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The Prevalence of *Helicobacter pylori* in Drinking Drinking Water Samples in Egypt

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ABSTRACT

Background: *Helicobacter pylori* infection is one of the most common infections worldwide. In Egypt, *H. pylori* prevalence has been reported to be 60% among patients greater than 6 years of age. However the environmental risk factors and sources of infection by this organism have not been extensively studied for this region.

Aim: The primary objective of the present study was to determine, for a population in which *H. pylori* is common, the potential environmental sources of infection by both filtration PCR and filtration culture PCR for water samples. Also we aimed to study the *cagA* and *vacA* genotypes of these strains.

Materials and Methods: In this study, fifty tap water samples were tested to investigate the presence of *H. pylori* from rural and urban regions in Alexandria. The detection of *H. pylori* in water was done by PCR using specific primers for urease gene *Ure C* (294 bp). Results: In this study, *H. pylori* *ure C* gene was detected in 4 (8%) of water samples. Two of these cases were detected using Filtration PCR and another two were detected using Filtration culture PCR. All positive samples were negative for *CagA* and *VacA* genes.

Conclusion: The present study confirms the presence of *H. pylori* in drinking water of Alexandria. Detection of *H.pylori* in water by both filtration PCR and filtration culture PCR in different samples confirm the presence of *H.pylori* in different forms in water, viable non culturable and culturable.

INTRODUCTION

H. pylori was first discovered and isolated in 1983 by Warren and Marshall. *H. pylori* infection is well known to be the most common human infection worldwide (Go, 2002). *H. pylorus* is one of the most common causes of chronic bacterial infection in humans, directly related to peptic ulcer and gastric cancer (Yingzhi *et al.*, 2002). The Center for Disease Control and Prevention (CDC) estimates that approximately two-thirds of the world's population harbors the bacterium, with infection rates much higher in developing countries than in developed nations (Sulakshana *et al.*, 2015). In Egypt, The prevalence of *H. pylori* infection among patients greater than 6 years of age is 60% and rising to 90% by adulthood (Katherine and John, 2001).

Detection of *H. pylori* in drinking water strengthens the argument for a possible route of transmission. It has been suggested that *H. pylori* can be acquired through different transmission routes, including water (Yingzhi *et al.*, 2002). In fact, several epidemiological studies have concluded that the drinking water source, or drinking water-related conditions, was a risk factor for *H. pylori* acquisition (Guimarães *et al.*, 2014).

An important stumbling block for *H. pylori* detection is the difficulty of isolating it from environmental samples using traditional culture methods. This is probably a consequence of the transformation of *H. pylori* in water samples from a culturable spiral-shaped form to a non-culturable coccoid one. To overcome this problem, a variety of culture-independent methods have been developed for the analysis of clinical and environmental samples, such as enzyme immunoassays, the urease activity test, and different DNA-based methods, including fluorescence in situ hybridization (FISH) and PCR (Yanez *et al.*, 2009).

Although PCR permits detection of single cells in a sample, environmental samples must be concentrated to detect concentrations of microorganisms of <1 cell/ml. Target genes must not be lost or destroyed during the concentration of cells from the environment, and substances used in the concentration procedure must not interfere with DNA amplification or detection.

In the present study, we used the filtration-PCR assay and Fluoropore filters (FHLF) described by Bej *et al.* (1991). The primary objective of this study was to determine, for a population in which *H. pylori* is common, the potential environmental sources of infection by both filtration PCR and filtration culture PCR for water samples. Also we aimed to study the *cagA* and *vacA* genotypes of these strains.

MATERIALS AND METHODS

Collection of water samples. Samples of 100 ml of tap water were collected from two

different regions of urban and rural areas in Alexandria, Egypt. Samples were collected in glass bottles containing 0.1% (w/v) of sodium thiosulphate to dechlorinate the water.

Filtration of water samples. Filtration was done through Millipore Fluoropore FHLF membranes (Millipore Corp) and a Swinnex filter holder. Each filter was soaked first in ethanol until it became transparent (≤ 1 minute) and then transferred with the laminated surface to the Swinnex filter holder to collect the bacterial cells under vacuum. In order to facilitate the filtration process, prefiltration of dechlorinated water samples was done by using filter with 0.8 μm pore size and 25 mm diameter. For PCR amplification, the water samples were filtered through membrane with 0.5 μm pore size and 13 mm diameter, whereas with 0.5 μm and 25 mm diameter for bacterial culture. After filtration, the filters were folded and transferred with a sterile forceps to 0.5 ml GeneAmp reaction tubes, containing 73 μl sterile distilled water, with the cell-coated surface facing the inside of the tubes, and were stored at -20°C until PCR processing.

Cultivation of *H. pylori*. After filtration, the filters were transferred with a sterile forceps to Dents media (Campygen CN0025, Oxoid Ltd) with the cell-coated surface facing the media and incubated at 37°C for 72 hours under microaerophilic conditions.

DNA extraction from samples for PCR of ure C gene. Boiling method was applied to extract the DNA from culture as described by Englen and Kelley (2000). It was used for bacterial colonies which isolated through Dent's medium. A loopful of bacterial growth was suspended in 300 μl of sterile distilled water in a microcentrifuge tube. The tube was boiled for 10 minutes and immediately cooled in ice for 30 minutes. After that, to precipitate PCR inhibiting particles, the tube was centrifuged at 5,000 rpm for 10 min and the supernatant was used as DNA templates for PCR amplification. Boiling method was also used to extract

crude DNA from water samples as described by Bej *et al.* (1991). After filtration (without removing the filters), the bacterial cells in the tubes were lysed and the DNAs were released by heating at 95° C for 5 minutes. The tubes were then cooled in ice for 30 minutes before the addition of PCR mixture.

PCR amplification for detection of *H. pylori* DNA (Labigne *et al.*, 1991). The PCR was performed using primers for urease gene Ure C (294 bp) 5' – AAG CTT TTA GGG GTG TTA GGG GTT T– 3' and 5' – AAG CTT ACT TTC TAA CAC TAA CGC – 3' indicative of *H. pylori* infection. Amplification was performed in a final volume of 150µl (for filter PCR) and 25µl (for bacterial culture DNA) of PCR mixture containing 0.8 µm of each primer, 10 mM of each deoxy nucleotide triphosphate (dATP,

dGTP, dTTP and dCTP), 10 mM tris HCl, 50 mM KCl, 0.1% triton X– 100, 1.5 mM MgCl₂, and 1 unit of DNA polymerase (Bioline). DNA amplification was applied as follow: denaturation at 94°C for 5 minutes, annealing at 55°C for 2 minutes and extension at 72°C for 2 minutes (for 35 PCR cycles), followed by a final 5 minutes extension at 72°C. Amplifications were performed with a thermal cycler (Genius Techne, England). PCR products were loaded on agarose (2%) in TBE (Tris/Borate/EDTA) buffer containing 0.5µg/ml ethidium bromide (Figure 1). After electrophoresis, the gels were photographed under UV light using black and white Polaroid film. Booster PCR assay was used for PCR negative samples with final volume 25 µl PCR mixture and 3 µl PCR products.

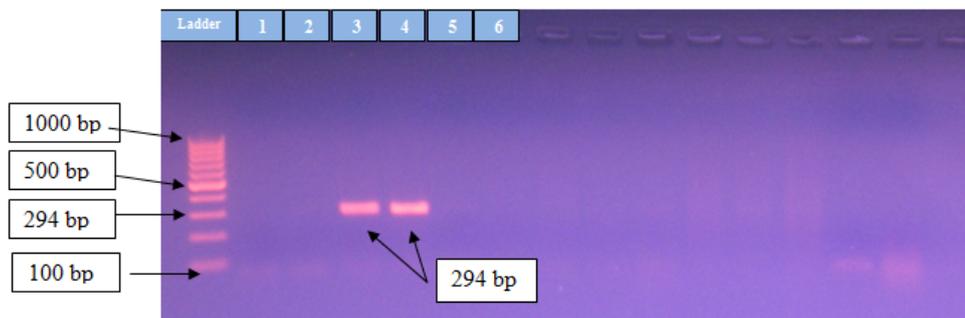


Fig. 1: Agarose gel showing specific bands of *H. pylori* ure C gene PCR products in lane 3 & 4 . Lane M: 100 bp DNA marker.

Multiplex PCR for detection of *H. pylori* genotypes (Atherton *et al.*, 1999; Mukhopadhyay *et al.*, 2002; Chattopadhyay *et al.*, 2004). PCR was performed to detect CagA and VacA (s1/s2, m1/m2) genes using primers shown in Table (1). Amplification was performed in a final volume of 50µl of PCR mixture containing 0.5µM of each primer, 10mM of each deoxynucleotide triphosphate (dATP, dGTP, dTTP and dCTP). Ten mM tris HCl, 50

mM KCl, 0.1% triton X– 100, 1.5 mM MgCl₂, 1 unit of Taq DNA polymerase (Maxima Hot Start Green PCR Master Mix, Thermo Scientific) and 10 µl of template DNA. DNA amplification was carried out under the following general conditions: 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min.

Statistical analysis was conducted by two ways ANOVA analysis.

Table 1: Primers used for multiplex PCR (Labigne *et al.*, 1991; Mukhopadhyay *et al.*, 2002; Chattopadhyay *et al.*, 2004).

Region	Primer Type	Primer sequence	PCR product size (bp)
CagA	cag5c-F	5'-GTTGATAACGCTGTCGCTTC-3'	350
	cag3c-R	5'-GGGTTGTATGATATTTTCCATAA-3'	
VacA s1 /s2	VAI-F	5'-ATG GAA ATA CAA CAA ACA CAC-3'	s1 259
	VAI-R	5'-CTG CTT GAA TGC GCC AAA C-3'	s2 286
VacA m1/m2	VAG-F	5'-CAATCTGTCCAATCAAGCGAG-3'	m1 567

RESULTS

This study was carried out on fifty water samples (tap water). Fifty two percent (52%) of these samples were from rural areas and 48% were from urban regions in

Alexandria. *H. pylori ureC* gene was screened by PCR after Filtration and after culture in all water samples, whereas only positive *H. pylori* samples were subjected to *cagA* and *vacA* genotypes (Table 2).

Table 2: Distribution of *H. pylori* *Ure C* gene detection among water samples by PCR method in relation to regions.

Regions	Samples examined and percentage		No. of positive samples			
	No.	%	No.	%	No.	%
Rural	26	52	1	3.8	1	3.8
Urban	24	48	1	4.2	1	4.2
Total	50	100	2	4	2	4
Test of significance			F = 0		p = 1	

In this study, *ure C* gene of *H. pylori* was detected in 4 (8%) of 50 samples of water. Furthermore, the source of water has no effect on the efficiency of PCR test since the p-value is larger than 0.05.

The efficiency of PCR test (after filtration against after culture) was evaluated using two ways ANOVA analysis. There was no statistical significance different between both ways of applying PCR test in detecting *H. pylori*, since the p-value of PCR test was larger than 0.05.

DISCUSSION

The detection of *H. pylori* in water samples could provide support for a waterborne route of *H. pylori* transmission and infection. In the present study, out of the 50 tested water samples in Alexandria city, *H. pylori* was detected in 4 (8%) of drinking water samples. Other nearly similar detection rates were reported by Khan *et al.* (2012), in Pakistan (Karachi) and by Bahrami *et al.* (2013) in Iran, which were 4%, 10%, respectively.

Higher results were reported by Hulten *et al.* (1998) in Sweden, by Watson *et al.* (2004) in England, by Samra *et al.* (2011) in Pakistan (Lahore) and by Hulten *et al.* (1996) in Peru, which were 12%, 15%, 40% and 50%, respectively. Increased rates of *H. pylori* infection in developing nations, which combat poverty, poor sanitary conditions and overcrowding indicate the role of

environmental factors in the transmission of infection (Khan *et al.*, 2012).

This variation in detection rates of *H. pylori* in drinking water samples presented by different investigators may be explained by several factors including the incidence rate of the bacteria in different environments, hygienic status of the city, low socioeconomic factors such as a lack of public health education, poverty, overcrowding, poor sanitation and unsafe water supplies (Khan *et al.*, 2012; Plonka *et al.*, 2014). Other possible factor that may account for the variation in prevalence rates is the detecting methods used for the etiological agent.

Egypt had the highest prevalence of *H. pylori* in the healthy asymptomatic population both in adults and the pediatric age group. Low socioeconomic status, low body weight and height, living in rural areas and lower educational status were risk factors for the acquisition of *H. pylori* in Egyptian studies (Eshraghian, 2014).

On the other hand failure to identify *H. pylori* in drinking water has been reported by Horiuchi *et al.* (2001) in Japan and by Yanez *et al.* (2009) in Spain. The lack of *H. pylori* detection in drinking water may reflect the fact that *H. pylori* is sensitive to chlorination and should therefore be controlled by disinfection practices normally employed in the treatment of drinking water (Eshraghian, 2014).

In this work, to compare the efficiency of PCR test directly after filtration against after culture, two ways ANOVA analysis was conducted on a sample of 50 observations of tap water. The result showed no significance different between both ways of applying PCR test on discovering *H. pylori* gene, since the p-value of PCR test was larger than 0.05. In addition, there was no effect of the source of water sample, whether it is from rural or urban region, on the efficiency of PCR test since the p-value of the test of significance is larger than 0.05.

It is worth notice that in the present study, the positive *H. pylori* UreC genes of water samples yielded by PCR assay after direct filtration were 2 of 50 (4%), whereas these two samples gave negative results by PCR assay after culture. The *H. pylori* DNA present in water samples could be from dead *H. pylori* cells or from VBNC forms, since culture is usually not possible. Guimarães *et al.* (2014) showed that after being exposed to water for 24 h at 25°C, *H. pylori* was no longer culturable. Studies have reported that when exposed to water, *H. pylori* enter a viable, but non-culturable state as a response to unfavourable environmental conditions, which means that even though *H. pylori* cannot be recovered by plating techniques, bacterial cells might remain viable (Guimarães *et al.*, 2014).

In conclusion, this study provides evidence of the presence of *H. pylori* in drinking water samples in Alexandria, Egypt. The development of PCR assay after filtration and after culture to detect *H. pylori* from water samples may be a significant tool for evaluating the ecological and public health aspects of the transmission of *H. pylori* through the environment.

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ARABIC SUMMARY

معدل إنتشار الحلزونية البوابية في مياه الشرب في مصر

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المقدمة: تعتبر عدوى الحلزونية البوابية من العدوى الأكثر شيوعاً في جميع أنحاء العالم. أفادة التقارير أن في مصر وصل معدل إنتشار الحلزونية البوابية الى 60٪ بين المرضى الذين تزيد أعمارهم عن 6 سنوات. ومع ذلك فإن مصادر العدوى والعوامل البيئية الخطرة لهذا الكائن لم تدرس في هذه المنطقة بشكل واسع.

الهدف من الدراسة: كان الهدف الأساسي من هذه الدراسة هو تحديد، لمجموعة من الحلزونية البوابية الشائعة، المصادر البيئية المحتملة للعدوى عن طريق تقنية تفاعل سلسلة البوليميريز (PCR) لكل من عينات الماء بعد الترشيح (Filtration PCR) وبعد الترشيح والزراعة (Filtration culture PCR). كما تهدف إلى دراسة الزمرة الجينية Cag A و VacA لهذه السلالات.

المواد والطرق: في هذه الدراسة تم اختبار خمسين عينة من مياه الشرب للتحقق من وجود الحلزونية البوابية من مناطق ريفية ومتحضرة في الإسكندرية. تم الكشف عن الحلزونية البوابية في الماء عن طريق تفاعل سلسلة البوليميريز باستخدام بادئات نوعية.

النتائج: في هذه الدراسة تم الكشف عن مورثات الحلزونية البوابية (*urease gene, Ure C*) في 4 (8٪) من عينات الماء. إثنان من هذه الحالات تم إكتشافهم باستخدام تقنية تفاعل سلسلة البوليميريز بعد الترشيح والإثنان الآخران بنفس التقنية بعد الترشيح والزرع. وكانت جميع العينات الإيجابية سلبية فيما يخص المورثات Cag A و VacA.

الخلاصة: تؤكد الدراسة الحالية وجود الحلزونية البوابية في مياه الشرب في الإسكندرية. ولقد أكد وجود الحلزونية البوابية في الماء من خلال تقنية تفاعل سلسلة البوليميريز بعد الترشيح وبعد الترشيح والزرع لعينات مختلفة على وجود الحلزونية البوابية في أشكال متعددة في الماء إما قابلة للزرع على منابت الأجار أو حية غير قابلة للزرع.