. The application of this technique to test for *H. pylori* has allowed the cloning and sequencing of important genes involved in colonization and pathogenesis, and for the development of simple patterns to determine a sequence of *H. pylori*-specific genes. It also allows for the detection of the protein CagA and VacA which are encoded by the pathogenic genes of *cagA* and *vacA* found in particular strains of *H. pylori* bacteria that cause serious gastric disorders including chronic gastritis, peptic gastric and duodenal diseases and /or even gastric cancer.

Despite the numerous research findings identifying *H. pylori* in water, it is important to consider the fact that the use of PCR and other molecular methods for the detection of pathogens in environmental samples has limitations. This is principally due to the inability of PCR to differentiate between naked DNA from dead and living cells. Consequently, to scientifically interpret data regarding the epidemiology of *H. pylori*, cultured bacteria from appropriate water sources are necessary (Fig. 4).

H. pylori rapidly transforms into VBNC state which is induced by low nutrient and hyperosmotic conditions [67 - 69]. Such stressed conditions are commonly found in water and the watery environment. Nayak and Rose [70] demonstrated that quantitative polymerase chain reaction (qPCR) could determine *H. pylori* concentrations in water. In this study real time qPCR was shown to be a specific, sensitive and rapid method to quantify *H. pylori* in sewage. Prior to these studies a two-stage *in vitro* method for detection of *H. pylori* in spiked water and fecal samples.

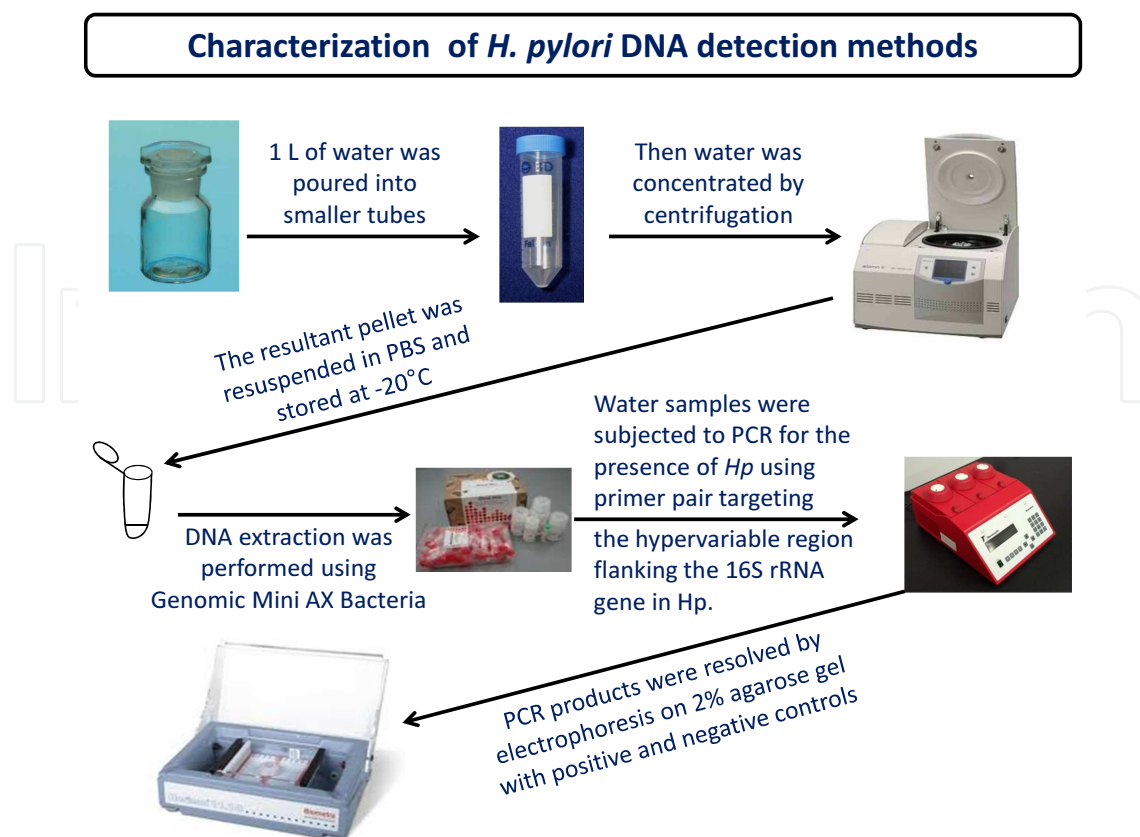


Figure 4. *H. pylori* detection by the assessment of *H. pylori* DNA and 16S rRNA by PCR technique. Proposed steps of detection of *H. pylori* in municipal water.

Janzon et al [71] developed tested and optimized two complementary *H. pylori* specific real-time PCR assays for quantification of *H. pylori* DNA in water. The minimum detection level of the assays including collection procedures and DNA extraction was shown to be approximately 250 *H. pylori* genomes per water sample. They analyzed samples of drinking and environmental water ($n = 75$) and natural water biofilms ($n = 21$) from a high-endemic area in Bangladesh. They could not identify *H. pylori* DNA in any of the samples, even though other pathogenic bacteria have been found previously in the same water samples by using the same methodology. A series of control experiments were performed to ensure that the negative results were not falsely caused by PCR inhibition, nonspecific assays, degradation of template DNA, or low detection sensitivity. Their results suggest that it is unlikely that the predominant transmission route of *H. pylori* in this area is waterborne.

3.2.2.1. *H. pylori* DNA is not detected in environmental and wastewater samples

Since *H. pylori* DNA was not detected in the household waters, the presence of *H. pylori* DNA in different environmental water sources in Dhaka was evaluated. A total of 15 water samples from ponds and lakes and 6 wastewater samples were collected in the larger Dhaka area between November 2005 and March 2006. The sample volumes ranged between 150 ml and 1,000 ml. Also, these samples were all negative for the presence of *H. pylori* DNA.

3.2.2.2. Determination of inhibitors in samples subjected for PCR.

To evaluate the possibility that the absence of *H. pylori* DNA in the water samples was caused by substances inhibiting the PCR process, two *H. pylori* strains, Hel513 and Hel703, were incubated at approximately 10⁷ bacteria per ml in water samples from three drinking water sources and one pond in Dhaka and in PBS as a control and sampled after 1 and 21 days. The detection rates of less than 100% compared to the level for the control incubated in PBS indicated that PCR inhibitors were present in one of the drinking water sources and in the pond water. However, ten-fold dilution of the purified DNA was shown to remove the effect of the PCR inhibitors.

To further determine the presence of inhibitory factors in the field study water and biofilm samples from Dhaka, the real-time PCR analysis was repeated on all field samples but spiked with 1,000 genomes of *H. pylori* strain J99 in each PCR. Detection rates lower than 30% were found in 41% of the drinking water samples. However, 10-fold dilution of the DNA was shown to remove the inhibitory effect in 90% of samples, but still without positive results. These analyses showed that levels of *H. pylori* DNA were below 250 genome copies in 38% of the samples and below at least 2,500 genome copies in another 52% of the samples. Similar results were found in biofilm and environmental water samples. Interestingly, fewer than 10% of the wastewater samples showed presence of inhibitors, presumably because these were extracted using the Qiagen stool kit, which removes PCR inhibitors but decreases the DNA yield.

3.2.2.3. *H. pylori* DNA and RNA stability during long-term incubation in tap water and seawater

Possible degradation of *H. pylori* DNA in water was studied using two different *H. pylori* strains, Hel513 and Hel703. Because of the possible confounding effect of inhibitors in the water in Dhaka, water was taken from Gothenburg, Sweden, for this experiment. Morphology, culturability, *hpaA* and *glmM* gene numbers, and RNA integrity were analyzed at different time points. A majority of the initially spiral-shaped *H. pylori* bacteria converted into the coccoid shape within 24 h, and on day 7, no spiral-shaped bacteria were detected and no viable bacteria were recovered. Real-time PCR assays revealed that the copy numbers of both *H. pylori* genes were constant in tap water for up to 35 days, followed by small decreases at 100 days, whereas copy numbers in seawater decreased up to 50-fold after 100 days. However, gel electrophoresis of total RNA showed that RNA was degraded in tap water within 7 days, with no visible 23S or 16S fragments.

4. Evidence that biofilms in water distribution systems may harbor *H. pylori*

It is well known that waterborne bacteria can attach to surfaces by aggregating in a hydrated exopolymer known as a biofilm [72, 73]. The association of bacteria, particularly pathogens, with biofilm communities within a water distribution system may offer vulnerable and susceptible bacteria protection from disinfection and protozoan predation [74]. In fact

microorganisms in drinking water are predominantly associated with biofilms rather than in the planktonic state [72, 73, 75]. There is evidence that biofilms in water distribution systems may harbor *H. pylori* [76-78]. In addition a study undertaken in Western Africa, utilizing 16S rDNA sequences, has shown evidence that *H. pylori* can be detected in natural biofilms [79]. A more recent study by Watson and colleagues [80] showed a close link between *Helicobacter* DNA in showerhead biofilm used in domestic households.

4.1. Cultivability of *H. pylori* in water and water-associated biofilms and implications for transmission

Adams et al. [81] have shown that in pure culture *H. pylori* cells remain cultivable longer at 15°C than at 20°C, but in their study it was not possible to recover cultivable *H. pylori* from water samples and biofilms. However, considering the shape of the cells detected by PNA-FISH (peptide nucleic acid fluorescence in situ hybridization) and considering that cultivable cells are spiral shaped, while coccoid cells are VBNC and therefore likely to be non-cultivable, the expectation is that there should be more cultivable *H. pylori* cells at 20°C. It is assumed demonstrating that the behavior of this pathogen in heterotrophic biofilms might be completely different than its behavior in pure culture. Additionally, the PNA probe used in this work targets sites on the 16S rRNA molecule, and it is known that the RNA content of a cell can be indicative of viability which suggests that the cells detected were still viable [82]. It has been shown that the concentration of all *H. pylori* cells in the biofilms formed in this work is either higher than or very similar to the concentrations found when pure-culture biofilms were formed [83]. In addition, the detection of *H. pylori* embedded in biofilms suggests that there is a close association with other bacteria present in the biofilms. These two factors, together with the persistence of a bright PNA-FISH signal, which is indicative of a high rRNA content, suggest that the heterotrophic bacteria present in the biofilms formed in this study were not a negative influence on *H. pylori* but only induced its transformation to the more robust coccoid morphology [84-86].

4.2. The existence of *H. pylori* in the drinking water

The evidence based medicine regarding *H. pylori* transmission is not convincing, making it difficult to avoid the notion that the burden/inconvenience accounts for this bacterial infection. The microbiological and epidemiological studies confirmed that in certain conditions the intake of water contaminated with *H. pylori* might be considered as potential source of human infection with this bug. The water could be considered as a reservoir of *H. pylori* [87].

In the U.S. the problem of drinking water pollution is handled by the Environmental Protection Agency (EPA). On the basis of a survey which was carried out, the Agency has developed and issued a list of microbiological contaminants of water, which are likely to significantly affect the public health of consumers of drinking water. The list includes three species of bacteria: *Aeromonas*, *Mycobacterium avium* and *H. pylori*.

The water contamination of bacteria was documented specially in suburban areas because the number of systems for discharging sewage to sewage treatment plants is inadequate. The reason of these bacterial contaminations is not fully understood but some mechanisms were

already proposed. Moreover, many farms and households either have their own small wastewater treatment plants, which do not always function properly, or in worse cases, waste is disposed of by releasing pollutants into nearby rivers, streams or roadside ditches. Some farms have leaky septic tanks often built near wells from which they derive all their household water.

Recent advances in the role of the particular ionic concentrations and the possible correlation between the presence or absence of contamination of water samples tested for *H. pylori* may be an important contribution for tracing the presence of this microorganism in environmental conditions and attempting to define the role of microelements regarding the inhibition or stimulation of *H. pylori* proliferation in water environment.

Determining the concentrations of the suggested ions and the possible correlation between the presence and absence of contamination of water samples tested for *H. pylori* may be an important contribution for tracing the development of this organism in environmental conditions and to attempt to define the role of microelements on the inhibition or stimulation of *H. pylori* proliferation.

4.2.1. Sample preparation

The samples were collected, cooled and stored in polyethylene containers prior to analyses. Before quantitative elemental analysis, if appropriate the samples were filtered and diluted adequately to fit the analytical signal to the linear range of calibration curve.

4.2.2. Quantitative determination of metals

Quantitative determinations of sodium and potassium were made using flame photometry method in air acetylene flame in standard conditions (Perkin Elmer AAS spectrometer Model 3110, USA).

Determinations of Fe, Mg and Zn were performed by means of atomic absorption spectrometry, flame technique (air acetylene flame) using Perkin Elmer AAS spectrometer Model 3110, USA. In both methods, analyses were preceded by thorough optimization of measurement conditions (flame characteristic, burner position, and nebulizer performance). Spectral conditions are given in Table 1.

Element	Method	Lamp type	Wavelength (nm)	Slit (nm)
K	AES		766.5	0.7
Na	AES		589.0	0.2
Fe	F AAS	HCL	248.3	0.2
Mg	F AAS	HCL	285.2	0.7
Zn	F AAS	HCL	213.9	0.7

Table 1. Spectral conditions of elements determination.

All measurements were made using the calibration curve technique. In case of Mg measurements, the samples characterized by high Na concentration were quantified using the method of standard additions calibration. Measurements were made in triplicate.

4.2.3. The methods of molecular assessment of *H. pylori*

We have collected 150 water samples from different municipal water distribution systems (n=49), rivers (n=48), water reservoirs (n=39) and drinking water tanks and wells (n=14) and they were analyzed between June and December 2012. Samples of 1000 ml water were poured into smaller tubes water and were processed to remove organic matter by centrifugation at 121xg for 5 min. The supernatant was concentrated by centrifugation at 7740xg for 15 min. The resultant pellet was dissolve in 1 ml of PBS, followed by centrifugation at 10 000Xg for 5 min. Finally the pellet was stored at -20°C. Total DNA from concentrated samples for PCR was purified using Genomic Mini or Genomic Mini AX Bacteria (A & A Biotechnology, Gdynia, Poland). DNA was stored at -20°C. All PCR amplifications were performed using the GoTaq DNA Polymerase (Promega, WI, USA) in Thermo cycler T3 (Biometra, Gottingen, Germany).

A negative control with sterile water and positive control with *H. pylori* 43504 DNA were included.

PCR with primers specific for *Helicobacter spp.* and *H. pylori* (Table 2) were used to screen samples for potential presence of *H. pylori*.

First, samples were analyzed for *Helicobacter* species with phosphoglucosamine mutase gene and 23S rRNA *Helicobacter spp.* and then were further tested with primer specific for 16S rRNA hyper variable flanking region of *H. pylori*.

For DNA visualization, electrophoresis or PCR products was performed through 2% agarose gel containing ethidium bromide and gels were photographed under UV light. A 100 bp and 50 bp ladder were used as a molecular weight marker.

Primer	Target site	Sequence (5'→3')	Ref*
Glmm-forward	phosphoglucosamine mutase gene	AGG CTT TTA GGG GTG TTA GGG GTT T	[85]
Glmm-reverse		AAG CTT ACT TTC TAA CAC TAA CGC	
Cluster2	16S rRNA hyper variable flanking region of <i>H. pylori</i>	GGC GTT ATC AAC AGA ATG GC	[86]
B1J99		CTC AGT TCG GAT TGT AGG CTG C	
HelGen-forward	23S rRNA <i>Helicobacter spp.</i>	AAC GGG GCT AAG ATA GAC	[87]
HelGen-reverse		TCT CAT CTA CCT GTG TCG	

*References

Table 2. PCR primer used in this study.

5. Conclusions that arise in relation to the presented results

On the basis of a chemometric analysis that involved the use of multidimensional data analysis known as multivariate data analysis it was possible to distinguish water samples containing DNA *H. pylori* and from samples that did not contain the DNA (Fig. 4). At the same time a correlation was demonstrated between the presence of *H. pylori* DNA in water samples and phosphate, ammonia and iron concentrations.

In analyzing the results of water sampling conducted in the framework of the project "Detection of *Helicobacter pylori* in drinking water samples. In what way is the water contaminated and what is the source of contamination?" we have reached the conclusion that we cannot yet say with certainty whether water can be considered as a source of *H. pylori* infection. Questions, therefore, arise to which the answers would be helpful in solving the abovementioned problem.

As the results of our study confirmed the presence of the bacteria in tap water only (i.e., after the process of purification/water treatment), but was not found in rivers, reservoirs and in wells where the water is completely untreated, the first question concerns the existence of a link between the amount of bacteria and the degree and process of the water treatment. The link between the amount of bacteria present in tap water and the distance from the point of chlorination can also be taken into consideration (the farther from the treatment, the smaller the concentration of bactericidal chlorine).

On the basis of these results it can be concluded that the presence of *H. pylori* in water is affected by the season. Confirmation of the presence of *H. pylori* in the water samples taken in June, while there was no evidence of it in October, may also result from the fact that each of the water treatment plants in Krakow uses a number of technological processes for water treatment, depending on the quality of the water collected. Accordingly, further examinations should be conducted to ascertain whether the presence of *H. pylori* is actually related to the time of year, or to other factors.

This raises the question - if the bacteria can survive for so long in distilled water, does this mean that the physiologically important endogenous pool of metals is enough for them?

If so we can expect that the metals in the water around them will be important for their survival? On the other hand, there is no doubt about the effects of toxic metals, but these levels are continuously monitored in water - as is the bacterial cell toxicity of heavy metals. And do the levels of acceptable standards for drinking water have anything to do with this.

6. Further research

1. Determination of *H. pylori* interaction with co-occurring elements in water by enriching their culture media with in the laboratory experiment.

2. Understanding the effects of dissolved organic carbon in the survival of bacteria during water treatment.
3. Taking into account the differences in the composition and concentration studied seasonal elements for the possible coexistence of *H. pylori*.

These relationships can be used to develop a better method of treating water in order to minimize the exposure of humans to infection

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