Original Article

Localization of *H.pylori* within the Vacuole of *Candida* Yeast by Direct Immunofluorescence Technique

Parastoo Saniee MSc[1], Farideh Siavoshi PhD•[1], Gholamreza Nikbakht Broujeni PhD[2], Mahmood Khormali MSc[2], Abdolfatah Sarrafnejad PhD[3], Reza Malekzadeh MD[4]

Abstract

Background: Reports indicate that *H.pylori* is able to invade the eukaryotic cells and establish inside their vacuoles. In this study, FITC-conjugated IgY-Hp was used to localize *H.pylori* inside the vacuole of *Candida* yeast. Presence of intracellular *H.pylori* inside the new generations of yeast cells was also examined by light microscopy and Live/Dead *Bac*Light staining method.

Methods: A single colony of fresh yeast culture was cultivated in a 100-µl medium containing yeast extract and N-acetylglucoseamine supplemented with fetal bovine serum. After 12-hr incubation at 37%, FITC-conjugated IgY-Hp was added. After 3 hours, 10 µL of yeast suspension was smeared on a glass slide, air-dried and examined by fluorescent microscopy. Wet mounts of yeast culture and Live/Dead *Bac*Light stained preparations were examined by light and fluorescent microscopy, respectively. Photographs were taken from the fast-moving *H.pylori* inside the yeast vacuoles.

Results: Fluorescent microscopy showed that FITC-conjugated IgY-Hp could enter yeast cells and specifically react with *H.pylori*, localizing the bacterium inside the yeast vacuole. Photographs taken from wet mounts observed by light and fluorescent microscopy showed fast-moving *H.pylori* cells in the vacuole of mother as well as daughter yeast cells. The intravacuolar *H.pylori* cells stained green, showing their viability.

Conclusion: Intracellular life of prokaryotes inside eukaryotes has been described as an evolutionary phenomenon with a great impact on bacterial persistence despite environmental stresses. Results of this study demonstrated the specific interaction of FITC-conjugated IgY-Hp with *H.pylori* cells and the bacterial localization inside the *Candida* yeast vacuole. The intracellular bacteria were viable and existed in the vacuole of next generations of yeast cells. It appears that *H.pylori* is well-equipped to establish within the vacuole of eukaryotic cells where it is protected from stressful conditions, including antibacterial therapy. Presence of *H.pylori* inside the vacuole of new generations of yeasts demonstrates the intimate relationship between the two microorganisms, resulting in bacterial inheritance as part of the vacuolar content of yeast cells.

Keywords: *H.pylori*, immunofluorescent microscopy, intracellular, yeast vacuole

Cite the article as: Saniee P, Siavoshi F, Nikbakht Broujeni G, Khormali M, Sarrafnejad A, Malekzadeh R. Localization of *H.pylori* within the Vacuole of *Candida* Yeast by Direct Immunofluorescence Technique. *Arch Iran Med*. 2013; **16(12):** 705 – 710.

Introduction

It is believed that persistence of *H.pylori* despite the hostile gastric environment and antibiotic therapy may be due to existence of an ecological niche inside the human epithelial cells.[1–3] Reports describe that *H.pylori* is capable of invading eukaryotic cells and establishing inside their vacuoles.[3–5] Many investigations on human gastric biopsies, using different staining and microscopic techniques have described the internalization of *H.pylori* inside epithelial cells[6,7] and immunocytes.[8–10] Internalization of *H.pylori* has also been reported in cultured cell lines[4,11,12] where the intracellular spiral or coccoid forms were observed within defined membrane-bound cytoplasmic vacuoles.[4] Inside the vacuole, the bacteria exhibited directional movements [13] and their viability was confirmed by acridine orange technique.[14] In nature, many pathogenic bacteria persist in stressful conditions by residing inside eukaryotic microorganisms. The intracellular life of prokaryotes has been considered a significant evolutionary phenomenon which has led to the adaptation of prokaryotes to a wide range of environmental niches. However, elucidating the details of this relationship has been hampered due to non-culturability of most endosymbiotic bacteria.[15]

In our previous studies, we demonstrated the endosymbiotic relationship of non-culturable *H.pylori* with oral and gastric *Candida* yeasts by light and flouresent microcopy and detected *H.pylori*-specific genes in total DNA extracted from yeasts.[16,17] The presence of *H.pylori*-specific proteins in the protein pool of oral and gastric *Candida* yeasts detected by western blotting and IgY-Hp revealed that the *H.pylori* residing inside the vacuole of yeast produces proteins and is viable[18] In this study, we investigated the intracellular localization of *H.pylori* inside the vacuole of a *Candida* yeast by direct immunofluorescence (IF) technique, using fluorescein isothiocyanate (FITC)-labled polyclonal anti-*H. pylori* egg yolk immunoglobulin Y (IgY-Hp) which was raised in hens and its specificity to *H.pylori* was confirmed by dot and western blotting. The presence of live intracellular *H.pylori* inside the vacuole of new generations of yeast cells was also examined by light and fluorescent microscopy, in the latter case with Live/Dead *Bac*Light staining method.

Authors' affiliations: [1]Department of Microbiology, School of Biology, University College of Sciences, University of Tehran, Tehran, Iran. [2]Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. [3]Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. [4]Digestive Disease Research Institute, Tehran University of Medical Sciences, Tehran, Iran.
•**Corresponding author and reprints:** Farideh Siavoshi PhD, Department of Microbiology, School of Biology, University College of Sciences, University of Tehran, P.O. Box 14176-14411, Tehran, Iran. Tel: +98-216-111-2460, Fax: +98-2166492992, E-mail: Siavoshi@khayam.ut.ac.ir
Accepted for publication: 29 October 2013

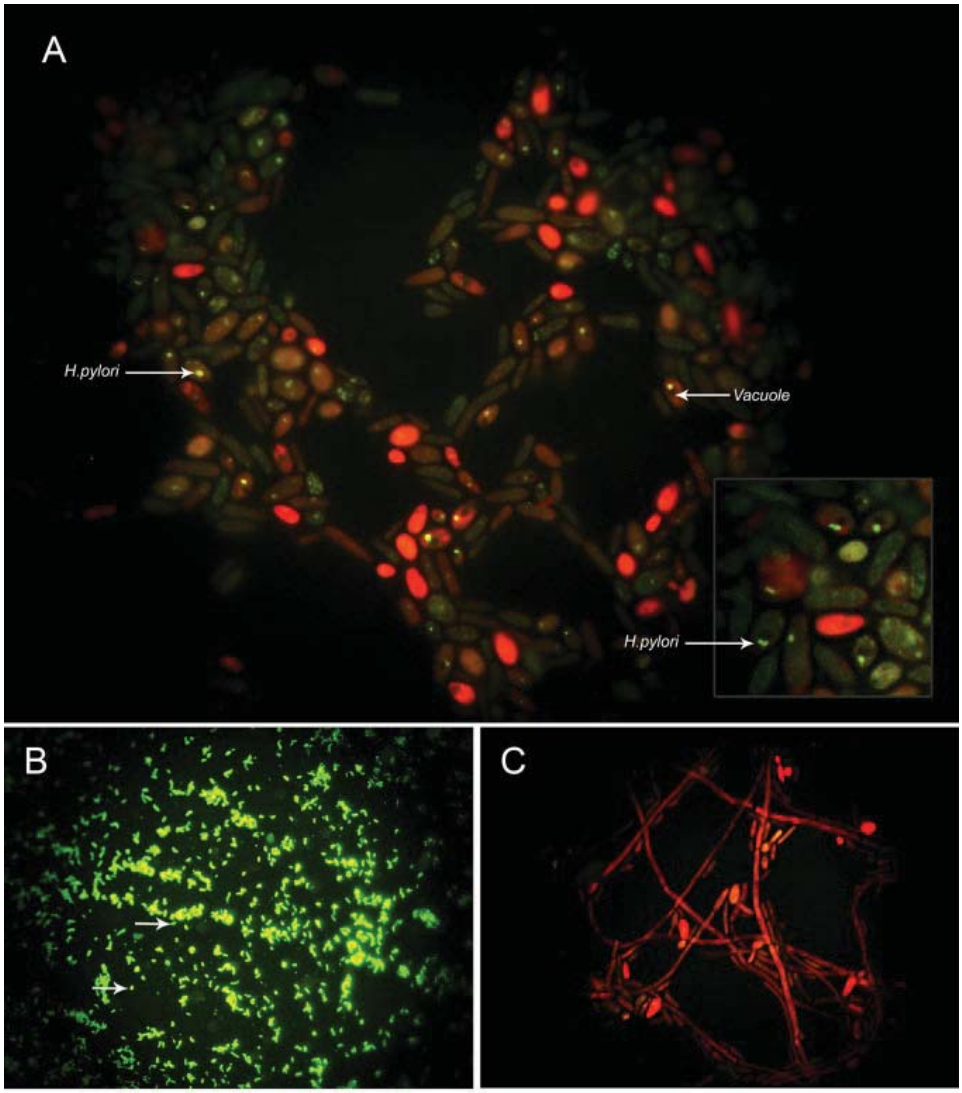

Figure 1. Direct immunofluorescent microscopy. **A)** Green fluorescent bacterial cells inside the G2 yeast vacuole show the intracellular localization of *H.pylori*. **B)** Green fluorescent bacterial cells indicate the specific reaction of FITC-conjugated IgY-Hp with free *H.pylori* (arrows). **C)** Heat-killed G2 yeast.

Materials and Methods

Yeast and bacterial strains

One yeast was isolated from a gastric biopsy which was cultured on the selective Brucella blood agar (without amphotericin B) for isolation of *H.pylori.* The recruited patient was a female, aged 54 years, with gastric ulcer. The gastric biopsy culture was positive for *H.pylori* and yeast after 3 days of microaerobic incubation at 37°C. *H.pylori* was identified based on microscopic observation of Gram negative spiral bacteria and the positive results of catalase, oxidase and urease tests. The identity of bacteria as *H.pylori* was confirmed by amplification of *16S rRNA, vacA s1, ureAB,* and *ahpC* genes and sequencing of the PCR product of *16S rRNA* gene. This *H.pylori* strain was used for preparation of IgY-Hp and as a positive control for IF assay. The isolated yeast was identified as *Candida* based on microscopic oval morphology and formation of blastoconidia on Subouraud dextrose agar. The *Candida* yeast was further identified as *Candida* spp. based on its interaction on Chromagar (CHROMagar, France) and production of white-pink colonies. It was nominated as G2 yeast.

Preparation of FITC-conjugated IgY-Hp

The reference *H.pylori* was suspended in normal saline with the turbidity of McFarland standard 3, heat-killed and its protein content was measured as 200 µg/mL. A 500-µL volume of whole cell lysate was mixed with complete Freund's adjuvant and injected into two Leghorn hens intramuscularly. After two booster injections at two-week intervals, IgY-Hp was extracted from collected eggs according to Nikbakht.[19] Dot blotting was performed to confirm the specific interaction of IgY-Hp with *H.pylori*-specific proteins[18] IgY-Hp was conjugated with FITC as described previously.[20] In brief, extracted IgY-Hp was dialyzed overnight against the carbonate/bicarbonate buffer. The protein content of antibody was measured by spectrophotometer and diluted to 10mg/mL with carbonate buffer 0.5M (pH 9.1). A 100-µL volume of FITC solution (1mg/mL in DMSO) was added to the antibody, mixed thoroughly and incubated in dark and at room temperature for 2 hr. To remove the unbound FITC, the mixture was passed through a Sephadex G-25 column equilibrated with the phosphate-buffered saline (PBS 1X).

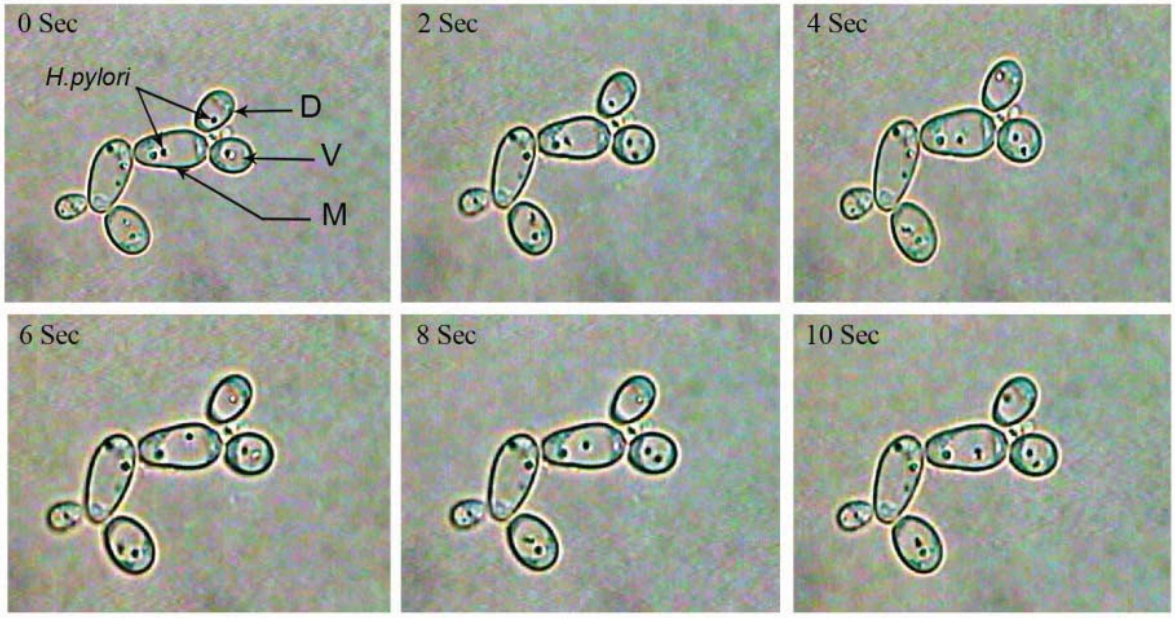

Figure 2. Light microscopy of the G2 yeast. Fast-moving intracellular *H.pylori* cells are demonstrated inside the vacuoles (V) of mother (M) and daughter (D) yeast cells. Photographs were taken at 6 time intervals (0, 2, 4, 6, 8, and 10 seconds). Original magnification ×1250.

Detection of *H.pylori* inside the yeast vacuole by direct immunofluorescence assay

This method was designed to identify the target *H.pylori* inside yeast vacuole. The fast-moving *H.pylori* cells observed inside the vacuole of G2 yeast had been previously identified as *H.pylori* by amplification of *16S rRNA*, *vacA*, *ureAB*, and *ahpC* genes from the whole DNA of yeast and observing 100% sequence homology between the amplified products of *16S rRNA* from the G2 yeast and the control *H.pylori* and those of the reference strains in Genbank.[18] A 100-µL medium containing yeast extract (5 g/L) and N-acetylglucoseamine (20 g/L) supplemented with equal volume of fetal bovine serum (Invitrogen, USA) was inoculated with a single colony of G2 yeast and incubated at 37°C for 12 hr. A 20-µL volume of 1:5 dilution of FITC-conjugated IgY-Hp was added and vortexed. A 5-µL volume of Evans blue solution (0.01% in PBS) was added to create color contrast. The mixture was incubated at 37°C for 3 hr while shaking at 200 rpm. A 10-µL volume of the yeast culture was smeared on a glass slide and air-dried. To remove unbound antibodies, slides were washed with PBS while shaken gently. Samples were covered with mounting medium and examined by fluorescent microscopy. Positive and negative controls included fresh culture of *H.pylori* and heat-killed G2 yeast, respectively.

Light and fluorescent microscopy

G2 yeast was subcultured on yeast extract glucose chloramphenicol agar for more than 10 times to ensure the absence of bacterial contamination. A wet mount was prepared from fresh culture of yeast and examined by light microscopy to observe intracellular *H.pylori* inside the vacuoles of mother and daughter yeast cells. Photographs were taken from fast-moving intravacuolar *H.pylori* cells at 6 time intervals. Fluorescent microscopy and Live/Dead *Bac*Light Bacterial Viability kit (Invitrogen, USA) were used to reveal the viability of the intravacuolar *H.pylori*, according to manufacturer´s instructions. Photographs were taken from live (green) and fast-moving *H.pylori* cells in the vacuoles of mother and daughter yeast cells at 3 time intervals.

Results

Detection of *H.pylori* inside yeast vacuole by direct immunofluorescence assay

Fluorescent microscopy demonstrated green fluorescent bacterial cells inside the vacuole of G2 yeast cells, showing that FITC-conjugated IgY-Hp reacted specifically with *H.pylori* inside the vacuole of yeast (Figure 1A). FITC-conjugated IgY-Hp also specifically reacted with free *H.pylori* cells (Figure 1B). No reaction was observed in heat-killed G2 yeast (Figure 1C).

Light and fluorescent microscopy

Light and fluorescent microscopy of G2 yeast cells showed fast-moving and live *H.pylori* cells inside the vacuole of yeast cells. *H.pylori* cells could be observed in yeast vacuoles after many subcultures. *H.pylori* cells were present inside the vacuoles (V) of mother (M) as well as daughter (D) yeast cells (Figures 2 and 3).

Discussion

Egg yolk antibodies have been used as powerful and specific tools in many diagnostic and biomarker discovery applications.[21] It has been demonstrated that IgY-Hp strongly reacts with *H.pylori*-specific proteins and can be used for effective inhibition or detection of *H.pylori*.[22] IgY-Hp inhibited attachment of *H.pylori* to cultured cells, the bacterial growth *in vitro* and its urease activity. It also decreased *H.pylori*-induced lymphocyte and neutrophil infiltration and thus mucosal injury.[22] Furthermore, IgY-Hp strongly reacted with immunodominant proteins of *H.pylori*, including Hsp 60, urease, peroxiredoxin and thiol peroxidase.[22] In our previous study, IgY-Hp was used for detection of *H.pylori*-specific proteins; vacuolating cytotoxin A (VacA), Urease, peroiredoxin,

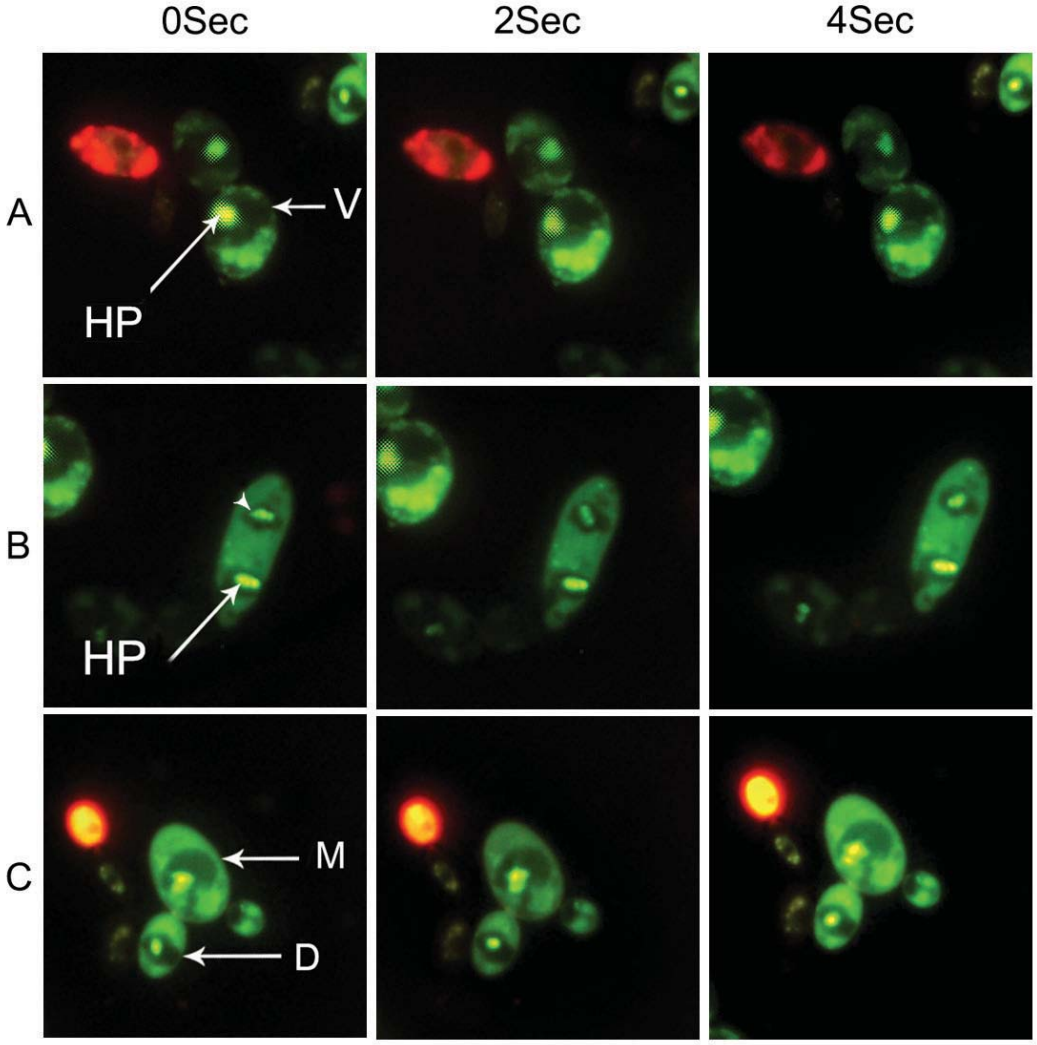

Figure 3. Three selected views of live *H.pylori* cells inside the vacoules of G2 yeast observed by fluorescent microscopy. Live and fast-moving *H.pylori* cells are demonstrated inside the vacuoles (V) of mother (M) and daughter (D) yeast cells. Photographs (A, B, and C) were taken at 3 time intervals (0, 2 and 4 seconds). Arrow head shows a moving *H.pylori* cell within the vacuole. Original magnification ×1000.

and thiol peroxidase in the protein pool of *Candida* yeast by western blotting [18]

In this study, fluorescent microscopy of yeast cells treated with FITC-conjugated IgY-Hp demonstrated the entry of conjugated antibody into yeast cell and its specific interaction with *H.pylori*, thus verifying the presence of *H.pylori* inside the yeast vacuole. FITC is a pH-sensitive fluorophore which quenches at acidic pH and brightens when the pH is neutralized.[24] Accordingly, one reason for observing some yeast cells with dark vacuoles could be the acidic pH of the vacuoles. Whole cell *H.pylori* and *H.pylori*-immunopositive materials were found in the lamina propria of stained gastric biopsies.[25] Electron microscopy observations on gastric biopsies fixed and stained with *H.pylori*-specific antibodies revealed the intracellular occurrence of *H.pylori* inside the epithelial cells.[26] Confocal microscopy of immunohistochemistry and fluorescence *in situ* hybridization preparations of cultured cells[8] as well as ultrastructural studies on cultured cells stained with immunogoldcytochemistry[9] revealed the specific identity of *H.pylori* and its intracellular localization. The intracellular bacteria were viable because they could express *H.pylori* mRNA and antigens and maintained their morphology.[8,9] By observing the multiplication of *H.pylori* inside the cultured epithelial cells, it was proposed that the intracellular niche could provide nutrients for the slow-growing bacterium and protect it against the hostile gastric environment, host immune system and the antibacterial therapy.[1]

Reports indicate that like many intracellular bacteria, *H.pylori* is well-equipped with peroxidases to detoxify oxygen metabolites formed, e.g., during the respiratory burst of immune cells.[27] Furthermore, the bacterial urease and VacA have been recognized as two important *H.pylori* virulence determinants to influence phago-lysosome fusion and bacterial survival in macrophages.[28,29] In our previous study, detection of *H.pylori*-specific proteins; peroxiredoxin, thiol peroxidase, urease, and VacA in yeast indicated that inside the yeast vacuole, *H.pylori* produces these proteins to stay alive.[18] These reports support the results of the present study which proposes that *H.pylori* has evolved to equip itself to invade eukaryotic cells and establish inside their vacuole.[3–5]

Reports describe that fungal vacuole is an acidic storage compartment with certain similarities to plant vacuoles and mammalian lysosomes. It is the storage site of essential nutrients including Ca^{2+}, phosphate and amino acids.[30,31] A considerable amount of ergosterol is also found in the membrane of vacuoles.[32] Vacuole receives membranes from biosynthetic, endocytotic and autopha-

gic pathways of the cell.[33] It is noteworthy that members of the genus *Helicobacter* incorporate a large amount of ergosterol in the bacterial cell membrane as their unique property.[34] *H.pylori* ergosterol content exceeds 70% of cellular neutral lipids in contrast to *E.coli* which accumulates only 17%.[35] It has been proposed that dependence of *H.pylori* on ergosterol could have developed alongside the symbiotic association of bacterium with eukaryotic hosts.[34] Accordingly, yeast vacuoles can be considered a unique and specialized niche for accommodation of *H.pylori*.

The viability of intracellular *H.pylori* was determined when fast-moving *H.pylori* cells were stained green with BacLight bacterial viability kit. The nonculturable *H.pylori* cells were observed inside the vacuole of mother as well as daughter yeast cells, indicating that the bacterial cells were present in the next generations of yeasts after several subcultures. Vertical transmission of nonculturable intracellular bacteria has been reported in animals[36] and fungi.[37] Studies on Arbuscular Mycorrhizal fungi have revealed that like other prokaryotic-eukaryotic endosymbioses, fugal endobacteria are localized inside the membrane-bound vacuole.[38] The intracellular bacteria have been observed in the vacuole or cytoplasm of AM fungi by light and electron microscopy[38,39] as well as confocal fluorescent[40] microscopy. Furthermore, their identity as *Candidatus* Glomeribacter gigasporarum was determined by detection of bacterial *16SrRNA* gene. [38,41] These reports suggest that fungal vacuole provides a nourishing and protective niche for the endosymbiotic bacterium, facilitating its replication and transmission to the next generation.[37]

The results of this study show the intracellular localization of *H.pylori* inside the vacuole of *Candida* yeast. The fast-moving intracellular *H.pylori* cells were viable and present in the new generations of yeast cells. It appears that, like many invasive pathogens,[42] *H.pylori* has developed strategies to protect its membrane-bound vacuole by disrupting normal endosomal maturation and fusion with lysosome, promoting intracellular survival thereby maintaining symbiotic relationship with its eukaryotic host. This mutual adaptation of intracellular bacteria and their eukaryotic hosts is so intimate that would often allow both partners to survive the entire lifespan and the intracellular bacterium is transmitted to the next generation as an integral part of its host system.[38,43] The intracellular occurrence of *H.pylori* inside the *candida* yeast, while describing an example of the magnificent phenomenon of symbiotic relationship between prokaryotes and eukaryotes, it could have a great impact on the persistence of *H.pylori* against stressful conditions, including antibacterial therapy.

Acknowledgments

This study was funded by the research council of University of Tehran and Digestive Disease Research Institute. Part of this study has been presented as a poster in the 26th International workshop on Helicobacter and related bacteria in chronic digestive inflammation and gastric cancer. The authors have declared that no competing interests exist. Authors wish to thank Kaveh Oskoei for his help in photography and Mehran Ahadi for his support throughout the preparation of the manuscript.

References

1. Chu YT, Wang YH, Wu JJ, Lei HY. Invasion and multiplication of *H.pylori* in gastric epithelial cells and implications for antibiotic resistance. *Infect Immun.* 2010; **78:** 4157 – 4165.
2. Engstrand L, Graham D, Scheynius A, Genta RM, El-Zaatari F. Is the sanctuary where *H.pylori* avoids antibacterial treatment intracellular? *Am J Clin Pathol.* 1997; **108:** 504 – 509.
3. Dubois A, Boren T. *H.pylori* is invasive and it may be a facultative intracellular organism. *Cell Microbiol.* 2007; **9:** 1108 – 1116.
4. Segal ED, Falkow S, Tompkins LS. *H.pylori* attachment to gastric cells induces cytoskeletal rearrangements and tyrosine phosphorylation of host cell proteins. *Proc Natl Acad Sci U S A.* 1996; **93:** 1259 – 1264.
5. BirknessKA, Gold BD, White EH, Bartlett JH, Quinn FD. In vitro models to study attachment and invasion of *H.pylori*. *Ann N.Y Acad Sci.*1996; **797:** 293 – 295.
6. Noach LA, Rolf TM, Tytgat GN. Electron microscopic study of association between *H.pylori* and gastric and duodenal mucosa. *J Clin Pathol.* 1994; **47:** 699 – 704.
7. Wyle FA, Tarnawski A, Schulman D, Dabros W. Evidence for gastric mucosal cell invasion by *C. pylori*: an ultrastructural study. *J Clin Gastroenterol.* 1990; **12(Suppl 1):** S92 – S98.
8. Semino-Mora C, Doi SQ, Marty A, Simko V, Carlstedt I, Dubois A. Intracellular and interstitial expression of *H.pylori* virulence genes in gastric precancerous intestinal metaplasia and adenocarcinoma. *J Infect Dis.* 2003; **187:** 1165 – 1177.
9. Necchi V, Candusso ME, Tava F, Luinetti O, Ventura U, Fiocca R, et al. Intracellular, Intercellular, and Stromal Invasion of Gastric Mucosa, Preneoplastic Lesions, and Cancer by *H.pylori*. *Gastroentrol.* 2007; **132:** 1009 – 1023.
10. Shousha S, Bull TB, Parkins RA. Gastric spiral bacteria. *Lancet.* 1984; **2:** 101.
11. Smoot DT, Resau JH, Naab T, Desbordes BC, Gilliam T, Bull-Henry K, et al. Adherence of *H.pylori* to cultured human gastric epithelial cells. *Infect Immun.* 1993; **61:** 350 – 355.
12. Petersen AM, Blom J, Andersen LP, Krogfelt KA. Role of strain type, AGS cells and fetal calf serum in *H.pylori* adhesion and invasion assays. *FEMS Immunol Med Microbiol.* 2000; **29:** 59 – 67.
13. Amieva MR, Salama NR, Tompkins LS, Falkow S. *H.pylori* enter and survive within multivesicular vacuoles of epithelial cells. *Cell Microbiol.* 2002; **4:** 677 – 690.
14. Wilkinson SM, Uhl JR, Kline BC, Cockerill FR, 3rd. Assessment of invasion frequencies of cultured HEp-2 cellsby clinical isolates of *H.pylori* using an acridine orange assay. *J Clin Pathol.* 1998; **51:** 127 – 133.
15. Ruiz-Lozano JM, Bonfante P. Identification of a putative P-transporter operon in the genome of a Burkholderia strain living inside the arbuscular mycorrhizal fungus Gigaspora margarita. *J Bacteriol.* 1999; **181:** 4106 – 4109.
16. Salmanian AH, Siavoshi F, Akbari F, Afshari A, Malekzadeh R. Yeast of the oral cavity is the reservoir of *H.pylori*. *J Oral Pathol Med.* 2008; **37:** 324 – 328.
17. Siavoshi F, Salmanian AH, Kbari FA, Malekzadeh R, Massarrat S. Detection of *H.pylori*-Specific Genes in the Oral Yeast. *Helicobacter.* 2005; **10:** 318 – 322.
18. Saniee P, Siavoshi F, Nikbakht Brujeni G, Khormali M, Sarrafnejad A, Malekzadeh R. Immunodetection of *H.pylori*-specific proteins in oral and gastric *Candida* yeasts. *Arch Iran Med.* 2013; **16:** 624 – 630.
19. Nikbakht Brujeni G, Jalali SAH, Koohi MK. Development of DNA-designed avian IgY antibodies for quantitative determination of bovine interferon-gamma. *Appl Biochem Biothech* 2011; **163:** 338 – 345.
20. Wisdom GB. Conjugation of antibodies to fluorescein or rhodamine. *Methods Mol Biol.* 2005; **295:** 131 – 134.
21. Kovacs-Nolan J, Mine Y. Egg Yolk Antibodies for Passive Immunity. *Ann Rev Food Sci Technol.* 2012; **3:** 163 – 182.
22. Shin JH, Yang M, Nam SW, Kim JT, Myung NH, Bang WG, et al. Use of egg yolk-derived immunoglobulin as an alternative to antibiotic treatment for control of *H.pylori* infection. *Clin Diagn Lab Immunol.* 2002; **9:** 1061 – 1066.
23. Shin JH, Nam SW, Kim JT, Yoon JB, Bang WG, Roe IH. Identification of immunodominant *H.pylori* proteins with reactivity to H. pylori-specific egg-yolk immunoglobulin. *J Med Microbiol.* 2003; **52:** 217 – 222.
24. Clarke M, Maddera L, Engel U, Gerisch G. Retrieval of the vacuolar H+-ATPase from phagosomes revealed by live cell imaging. *PloS one.* 2010; **5(1):** e8585.
25. Andersen LP, Holck S. Possible evidence of invasiveness of *H.pylori*. *Eur J Clin Microbiol Infect Dis.* 1990; **9:** 135 – 138.
26. Ko GH, Kang SM, Kim YK, Lee JH, Park CK, Youn HS, et al. Invasiveness of *H.pylori* into human gastric mucosa. *Helicobacter.* 1999;

4(2): 77 – 81.
27. Mauel J. Mechanisms of intracellular microbicide. *Bull Eur Physiopathol Respir.* 1983; **19:** 115 – 122.
28. Zheng PY, Jones NL. *H.pylori* strains expressing the vacuolating cytotoxin interrupt phagosome maturation in macrophages by recruiting and retaining TACO (coronin 1) protein. *Cell Microbiol.* 2003; **5:** 25 – 40.
29. Schwartz JT, Allen LA. Role of urease in megasome formation and *H.pylori* survival in macrophages. *J Leukoc Biol.* 2006; **79:** 1214 – 1225.
30. Klionsky DJ, Herman PK, Emr SD. The fungal vacuole: composition, function, and biogenesis. *Microbiol Rev.* 1990; **54:** 266.
31. Kane PM. The where, when, and how of organelle acidification by the yeast vacuolar H+-ATPase. *Microbiol Molecul biol Rev.* 2006; **70:** 177 – 191.
32. Schneiter R, Brugger B, Sandhoff R, Zellnig G, Leber A, Lampl M, et al. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis of the lipid molecular species composition of yeast subcellular membranes reveals acylchain-based sorting/remodeling of distinct molecular species en route to the plasma membrane. *J Cell Biol.* 1999; **146:** 741 – 754.
33. Maxfield FR, Tabas I. Role of cholesterol and lipid organization in disease. *Nature.* 2005; **438:** 612 – 621.
34. Haque M, HiraiY, Yokota K, Mori N, Jahan I, Ito H, et al. Lipid profile of *Helicobacter* spp.: presence of cholesteryl glucoside as a characteristic feature. *J Bacteriol.* 1996; **178:** 2065 – 2070.
35. Haque M, Hirai Y, Yokota K, Oguma K. Steryl glycosides: a characteristic feature of the *Helicobacter* spp. *J Bacteriol* 1995; **177:** 5334 – 5337.
36. Mira A, Moran NA. Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria. *Microb Ecol.* 2002; **44:** 137 – 143.
37. Bianciotto V, Genre A, Jargeat P, Lumini E, Becard G, Bonfante P. Vertical transmission of endobacteria in the arbuscular mycorrhizal fungus Gigaspora margarita through generation of vegetative spores. *Appl Environ Microbiol.* 2004; **70:** 3600 – 3608.
38. Bianciotto V, Bandi C, Minerdi D, Sironi M, Tichy HV, Bonfante P. An obligately endosymbiotic mycorrhizal fungus itself harbors obligately intracellular bacteria. *Appl Environ Microbiol.* 1996; **62(8):** 3005 – 3010.
39. MacDonald RM, Chandler MR, Mosse B. The occurrence of bacterium-like organelles in vesicular-arbascular mycorizal fungi. *New phytol.*1982; **90:** 659 – 663.
40. Bianciotto V, Lumini E, Lanfranco L, Minerdi D, Bonfante P, Perotto S. Detection and identification of bacterial endosymbionts inarbuscular mycorrhizal fungi belonging to the family Gigasporaceae. *Appl Environ Microbiol.* 2000; **66:** 4503 – 4509.
41. Scannerini S, Bonfante P. Bacteria and bacteria like objects in endomycorrhizal fungi (Glomaceae). In: Margulis L, ed. *Symbiosis as Source of Evolutionary Innovation: Speciation and Morphogenesis.* Cambridge: MIT press; 1991: 273 – 287.
42. Swanson MS, Hammer BK. *L.pneumophila* pathogesesis: a fateful journey from amoebae to macrophages. *Annu Rev Microbiol.* 2000; **54:** 567 – 613.
43. Ewald PW. *Evolution of Infectious Disease*. USA: Oxford University Press; 1994.