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Original Article

Molecular Diagnosis of *Helicobacter pylori* Endosymbiont in *Acanthamoeba*-Positive Samples in Laboratory Conditions and in the Hospital Environments

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Abstract

Background: We aimed to identity *Helicobacter pylori* endosymbiont in *Acanthamoeba*-positive samples in natural and laboratory conditions.

Methods: Overall, 134 samples were collected from hospital environments. Microscopic and PCR test were used for detection of *Acanthamoeba* and *H. pylori*. The real-time PCR method was used to check the active presence of *H. pylori* within *Acanthamoeba* under natural conditions from hospital samples and in co-culture laboratory conditions.

Results: The rate of contamination of hospital samples with *Acanthamoeba* was 44.7%. Out of 42 *Acanthamoeba* PCR-positive samples, 13 isolates (31%) were positive in terms of *H. pylori* endosymbiont according to sampling location. *H. pylori* is able to penetrate and enter the *Acanthamoeba* parasite.

Conclusion: *H. pylori* is able to contaminate *Acanthamoeba* in natural and laboratory conditions. The presence of pathogenic *Acanthamoeba* in various hospital environments and the hiding of *Helicobacter* as an endosymbiont inside it can pose a serious threat to the health of hospitalized patients.

Introduction

Acanthamoeba (Amoebozoa: Centramoebida) is naked amoebae and as a member of the Free Living Amoeba (FLA) group is one of the most abundant protozoa in the environment. It has been isolated from soil, water (fresh & brackish) and air samples all over the world. *Acanthamoeba* is very resistant in harsh environmental conditions (1), extensive life and play the role of main microbial predators in different environments (2). Some species of *Acanthamoeba* can cause granulomatous amoebic encephalitis (GAE), as well as amoebic keratitis (AK). So far, 20 types of *Acanthamoeba* species (T1-T20) have been reported. The T4 genotype is the most dominant type in environmental and clinical cases around the world, it possesses the most pathogenic ability (3). Amoebae usually phagocytose some bacteria as food and these bacteria usually enter the phagolysosome of the parasite, where the ingested bacteria face acidification, oxidative stress, lack of nutrients and small antimicrobial molecules (4). However, some intracellular bacteria use two strategies to survive and exploit cell resources. Some of them, such as *Mycobacterium marinum* and *M. tuberculosis*, can escape from the phagosome into the cytosol. Some other bacteria such as *Legionella pneumophila* remain inside the phagosomal vacuole but change its antimicrobial mechanisms. In this way, they create a safe intracellular environment called the vacuole containing *Legionella*, which allows intracellular reproduction (5). Usually, the place of bacteria in *Acanthamoeba* is ectoplasm or endoplasm (6).

The endosymbiont bacteria that have been reported within this amoeba include *Francilla tularensis, M. avium, Burkholderia spp., Vibrio cholerae, Listeria monocytogenes, Helicobacter pylori and Escherichia coli* (6). Among these, *H. pylori* can accumulate in the human stomach and cause chronic gastritis, duodenal ulcers, gastric adenocarcinoma, and gastric non-Hodgkin's lymphoma (mucosal lymphoid tissue [MALT] lymphomas) (7). It is considered the only bacterium that has been classified as a Class I human carcinogen due to its strong association with gastric cancer and gastric ulcer by the International Agency for Research on Cancer (IARC) (8). In addition, it is reported as a rare complication in patients with unspecified gastric infection (7). This bacterium is one of the most common pathogenic bacteria, with half of the world's population infected with it. Humans are the only important reservoir of *H. pylori*, and transmission is through close personal contact, consumption of contaminated food and water. The presence of *H. pylori* in water samples is a good reason that we should focus on environmental factors to better understand the transmission pattern of this bacterium (9). *Acanthamoeba* also lives in water and soil and its presence along with *H. pylori* has been reported by different researchers.

We aimed to identify the *H. pylori* endosymbiont in *Acanthamoeba* positive samples in the hospital environment.

Materials and Methods

Ethics Approval

This study was approved by the ethics committee of Tarbiat Modares University with code number IR.MODARES.REC.1397.074.

Sampling

This study was a cross-sectional type. Overall, 134 samples from the hospital environment in Markzi Province, Iran including 7 hospitals located in Arak City, one hospital from Farahan City and one hospital from Komijan City were collected (Fig. 1). The environmental samples consisted of water, soil, and dust collected from different clinical units of the hospital. Dust samples were taken with sterile swabs from different surfaces of medical equipment, ventilation system, windows, floors, sinks, surfaces of patient rooms, etc. In addition, soil samples from the hospital yards.

Fig. 1: Geographical location of sampling location (Markzi Province, Central Iran)

Cultivation of Acanthamoeba

Non-nutritive agar culture medium (NNA) dissolved in page saline of 24-hour culture of inactive *E.coli* bacteria for feeding (Xenic culture) was used for the proliferation of *Acanthamoeba* (10, 11).

Soil samples mixed with sterile water and water samples filtered with a vacuum pump and membrane filters were inverted in NNA environment, as well as swabs of dust samples were inoculated in the saline screw in the center of the mentioned non-nutritive environment (1). First they were examined microscopically (12) for observation of cysts, and *Acanthamoeba* positive samples were selected to extract DNA and examine the endosymbiont.

DNA extraction

Parasite DNA was extracted based on protocol of the DNGTM-Plus kit (Cinagen, Iran) and stored at -20 °C until PCR. The DNA of T4 *Acanthamoeba* sp suspension was used as a positive control and sterile distilled water as a negative control (12) and was evaluated qualitatively with 1% gel electrophoresis and quantitatively with a Nano drop device (13).

PCR test

For *H. pylori,* a primer was designed from the vacA gene (14). The designed primer (20bp length and Tm=49.7 °C and for detecting the 319bp fragment) included: forward primer vacA-F(GCTATCGGCAATCAAAGCAT) and reverse primer vacA-R(AGCGGTTAGCCAATTCAAAC). For *Acanthamoeba*, the PCR reaction was performed using the pair of diagnostic primers JPD1 and JPD2 (10).

Parasite contamination in laboratory conditions:

At this stage, the contamination of *Acanthamoeba* with *H. pylori* in laboratory conditions (In vitro) and the possibility of bacteria entering into *Acanthamoeba* was measured by Real Time-PCR method.

Anaerobic cultivation of bacteria

Columbia agar-blood agar was used to culture *H. pylori* (16). Then it was incubated at 37-42 ◦C and after 48-72-96 hours the growth of colonies was checked and microscopic diagnosis was made by warm staining. Then 3-5 colonies were dissolved in 3-5 ml of physiological serum and its turbidity was evaluated with half McFarland concentration (107-108 cfu/ml) and mixed with *Acanthamoeba* (17, 18).

Axenic culture of Acanthamoeba

To investigate the entry of endosymbionts into bacteria-free *Acanthamoebas* in laboratory conditions, the parasite must first be transformed into a trophozoite (19, 20). First, the sample of *Acanthamoeba* genotype T4 (negative PCR bacteria) was cultured and purified by migration or sub-culturing in E. coli-coated NNA medium. To remove any contamination, antibiotics (100 µg/ml penicillin plus 10 µg/ml streptomycin)/antifungal fluconazole (64 µg/ml) were used in NNA medium (21) or the sample washed with 3% HCl three times and then with Page's saline/PBS three times, 10 minutes at 750 rpm and washed again in NNA (22). Some of the cysts treated with 3% HCl in the previous step were transferred with 2-3 ml of the killed *E.coli* bacteria suspension to the NNA culture plate containing 200-500 µl of PYG (17) axenic medium and the antibiotic gentamicin (100 µg/ml) and antifungal fluconazole (64 µg/ml) were used to remove the contamination, and after 2-3 days, the cysts turned to trophozoites and some of the axenic liquid medium (PYG) was added to the culture. By repeatedly changing the culture medium every 24 hours and providing food, the trophozoite reached the logarithmic growth phase. Then add about 3-5 ml of Page's saline to the Petri dish and put it in the freezer for a few minutes and with a small tap and aspiration, the parasite was separated from the medium and slowly collected from the culture medium, then it was centrifuged (1000-3000 rpm, for 10 minutes) and the supernatant was discarded and a suspension was prepared again with Page's saline (18).

Proximity of bacteria and parasites

First, 1ml of *Acanthamoeba* suspension was poured into each well of a sterile 24-well plate and incubated at 37-28 °C for 1 hour until the parasite adheres to the bottom of the well. Unbound amoebae were removed by one wash with PAS. Then, 100-200 ml of bacterial suspension (half of McFarland and one-tenth MOI ratio of *Acanthamoeba* to bacteria) was added to the wells and incubated for 24 hours at 28-30 °C to allow bacteria to enter *Acanthamoeba*. To kill extracellular bacteria, it was first incubated with PAS containing gentamicin 100 µg/ml for one hour at 28-30 °C and washed 3 times with saline without antibiotics (17, 18) and if bacteria were not removed, acid wash fan (3% hydrochloric acid) was used overnight and finally the sample was kept at -20°C until DNA extraction (22). DNA extraction from *Acanthamoeba* co-culture with bacteria was done by DNGTM-Plus kit (Sina Clone).

Real-Time PCR reaction

For *H. pylori,* a primer from the *ureA* gene (23) was designed according to the method of the previous step (length 20bp and Tmf & r=56&54 °C and to detect the 218bp fragment), which includes: forward primer ureA-F(AAAAATGTTGGCGACAGACC) and reverse primer A-R(TTACCGCCAATGTCAATCAA). And for *Acanthamoeba*, the pair of diagnostic primers JPD1 and JPD2 was used (12). The bacterial primers were prepared by Gene Fanavaran (Denmark) and the stock solution was 100 pmol/µl and the working solution was 10 pmol/µl and stored at -20 °C.

Statistical analysis

The data was entered into Excel and SPSS software (version 26) was used for statistical analysis. The relationship between the infection rate of *Acanthamoeba* and *H. pylori* was analyzed by Chi-square, Pearson's correlation coefficient test, and simple linear regression (*P* value >0.05).

Results

Microscopic examination

Out of 134 hospital samples, 112 (83.5%) were dust, 9 (7.6%) were soil and 11 (8%) were water of tank and plumbing samples. Microscopic examination showed that 94/134

(71.14%) of hospital samples were contaminated with *Acanthamoeba*.

Acanthamoeba molecular identification:

PCR reaction was performed on 94 hospital positive microscopic samples, of which 42/94 (44.7%) were found to be positive, and *Acanthamoeba* T4 was the most common genotype.

H. pylori molecular identification:

Out of 42 *Acanthamoeba* PCR positive samples in terms of *H. pylori* endosymbiont, 13 isolates (31%) were positive. Based on statistically analysis, the results of the endosymbiont in *Acanthamoeba* positive samples were significant (P<0.05) in different location (Table 1).

Table 1: Frequency of *H. pylori* from *Acanthamoeba* isolates based on sampling location

Hospitals	*H. pylori*	*Acanthamoeba*	
	Positive No. (%)	Positive No. (%)	Negative No. (%)
Kemijan	2 (3.33)	0	2 (100)
Farahan	1 (12.5)	1 (100)	0
Amirolmomenin Arak	10 (62.5)	5 (50)	5 (50)
Taleghani Arak	7 (38.9)	2 (28.6)	5 (71.4)
Amir kabir Arak	6 (37.5)	0	6 (100)
Khansari Arak	6 (75)	2 (33.3)	4 (66.7)
Valieasr Arak	5 (50)	2 (40)	3 (60)
Ghods Arak	2 (40)	1 (50)	1 (50)
Gerdo Arak	3 (42.8)	0	3 (100)
Total	42 (44.7)	13 (31)	29 (69)

Out of 42 *Acanthamoeba* PCR positive samples in terms of *H. pylori* type and sampling units, 13 isolates (31%) were positive and 29 isolates (69%) were negative. Hospital yard soils and dust from the service department, have the highest simultaneous *Acanthamoeba* and *H. pylori* contamination with 100% and 40%, respectively. In the microscopic and molecular testing of the water samples of the studied hospitals, no contamination with *Acanthamoeba* and *H. pylori* was observed. Based on statistically analysis, the results of the endosymbiont in *Acanthamoeba* positive samples were significant (P<0.05) in different unit (Table 2).

Table 2: *H. pylori* frequency of *Acanthamoeba* isolates according to type and sampling units

Samples		*H. pylori*		*Acanthamoeba*
		Positive No. (%)	Positive No. (%)	Negative No. (%)
Dust from	General wards*	15 (52.6)	3 (20)	12 (80)
	Specialized wards**	20 (45.4)	6 (30)	14 (70)
	Service departments ***	5 (41.6)	2 (40)	3 (60)
	Total	40 (48.2)	11 (27.5)	29 (72.5)
Soil		2 (25)	2 (100)	0 (0)
Water		0 (0)	0 (0)	0 (0)
Total		42 (44.7)	13 (31)	29 (69)

*__General wards:__ Admission, triage area, injection room, laboratory, drug store, hemodialysis, radiology, hematology.

**__Specialized wards:__ Department of psychiatry, orthopedic, heart & lung, children, infants, midwifery, ICCU, CCU, ENT, surgery, ophthalmology, mammography.

***__Service departments__: Kitchen, laundry, CSR, equipment and emergency wards

The correlation between *Acanthamoeba* and *H. pylori* genotyping results is shown in Table 3. The *H. pylori* within *ProtoAcanthamoeba bohemica* was observed more prevalent than other

genotypes. Based on on statistically analysis, a significant correlation (P<0.05) was observed between genotype type and *H. pylori* endosymbiont.

Table 3: Frequency percentage of *Acanthamoeba* genotypes based on the occurrence of *H. pylori*

Enotypes	*H. pylori contamination*		*Total No. (%)*
	Positive No. (%)	**Negative No. (%)**	
ProtAcanthamoeba bohemica	7 (70)	3(30)	10 (40)
T2 Acanthamoeba	0	2 (100)	2 (8)
T11 Acanthamoeba	2 (100)	0 (0)	2 (8)
T4 Acanthamoeba	1 (10)	9 (90)	10 (40)
T5 Acanthamoeba	0 (0)	1 (100)	1 (4)
Total	8 (32)	17 (68)	25 (100)

Real-Time PCR results of coculture culture

The penetration of *H. pylori* bacteria into *Acanthamoeba* trophozoites, was assesed by Real-Time-PCR tests in a laboratory condition and repeated 5 times, and the results are shown in Table 4. Molecular result showed that *H. pylori* has the ability to penetrate and enter the *Acanthamoeba* parasite. According to Table 4, the average amount of bacterial penetration was (3.26×10^7).

Table 4: *Acanthamoeba* & *H. pylori* DNA copy numbers in coculture (Real-Time-PCR results)

Test	*DNA copy number*		
	H. pylori	***Acanthamoeba* spp.**	**Ratio**
1	1.61×10^7	3.01×10^7	0.21
2	1.63×10^7	7.78×10^7	0.49
3	3.22×10^7	6.53×10^6	0.02
4	2.11×10^7	8.35×10^8	2.49
5	7.76×10^7	3.10×10^7	2.49
Mean	3.26×10^7	20.79×10^7	0.07
Positive Control	6.85×10^{10}	6.79×10^{12}	

The average ratio of penetration of *H. pylori* into *Acanthamoeba* was calculated 0.07 (the ratio in Table 4). According to Pearson's correlation coefficient (-0.276 and *P*>0.05), there was no significant correlation between the number of *Acanthamoeba* and bacteria and no linear correlation.

The amplification and melting curves of *H. pylori* amplification in real time PCR are shown in Figs. 1-3.

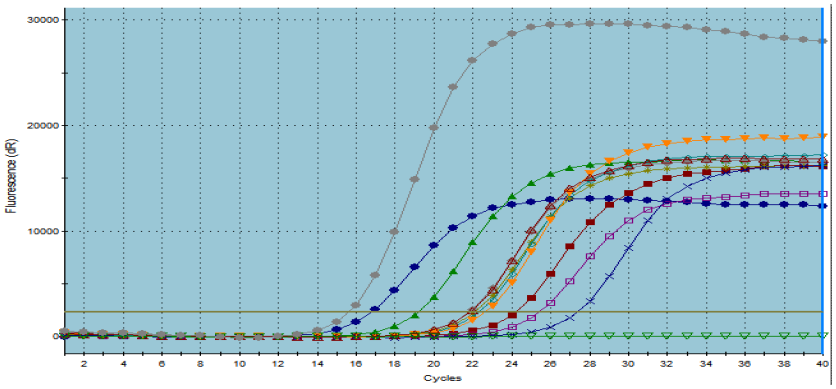

Fig. 1: Amplification curve of *H. pylori* in real time PCR

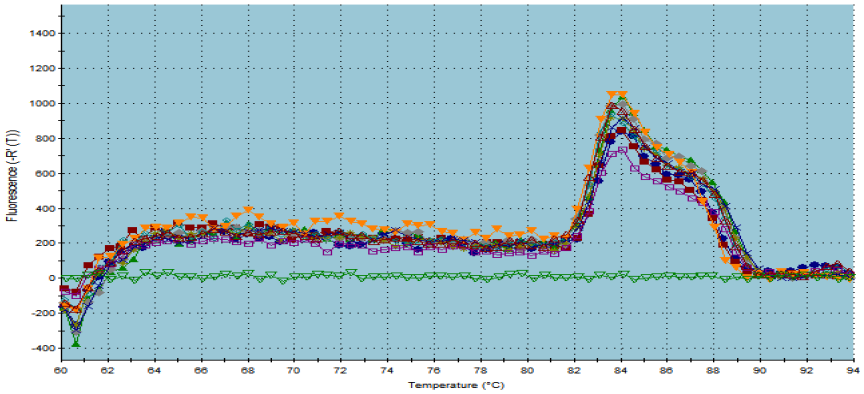

Fig. 2: Melting curve analysis of *H. pylori* amplification in real time PCR

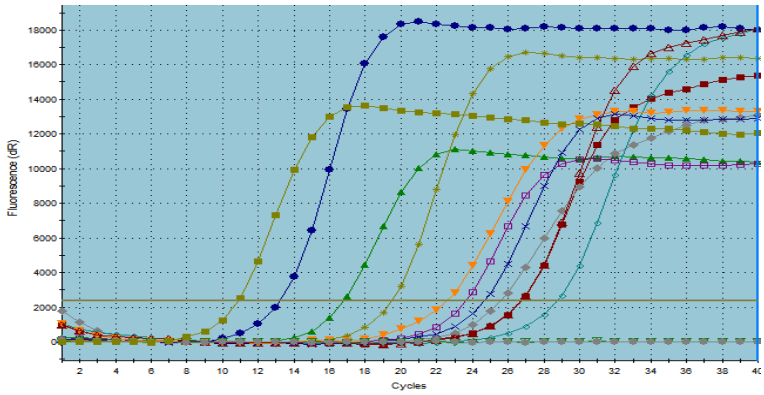

Fig. 3: Amplification curve of *Acantamoeba* in real time PCR

Discussion

In the present study, *Acanthamoeba* infection was detected based on microscopic and PCR methods (using 18s rRNA gene) in hospital samples from the Markazi Province of Iran. In total, the contamination rate in hospital samples was 44.7% & 71.14% with PCR & microscopic methods respectively.

Generally, *Acanthamoeba* feed on bacteria, algae and yeast. However, they can feed on almost anything (24). Out of 42 *Acanthamoeba*

PCR positive samples in terms of *H. pylori* endosymbiont, 13 isolates (31%) were positive. According to the type of sample, the highest amount of contamination was in soil samples (100%) then, dust samples (40%) from service departments consist of Kitchen, laundry, CSR, equipment and emergency wards of the hospitals.

In the microscopic and molecular testing of the water samples of the studied hospitals, no contamination with *Acanthamoeba* and *H. pylori* was observed. *H. pylori* may exist in water, and it has been shown that free-living amoebae can act as reservoirs for these tough microorganisms. This hypothesis has been proven by pointing out that *H. pylori* is able to grow when co-cultivated with *A. castellani* (25). In a study that carried out on river water samples (51 samples) and soil samples (75 samples) in Sapporo City, Japan to investigate the relationship between *H. pylori* and *Acanthamoeba* by PCR method, *H. pylori* was 88% and 0% in river water and soil samples respectively (26). In another study, *Acanthamoeba* in the river and soil samples were found 61% and 96% respectively, but the author did not find a statistical relationship between the prevalence of *Helicobacter* and *Acanthamoeba*, which indicates the limited role of *Acanthamoeba* in the survival of *Helicobacter* and the random presence of both of these microorganisms in the environment (27).

The presence of *A. castellani* increases the survival of *H. pylori* in the extracellular part (25). This extracellular connection suggests a reciprocal interaction between *H. pylori* and *Acanthamoeba* in the environment. In fact, parasites supporting the survival of bacteria in different environments may prevent not only *H. pylori* but also other pathogenic bacterial agents in the environment, for this reason, *H. pylori* is unable to survive and grow inside the amoeba (25). Of course, this result is different compared to the results of our study which was done by molecular method. This difference in molecular techniques and culture method may be due to the difference in *H. pylori* strains. Conventional molecular methods are not able to distinguish between live and dead bacteria, and Moreno-Mesonero's study confirms this issue (27). But using Real time PCR method, only live bacteria can be detected.

In the present study, based on the genotypes of *Acanthamoeba*, *ProtoAcanthamoeba bohemica*, genotype T2, genotype T11 (with *H. pylori*) and genotype T4 of *Acanthamoeba* also had *H. pylori* endosymbiont, but genotype T5, unlike other genotypes, did not have it.

The results of the molecular investigation of the mentioned endosymbiont in laboratory conditions (co-culture) in a present study showed that *H. pylori* has the ability to penetrate and enter the *Acanthamoeba* parasite. But according to their quantification based on Real-Time PCR, there was no significant difference between the number of *Acanthamoeba* and bacteria in the correlation test.

Overall, there are 539 species of bacteria listed by the Environmental Protection Agency (EPA). Of these, a comprehensive review of the literature has shown that 102 species (18.9%) can be identified alive when in contact with different species of amoeba (28), which in previous studies, *H. pylori* has been reported with the ability to remain infected and survive intracellularly (28). In the present study, the coexistence of bacteria and *Acanthamoeba* was obtained in laboratory conditions, so it can be claimed that the bacterium has at least the ability to survive inside *Acanthamoeba* and confirms the findings of the above study to some extent.

Interestingly, in some studies, bacteria such as *H. pylori,* which require microaerophilic conditions for cultivation in artificial media, can also remain and grow in amoebae. Therefore, probably protozoa provide an ecological environment that can respond to the needs of different and very different species of bacteria (28). Also, in a study with the co-culture of *H. pylori* with *A. castellani*, the parasite provided microaerophilic conditions and a large amount of nutrients for growth without which the

bacteria cannot survive and *H. pylori* had the ability to multiply in the presence of amoeba in laboratory conditions (9). It survives for several weeks and active bacteria can be shown in the vacuole of the parasite. The possible dependence of *H. pylori* on free amoebae in nature can be considered due to its transmission and prevalence because it acts as a vector and incubator and reproduces in the parasite by unknown mechanisms (9).

H. pylori multiplies in *Acanthamoeba* and is seen free in vesicles (29). Under laboratory conditions, *H. pylori* is able to multiply and survive in the presence of *Acanthamoeba* for several weeks. So, *Helicobacter*, as well as other pathogenic agents, can survive by "hiding" in amoebae, thus protecting them from environmental stress, and amoebae can be a natural reservoir of *Helicobacter* (30). *H. pylori* survives in amoeba for 24 hours and this was confirmed by vital staining, FISH, DVC-FISH and PMA-qPCR (8). Amoeba can create a suitable nutrient and anaerobic environment for bacteria.

H. pylori can be released after 4 days of co-culture with *A. astronyxis* without amoeba lysis (29). In addition to these reports, some studies indicate that *H. pylori* survives in co-culture with extracellular *Acanthamoeba castellani* but does not enter the parasite (25).

Acanthamoeba promotes the survival and proliferation of human microbial pathogens (26). The survival of *Helicobacter* is often increased in laboratory in the presence of *Acanthamoeba*. Regarding the intracellular or extracellular interaction of *Acanthamoeba* and *H. pylori,* there are contradictions that may depend on the strain of microorganisms and methods used (31). On the other hand, it is not clear whether *H. pylori* is capable of multiplying inside the amoeba or whether the bacteria survive only in the viable but nonculturable (VBNC) state until a more suitable environment or host is found (8). Our results indicated that the *H. pylori* endosymbiont is able to penetrate into inside *Acanthamoeba* in laboratory conditions. But more research is needed, especially in the field of genetic characteristics of amoeba that help its interaction and survival, to increase the understanding of the risks of infection.

Conclusion

Our results in natural environments and laboratory conditions support the hypothesis that *Acanthamoeba* acts as an environmental reservoir for *H. pylori* acts and provides the ability of the bacteria to survive or reproduce within itself. Therefore, special attention should be paid to the presence of free amoebas in the hospital environment, which in addition to the risk of *Acanthamoeba* as a pathogen, *H. Pylori* keeps and reproduces inside itself. As a result, it can provide a threat to the health of patients and hospital personnel.

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Conflict of Interest

There were no specific limitations that precluded the work, and the authors declare that there are no conflicts of interest.

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