Original Article

Immunodetection of *Helicobacter pylori*-specific Proteins in Oral and Gastric *Candida* Yeasts

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

Abstract

Background: Human gastric epithelium and immunocytes have been recognized as the sole specialized eukaryotic cells that host *Helicobacter pylori (H. pylori)*. The aim of this study was to provide further evidence for our previous proposal regarding the occurrence of *H.pylori* inside the yeast vacuole, verifying the viability of the intravacuolar *H.pylori* by western blotting.

Methods: Light microscopy and polymerase chain reaction (PCR) were used for primary detection of nonculturable *H.pylori* in 11 *Candida* yeasts (six oral and five gastric). Boiling was used for extraction of proteins from yeasts and the control *H.pylori*. Western blot analysis was recruited to assess the occurrence of *H.pylori*-specific proteins in protein pool of yeasts, using IgY-Hp raised in hens and IgG1-Hp raised in mice.

Results: The fast-moving bacterium-like bodies (BLBs) were identified as *H.pylori* by amplification of *H.pylori 16S rRNA*, *ureAB*, *vacA s1,* and *ahpC* genes from the whole DNA of yeasts. Analysis of the sequenced products of *16S rRNA* gene amplified from the yeast and *H.pylori* isolates of patient #2 showed 100 % homology with the corresponding sequences of the reference *H. pylori* strains in GenBank. According to published data, it was plausible to assign the *H.pylori*-specific proteins, detected by western blot analysis, as thiol peroxidase (21 kDa), peroxiredoxin (AhpC) (26 kDa), urease-A subunit (UreA) (32 kDa), vacuolating cytotoxin A (VacA) small subunit (36 kDa), and VacA large subunit (56 kDa).

Conclusion: Results of this study show that inside yeast, *H.pylori* expresses proteins and is viable. These proteins appear to serve as powerful tools to help *H.pylori* to establish in the vacuole of yeast where it can reach nutrients and multiply. The intimate relationship between *H.pylori* and *Candida* yeast which began long time ago, could have led to the establishment of *H.pylori* inside the yeast vacuole before invading human cells.

Keywords: *Candida* yeast, *H.pylori,* immunodetection, proteins, vacuole

Archive of SID

Cite this article as: Saniee P, Siavoshi F, Nikbakht Broujeni G, Khormali M, Sarrafnejad A, Malekzadeh R. Immunodetection of *Helicobacrer pylori*-specific proteins in oral and gastric *Candida* yeasts. *Arch Iran Med*. 2013; **16(11):** 624 – 630.

Introduction

H.pylori has been known as a noninvasive pathogen being evolved to specifically colonize the mucous surface of human stomach where no competing organism could withstand.[1] However, a large body of evidence now demonstrates that *H.pylori* is a facultative intracellular organism,[1,2] being able to multiply in the epithelial cells,[3] macrophages,[4] and dendritic cells.[5] Many early ultrastructural studies used gastric biopsies of patients with gastritis and ulcers to demonstrate that in addition to intimately adhering to epithelial cell surface, *Campylobacter*-like organisms invaded the intracellular spaces and were present within some gastric and duodenal epithelial cells, parietal cells, and immunocytes.[6–8] *H.pylori* cells were usually located within defined membrane-bound vacuoles.[9,10]

In many chronic bacterial infections, invasion of the epithelial cells is of major significance in the survival and multiplication of microorganisms.[11] Bacterial pathogens can enter mammalian cells and become adapted to live and multiply inside the membrane-bound vacuoles.[12] It has been proposed that success of invasion and survival strategies exploited by intracellular bacteria is determined by resisting destruction in the phagosome which is believed to have been learned in free-living amoebae as a preadaptation to survive within phagocytic cells.[13] This reflects the long coevolution process of bacteria and free-living amoebae which could have begun more than one billion years ago.[14] Mutualistic and symbiotic interactions between intracellular bacteria and eukaryotes are believed to have driven the evolution of eukaryotes,[15,16] however the details of these interactions are not clear mainly due to nonculturability of many of the endosymbiotic bacteria.[17–19]

In contrast to many other eukaryotic cells such as protozoa, bivalves, and insects[20] that could harbor intracellular bacteria, fungi offer only a limited number of examples.[19] Intracellular establishment of bacteria inside fungi has been regarded as an unusual evolutionary phenomenon because presence of cell wall restricts endocytosis and bacterial uptake by fungi.[21] Like other prokaryotic-eukaryotic endosymbioses[22] fungal endobacteria are localized inside the membrane-bound vacuole.[18] Accordingly, vacuole

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 21 61112460, Fax: + 98 21 66492992, E-mail: Siavoshi@khayam.ut.ac.ir.

Accepted for publication: 9 October 2013

www.SID.ir

of eukaryotic cells needs to have unique properties to provide a sophisticated niche for nourishing and protecting the endosymbiotic bacteria.

Examples of bacterial-fungal interactions range from complex microbial communities in soil to the rich microbiota of mammalian gastrointestinal (GI) tract.[15] GI tract of humans is an important niche for *Candida albicans*[23] where it encounters varying pH, oxygen, and nutrient levels[24] and could rapidly adapt itself to sudden changes by changing the expression of hundreds to thousands of genes.[25] Association of *H.pylori* with *Candida* yeast is becoming the focus of interest because of their concomitant occurrence in the human GI tract which indicates that both microorganisms are well-adapted to establish in this unique niche.[26–29] Despite the coexistence of *Candida* yeast and bacteria for billions of years, the basis and importance of their symbiotic relationship have not been studied in detail.[30] In our previous studies, the intracellular occurrence of nonculturable *H.pylori* inside the vacuole of oral and gastric *Candida* yeasts was demonstrated by light and fluorescent microscopy observations and detection of *H.pylori*-specific genes; *16S rRNA* and *cagA*[31] and *vacA* and *ureAB*[32] in total DNA of yeasts.[31,32] In this study, intracellular occurrence of *H.pylori* inside the vacuole of oral and gastric *Candida* yeasts was demonstrated by light microscopy and detection of *H.pylori*-specific genes; *16S rRNA, ureAB, vacA s1/s2, and ahpC* in the whole DNA of yeasts. To confirm the viability of intracellular *H.pylori*, polyclonal anti-*H.pylori* egg yolk immunoglobulin Y (IgY-Hp) raised in hens and monoclonal anti-*H.pylori* IgG1 raised in mice (IgG1-Hp) were recruited for detection of *H.pylori*-specific proteins in the whole-cell lysate of yeasts by western blotting analysis.

Materials and Methods

Isolation and identification of yeasts and H.pylori isolates

Eleven yeasts (six oral and five gastric) were included in this study. These yeasts were isolated from five patients who were referred to the Endoscopy Room of Digestive Disease Research Institute, Tehran University of Medical Sciences (Tehran, Iran). The patients signed an informed consent and the study was approved by the Research Ethics Committee of Tehran University of Medical Sciences. All patients had gastric ulcer and the positive result of rapid urease test showed the occurrence of *H.pylori* infection in their stomach. Gastric yeasts were isolated from gastric biopsies which were cultured on the selective Brucella blood agar for isolation of *H.pylori.* Oral swabs were also taken from the patients by rubbing the sterile cotton swab against tongue surface, oral mucosa, and gingiva. Yeast extract Glucose Chloramphenicol (YGC) agar was used for isolation of oral yeasts as well as subculturing of all yeasts. Biopsy cultures were incubated under microaerobic conditions at 37 °C and yeast cultures at 37 °C. The 11 isolated yeasts were identified as *Candida* according to their oval morphology and formation of blastoconidia, observed by light microscopy. Yeasts were further identified on Chromagar (CHROMagar, France) as *C.albicans* (green colonies), *C.tropicalis* (blue colonies), and *Candida* spp. (white-pink colonies). To ensure the absence of bacterial contamination in yeasts cultures, single colonies of yeasts were subcultured on YGC for more than 10 times. Bacterial isolates from the five patients were identified as *H.pylori* on the basis of Gram stain and spiral microscopic appearance as well as positive activities of urease, oxidase, and catalase. The identity of bacterial isolates was confirmed by amplification of *H.pylori*-specific genes. The reference strain used for PCR, raising IgY-Hp in hens, and immunoblot analysis was a previously PCR-confirmed *H.pylori* whose *16S rRNA*, *ureAB*, *cagA*, *vacA s1,* and *vacA m1* genes were sequenced and showed more that 99 % homology with those from the reference strain in GenBank.

Light microscopy

Wet mounts were prepared from fresh cultures of yeasts on YGC and examined by the light microscope to observe bacterium-like bodies (BLBs) inside the vacuoles of yeast cells. Photographs were taken from fast-moving BLBs inside the G2 yeast at two-second time intervals.

Recovery of intracellular H.pylori from yeasts

A 0.5 mL volume of acid-washed glass beads was added to 2 mL of 24- h cultures of yeasts in Brain Heart Infusion (BHI) broth and vortexed three times for 20 seconds. Crude extracts of the disrupted yeasts were cultured on Brucella blood agar with antifungal amphotericin (4 mg/L) and incubated under microaerobic conditions at 37°C and examined for *H.pylori* growth for more than three weeks. Since attempts to culture *H.pylori* from disrupted yeasts were not successful, molecular biology and immunologic methods were recruited for detection of *H.pylori* inside the vacuole of yeasts.

Amplification of H. pylori-specific genes from yeasts

DNA was extracted from pure cultures of yeasts and *H.pylori* isolates according to Sambrook and Russell.[33] Amplification of the *H.pylori*-specific genes from yeasts as well as *H.pylori* isolates was performed using appropriate primers for *16S rRNA*,[34] *ureAB*,[32] *vacA s1/s2* alleles,[35] and *ahpC*.[36] Amplification steps included: an initial denaturation at 94 °C for 3 min, followed by 33 cycles of 94 °C for 1 min, annealing at 56 °C (*16S rRNA*), 57 °C (*ureAB and vacA s1/s2*), or 54 °C (*ahpC*) for 1 min, 72 °C for 1 min, and the final extension at 72 °C for 5 min. PCR products were visualized on agarose gel and amplified products of *16S rRNA* from G2 and O2 yeasts and the corresponding *H.pylori* were sent for sequencing. Sequence homologies were determined by BLAST program (http://www.ncbi.nlm.nih.gov).

Production of IgY-Hp and dot blotting

Bacterial suspension was prepared from fresh culture of a reference *H.pylori* isolate and the turbidity was adjusted to McFarland standard 3. Bacterial cells were heat-killed and supernatant was collected and protein content in whole cell lysate (200 μg mL^{-1}) was measured by Bradford method. In the next step, a 500-μL volume of whole cell lysate was mixed with equal volume of complete Freund's adjuvant. Two leghorn hens were injected intramuscularly at two different sites of breast muscle. Two booster injections with Freund's incomplete adjuvant were performed at two-week intervals. Eggs were collected 10 days after the last immunization and IgY-Hp was extracted according to Nikbakht, et al.[37] Dot blotting was performed to assess the anti-*H.pylori* activity of extracted IgY-Hp. Briefly, 2 μL of *H.pylori* whole-cell lysate was spotted onto a polyvinylidene fluoride (PVDF) membrane and air- dried. The membrane was then incubated with 1 : 10 diluted IgY-Hp, washed with washing buffer, and incubated with 1 : 100 diluted horseradish peroxidase- conjugated goat anti-chicken IgY (abD Serotec, USA) for 1hr. Specific binding of IgY-Hp to *H.pylori*-specific proteins was observed as

Archive of SID

www.SID.ir

colored spots on the membrane, confirming production of IgY-Hp in the egg yolks. IgY obtained from the egg yolk of a nonimmunized hen was used as a negative control. Whole cell lysates of *Escherichia coli* and *Salmonella enterica* were used to ensure the absence of cross-reactivity of IgY-Hp.

Western blotting analysis

Fresh cultures of yeasts and the reference *H.pylori* were suspended in 50 µL of normal saline (McFarland standard 3), mixed with equal volume of sample buffer (0.06 M Tris–HCl, pH 6.8, 5 % glycerol, 2 % SDS, 4 % β-mercaptoethanol, and 0.0025 % bromophenol blue), and boiled for 5 min. Supernatants were used for western blotting analysis, using two sets of antibodies; one set included IgY-Hp and goat anti-chicken IgY and the other set IgG1-Hp (Clone Number 1.B.304, Thermo Scientific, USA) and goat anti-mouse IgG (abD Serotec, USA). A 20-µL volume of extracted proteins from yeasts and *H.pylori* were subjected to SDS-PAGE under reducing condition, using 7 % stacking gel and 14 % resolving gel. The proteins were then transferred onto a PVDF membrane at 35V for 1 h. The membrane was exposed to a 1 : 10 diluted IgY-Hp at 25 °C overnight. After the washing steps, membrane was incubated with 1 : 100 diluted goat anti-chicken IgY for 2 h. Colorimetric detection was performed using 100 µL of 0.03 % chloronaphtol in absolute ethanol and 10 µL of H_2O_2. Western blotting with IgG1-Hp and goat anti-mouse IgG was performed as described above.

Results

Isolation and identification of yeasts and H.pylori isolates

All the five patients had one gastric yeast and at least one oral yeast. Patient #1 (O1: *C.albicans*, G1: *C.albicans*), patient #2 (O2: *Candida* spp., G2: *Candida* spp.), patient #3 (O3: *C.tropicalis*, G3: *Candida* spp.), patient #4 (O4a: *C.albicans*, O4b: *Candida* spp., and G4: *Candida* spp.), and patient #5 (O5: *C.albicans*, G5: *Candida* spp.). *H. pylori* culture from gastric biopsies of all five patients was positive (Table 1).

Light microscopy

Light microscopy of oral and gastric yeasts showed fast-moving and live BLBs inside the vacuoles of yeast cells. BLBs were observed in yeasts vacuoles after more than ten subcultures. They were present inside the vacuoles (V) of mother (M) as well as daughter (D) yeast cells (Figure 1). The intracellular *H.pylori* was not recovered from disrupted yeasts after three weeks of microaerobic incubation.

Amplification of H. pylori-specific genes from yeasts

Electrophoresis of PCR products showed that 9/11 yeasts harbored genes homologous to *H. pylori*–specific genes; *16S rRNA* (519bp), *ureAB* (406bp), *vacA s1* (259bp), and *ahpC* (298bp) (Figure 2). Analysis of the sequenced products of *16S rRNA* gene from G2 and O2 yeasts as well as the corresponding *H.pylori* of patient #2 showed 100 % homology with the corresponding sequences of the reference *H. pylori* strains in GenBank. Furthermore, no homology was found between *H.pylori*-specific genes and yeast-specific genes published in GenBank (data not shown).

Western blotting analysis

The nine yeasts which contained *H.pylori*-specific genes were further analyzed for the occurrence of *H.pylori*-specific proteins. The SDS-PAGE of extracted proteins from *H.pylori* and subsequent western blotting using IgY-Hp, revealed six different and strongly reactive proteins (21, 26, 32, 36, 40, and 56 kDa). The *H.pylori*-specific proteins were detected in five yeasts (three oral and two gastric). The yeasts O1, O2, and G2 had all the six *H.pylori*-specific proteins and O3 and G4 had four of six proteins (26, 32, 36, and 56 kDa) (Figure 3a, Table 1). Western blotting using IgG1-Hp, revealed one distinct protein band with a molecular weight of 26 kDa. This protein was detected in three yeast isolates (O2, G2, and G4) (Figure 3b, Table 1).

Discussion

Light microscopy showed fast- moving BLBs inside the yeast vacuole. The high homology determined between the amplified products of *H.pylori*-specific genes; *16S rRNA*, *ureAB*, *vacA s1*, and *ahpC* from the nine yeasts with those amplified from the control *H.pylori*, showed that BLBs could represent the occurrence of *H.pylori* inside the yeast. Several reports used Live/Dead *BacLight* bacterial viability kit and molecular biology methods to demonstrate the existence of nonculturable endosymbiotic bacteria in arbuscular mycorrhizal (AM) fungi,[18] *Geosiphon pyriforme*,[38] *Laccaria bicolor*,[39] and *Tuber borchii*.[40] The identity of endobacteria of AM fungi was revealed on the basis of their ribosomal sequences as a new bacterial taxon, *Candidatus* Glomeribacter gigasporarum[18] which is related to *Burkholderia cepacia*. *Burkholderia cepacia* like many pathogenic bacteria such as *Legionella*,[13] *Pseudomonas,*[41] and *Mycobacteria*[42] survives in the vacuole of the eukaryotic cells, ranging from free-living amoebae[43] to macrophages,[44] epithelial cells,[45] and pneumocytes.[46] It has been described that AM fungi are very ancient, dating back to 350 – 400 million years ago[47] and establishment of endobacteria in these fungi appears to have happened very early in evolution. Although the basis of bacterial-fungal interaction is not clear,[48] it has been proposed that intracellular establishment could protect the bacterium against environmental stresses or immune system of the host.[49]

In this study, dot blotting analysis demonstrated that the IgY-Hp produced in large amounts in hens could be recruited as a specific and powerful probe for detection of *H.pylori*-specific proteins present in yeast. Egg yolk antibodies have been used as powerful and specific tools in many diagnostic and biomarker discovery applications.[50] Western blotting analysis using IgY-Hp, demonstrated the occurrence of four to six immunodominant *H.pylori*-specific proteins in 5/9 yeasts. Three yeasts contained six proteins with molecular weights of 56, 40, 36, 32, 26, and 21 kDa. The remaining two yeasts contained four proteins of 56, 36, 32, and 26 kDa size. The IgG1-Hp detected the 26 kDa protein in three yeasts, two harboring six-protein profile and one carrying four-protein profile. IgG-Hp has been confirmed suitable for use in immunofluorescence and ELISA and specifically reacts with *Helicobacter* bacteria.[51] No relationship could be established between *Candida* spp. and the profile of the immunodominant *H.pylori*-specific proteins they carried. In a report, seven *H.pylori* protein fractions have been eluted from bacterial whole-cell lysate, among these proteins, urease B-subunit (62 kDa), Hsp60 (60 kDa), urease A- subunit (26 kDa), peroxiredoxin (22 kDa), and thiol peroxidase (18 kDa) were immunodominant and strongly reactive to IgY-Hp.[52]

Archive of SID

www.SID.ir

Table 1. Classification of the five patients according to the # and identity of their yeasts (O: oral, G: gastric), *H.pylori* genes amplified from whole DNA of yeasts and the # of immunodominant proteins detected by western blot analysis. O5 and G5 yeasts were not included in western blot analysis (NWB)

Patients #	Yeast isolates #	Chromagar results	*H.pylori* genes				Number of immunodominant proteins detected by western blot analysis	
			16S rRNA (519bp)	*ureAB* (406bp)	*vacA s1* (259bp)	*ahpC* (298bp)	IgY-Hp	IgG1-Hp
1	O1	*C.albicans*	+	+	+	+	6/6	-
	G1	*C.albicans*	+	+	+	+	-	-
2	O2	*Candida* spp.	+	+	+	+	6/6	+
	G2	*Candida* spp.	+	+	+	+	6/6	+
3	O3	*C.tropicalis*	+	+	+	+	4/6	-
	G3	*Candida* spp.	+	+	+	+	-	-
4	O4a	*C.albicans*	+	+	+	+	-	-
	O4b	*Candida* spp.	+	+	+	+	-	-
	G4	*Candida* spp.	+	+	+	+	4/6	+
5	O5	*C.albicans*	-	-	-	-	NWB	NWB
	G5	*Candida* spp.	-	-	-	-	NWB	NWB

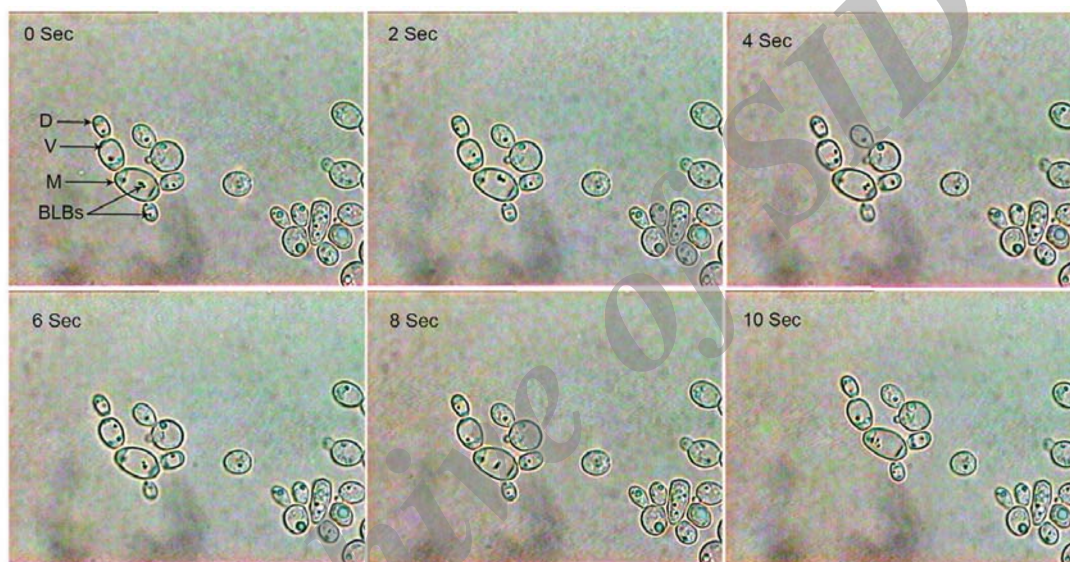

Figure 1. Light microscopy of the gastric yeast G2. Fast-moving intracellular bacterium-like bodies (BLBs) are demonstrated inside the vacuoles (V) of mother (M) and daughter (D) yeast cells. Photographs were taken at six-time intervals (0, 2, 4, 6, 8, and 10 seconds). Original magnification x1250.

Archive of SID

A western blotting study on whole cell lysate of *H.pylori*, recruiting IgG antibodies from *H.pylori*-infected patients detected 15 antigens. The highest seropositivity rates (> 80 %) were observed for p19 (OMP), p26, p29 (UreA), and p60 (UreB).[53] It was plausible to assign the immunodominant proteins recognized by IgY-Hp in this study, according to published data. The 56 kDa protein could be related to flagellin,[54] Hsp,[52] and Vacuolating cytotoxin A (VacA) large subunit,[55,56] 36 kDa to VacA small subunit,[55,56] 32 kDa to urease A- subunit,[54] 26 kDa to peroxiredoxin (AhpC), and 21 kDa to thiol peroxidase.[52] No homologue candidate could be found for the 40 kDa protein in the published references. Since VacA, urease, and AhpC encoded genes were also amplified from the whole DNA of yeasts by PCR, here VacA 56 and 36 kDa subunits, UreA (32 kDa), and AhpC (26 kDa) as well as thiol peroxidase (21 kDa) are discussed below, focusing on their probable roles in *H.pylori* survival in the vacuole of yeasts cells.

VacA being present in all strains of *H.pylori*[57] is a unique pore-forming toxin with the capacity to act on multiple intracellular sites.[56] The most well-studied effect of VacA on mammalian cells is induction of vaculation.[58,59] The 88 kDa VacA toxin consists of two domains, p33 and p55, which are both required for the activity of toxin.[60] The immunoprecipitation experiments indicated that p33 and p55 can interact with each other to form an active protein complex which inserts into the mammalian cell membrane, produces ion channels, internalizes, and induces vacuolation.[56] Infection of cultured epithelial cells with VacA mutant *H.pylori* did not produce any discernible vacuolar compartment.[61] It has been proposed that VacA traffics to late endosomal-lysosomal compartments where it induces vacuolation through a mechanism dependent on the small GTPase Rab7.[62] VacA also disrupts the degradative capacity of lysosomes by affecting lysosomal hydrolases such as cathepsin [59]. *H.pylori* urease comprises 10 % – 15 % of the bacterial cell protein and reacts strongly with IgY-Hp.[63] It has been demonstrated that urease-derived ammonia not only elevates phagosome pH and retards phagosome maturation[61] but also enhances phagosome clustering and fusion needed for megasome formation.[64] Furthermore, urease-derived ammonia enhances VacA activity[65] to disrupt membrane trafficking at the late endosome stage, thereby preventing phagosome maturation.[66] It appears that formation of VacA pores in the cell plasma membrane

www.SID.ir

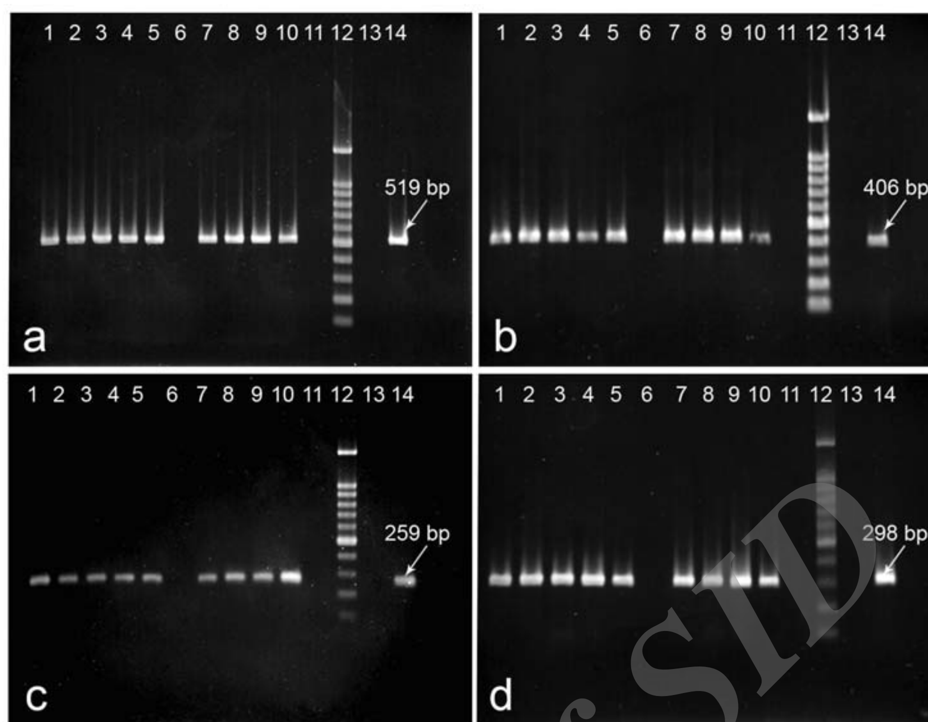

Figure 2. Electrophoresis of PCR products of *H.pylori* genes amplified from oral and gastric yeasts. **a)** *16S rRNA* (519 bp), **b)** *ureAB* (406 bp), **c)** *vacA s1* (259 bp), and **d)** *ahpC* (298 bp). In all photographs; lanes 1–5 and 7–10: PCR products amplified from oral (O1-O4b) and gastric (G1-G4) yeasts, lanes 6 and 11: no amplified products from O5 and G5 yeasts, lane 12: 100-bp ladder, lane 13: no template, lane 14: control *H. pylori.*

Archive of SID

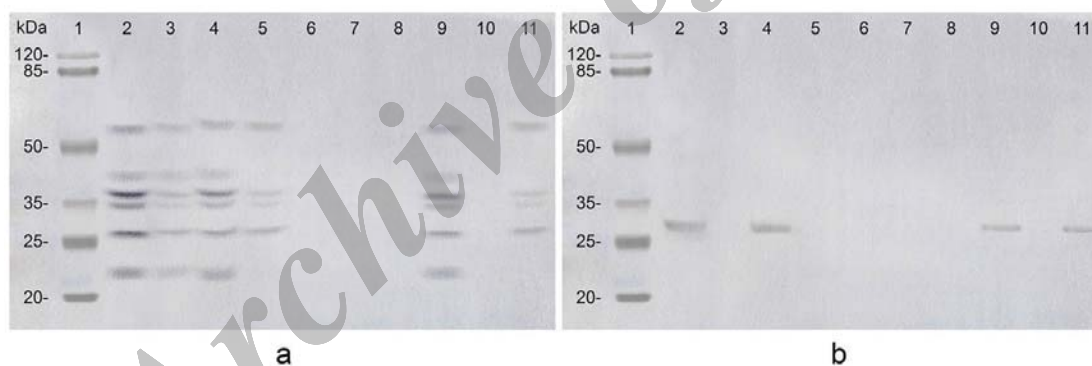

Figure 3. Western blotting analysis. **a)** Reaction with IgY-Hp; lane 1: molecular weight marker (20–120 kDa), lane 2: Control *H.pylori,* lanes 3, 4, and 9: yeasts (O1, O2, and G2) which contained six *H.pylori*-specific proteins, lanes 5 and 11: yeasts (O3 and G4) which Contained four proteins, lanes 6, 7, 8, and 10: yeasts (O4a, O4b, G1, and G3) containing no proteins. **b)** Reaction with IgG1-Hp; lane 1: molecular weight marker, lane 2: Control *H.pylori,* lanes 4, 9, and 11: yeasts (O2, G2, and G4) Containing a 26 kDa protein, lanes 3, 5, 6, 7, 8, and 10: yeasts (O1, O3, O4a, O4b, G1, and G3) Containing no protein.

is responsible for the increased urea permeability.[67] Accordingly, bacterial urease and VacA could be regarded as the main *H.pylori* virulence determinants to influence phago-lysosome fusion and bacterial survival in macrophages.[64] The important roles of VacA and urease in intracellular survival of *H.pylori* were demonstrated by disruption of *ureAB* or *vacA* which led to enhanced phagosome maturation.[67] Peroxiredoxin and thiol peroxidase belong to the family of antioxidant enzymes.[68] Peroxiredoxin is a conserved species-specific[69] and the most abundant antioxidant protein found in *H.pylori*.[70] It has been proposed as a useful diagnostic antigen in enzyme immunoassay tests for the detection of *H.pylori* infection.[71] Peroxiredoxin and thiol peroxidase may play a crucial role in *H.pylori* resistance against the oxidants generated in macrophages and neutrophils.[72] Being equipped with enzymes to detoxify oxygen metabolites formed e.g., during the respiratory burst of immune cells has been considered as an important feature of intracellular bacteria.[73]

In our previous studies, we examined the intracellular occurrence of *H.pylori* in *Candida* yeast and proposed that yeast which is remarkably resistant to stressful conditions[74] could protect *H.pylori* against hostile conditions, provide nutrients for its multiplication, and act as a vehicle for the spread of bacterium in the environment and within the human populations.[31,32] In this study, microscopic photographs taken from yeasts after several subcultures showed

www.SID.ir

the occurrence of *H.pylori* cells in the consecutive generations of yeasts, indicating that they are viable, being able to replicate, and transmit to the next generations of yeasts. Furthermore, detection of *H.pylori*-specific proteins; VacA, urease, peroxiredoxin, and thiol peroxidase in the protein pool of yeasts revealed that inside the yeast *H.pylori* expresses proteins and is viable. These results show that *H.pylori* is equipped with powerful tools to establish in the vacuole of eukaryotic cells. Fungal vacuole has been considered as an acidic storage compartment with certain similarities to plant vacuole and mammalian lysosome. The various functions of vacuole include glycoprotein turnover and hydrolysis, storage of Ca^{2+}, phosphate and amino acids, pH and osmotic regulation, ion homeostasis, and cytoplasmic detoxification.[75,76] Furthermore, fungal vacuole as the center of transport networks facilitates degradation of nutrient resources, their sorting, transport, and redistribution. Fungal vacuole has been considered as a vital organelle at the heart of fungal physiology.[77] Accordingly, results of this study propose yeast vacuole as an important storage site of essential nutrients needed for *H.pylori* survival and multiplication.

Human gastric epithelial and immune cells have been recognized as the sole specialized eukaryotic cells that host *H.pylori*. However, the very intimate relationship between *H.pylori* and *Candida* yeast in human GI tract could indicate their old and evolutionary association which began long time ago and has led to the establishment of *H.pylori* inside the yeast vacuole as a preadaptation of *H.pylori* for invading and persisting in the vacuole of human cells. This is in agreement with the proposal that association of *H.pylori* with humans likely postdated the evolution of humans and could have happened as a result of host jump from an unknown, nonhuman species which occurred approximately 100,000 years ago or earlier.[78] Accordingly, it is plausible to propose that intracellular life of *H.pylori* in *Candida* yeast could play a crucial role in its colonization in human GI tract as well as persistence in a variety of environmental conditions.

Acknowledgments

This study was funded by the Research Council of University of Tehran and Digestive Disease Research Institute, Tehran University of Medical Sciences. Parts of this study have been presented as three posters in the XXVth International workshop on Helicobacter and related bacteria in chronic digestive inflammation and gastric cancer (2012). The authors have no conflict of interest to declare. The 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. Petersen AM, Krogfelt KA. Helicobacter pylori: an invading microorganism? A review. *FEMS Immunol Med Microbiol.* 2003; **36(3):** 117 – 126.
2. Dubois A, Boren T. Helicobacter pylori is invasive and it may be a facultative intracellular organism. *Cell Microbiol.* 2007; **9(5):** 1108 – 1116.
3. Chu YT, Wang YH, Wu JJ, Lei HY. Invasion and multiplication of Helicobacter pylori in gastric epithelial cells and implications for antibiotic resistance. *Infect Immun.* 2010; **78(10):** 4157 – 4165.
4. Wang YH, Wu JJ, Lei HY. The autophagic induction in Helicobacter pylori-infected macrophage. *Exp Biol Med.* 2009; **234(2):** 171 – 180.
5. Wang YH, Gorvel JP, Chu YT, Wu JJ, Lei HY. Helicobacter pylori impairs murine dendritic cell responses to infection. *PloS One.* 2010; **5(5):** e10844.
6. Thomas JM, Poynter D, Gooding C, Woodings DF, Selway S, Cook AR, et al. Gastric spiral bacteria. *The Lancet.* 1984; **324(8394):** 100 – 101.
7. Chen XG, Correa P, Offerhaus J, Rodriguez E, Janney F, Hoffmann E, et al. Ultrastructure of the gastric mucosa harboring Campylobacter-like organisms. *Am J Clinic Pathol.* 1986; **86(5):** 575.
8. Bode G, Malfertheiner P, Ditschuneit H. Pathogenetic implications of ultrastructural findings in Campylobacter pylori- related gastroduodenal disease. *Scand J Gastroenterol.* 1988; **23(S142):** 25 – 39.
9. Birkness KA, Gold BD, White EH, Bartlett JH, Quinn FD. In vitro models to study attachment and invasion of Helicobacter pylori. *Ann N.Y Acad Sci.* 1996; **797(1):** 293 – 295.
10. Segal ED, Falkow S, Tompkins LS. Helicobacter pylori attachment to gastric cells induces cytoskeletal rearrangements and tyrosine phosphorylation of host cell proteins. *Proc Natl Acad Sci U S A.* 1996; **93(3):** 1259 – 1264.
11. Finlay BB, Falkow S. Common themes in microbial pathogenicity revisited. *Microbiol Molecul Biol Rev.* 1997; **61(2):** 136 – 169.
12. Garcia-del Portillo F, Finlay BB. The varied lifestyles of intracellular pathogens within eukaryotic vacuolar compartments. *Trends Microbiol.* 1995; **3(10):** 373 – 380.
13. Bozue JA, Johnson W. Interaction of Legionella pneumophila with Acanthamoeba castellanii: uptake by coiling phagocytosis and inhibition of phagosome-lysosome fusion. *Infect Immun.* 1996; **64(2):** 668 – 673.
14. Greub G, Raoult D. History of the ADP/ATP-translocase-encoding gene, a parasitism gene transferred from a Chlamydiales ancestor to plants 1 billion years ago. *Appl Environ Microbiol.* 2003; **69(9):** 5530 – 5535.
15. Valdivia RH, Heitman J. Endosymbiosis: The evil within. *Curr Biol.* 2007; **17(11):** R408 – R410.
16. Margulis L, Fester R. *Symbiosis as a Source of Evolutionary Innovation: Speciation and Morphogenesis*. Cambridge: MIT Press; 1991.
17. Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev.* 1995; **59(1):** 143 – 169.
18. 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.
19. Scannerini S, Bonfante P. Bacteria and bacteria- like objects in endomycorrhizal fungi (Glomaceae). *Symbiosis as a Source of Evolutionary Innovation: Speciation and Morphogenesis.* 1991: 273 – 287.
20. Douglas AE. *Symbiotic Interactions*: Oxford University Press Oxford; 1994.
21. Gehrig H, SchÜßler A, Kluge M. Geosiphon pyriforme, a fungus forming endocytobiosis with Nostoc (Cyanobacteria), is an ancestral member of the glomales: Evidence by SSU rRNA analysis. *J Molecul Evol.* 1996; **43(1):** 71 – 81.
22. Moran NA, Degnan PH, Santos SR, Dunbar HE, Ochman H. The players in a mutualistic symbiosis: insects, bacteria, viruses, and virulence genes. *Proc Natl Acad Sci U S A* 2005; **102(47):** 16919 – 16926.
23. Odds FC. Candida infections: an overview. *Critic Rev Microbiol.* 1987; **15(1):** 1 – 5.
24. Rosenbach A, Dignard D, Pierce JV, Whiteway M, Kumamoto CA. Adaptations of Candida albicans for growth in the mammalian intestinal tract. *Eukaryotic Cell.* 2010; **9(7):** 1075 – 1086.
25. Gasch AP, Werner-Washburne M. The genomics of yeast responses to environmental stress and starvation. *Func Integ Genomics.* 2002; **2(4):** 181 – 192.
26. Zwolinska-Wcisło M, Budak A, Bogdał J, Trojanowska D, Stachura J. Fungal colonization of gastric mucosa and its clinical relevance. *Medical Science Monitor: Int Med J Exp Clinic Res.* 2001; **7(5):** 982.
27. Kalogeropoulos NK, Whitehead R. Campylobacter-like organisms and Candida in peptic ulcers and similar lesions of the upper gastrointestinal tract: a study of 247 cases. *J Clinic Pathol.* 1988; **41(10):** 1093 – 1098.
28. Karczewska E, Wojtas I, Sito E, Trojanowska D, Budak A, Zwolinska-Wcislo M, et al. Assessment of coexistence of Helicobacter pylori and Candida fungi in diseases of the upper gastrointestinal tract. *J Physiol Pharmacol.* 2009; **60(6):** 33 – 39.
29. Loster BW, Majewski SW, Czesnikiewicz-Guzik M, Bielanski W, Pierzchalski P, Konturek SJ. The relationship between the presence of Helicobacter pylori in the oral cavity and gastric yeast in the stomach. *J Physiol Pharmacol.* 2006; **57(3):** 91 – 100.
30. Hogan DA, Kolter R. Pseudomonas-Candida interactions: an ecological role for virulence factors. *Science.* 2002; **296(5576):** 2229 – 2232.
31. Siavoshi F, Salmanian AH, Kbari FA, Malekzadeh R, Massarrat S. Detection of Helicobacter pylori-specific genes in the oral yeast. *Helicobacter.* 2005; **10(4):** 318 – 322.
32. Salmanian AH, Siavoshi F, Akbari F, Afshari A, Malekzadeh R. Yeast of

Archive of SID

www.SID.ir

the oral cavity is the reservoir of Heliobacter pylori. *J Oral Pathol Med.* 2008; **37(6):** 324 – 328.
33. Sambrook J, Russell DW. *Molecular Cloning: A Laboratory Manual.* New York: Cold Spring Harbor Laboratory Press; 2001.
34. Lu Y, Redlinger TE, Avitia R, Galindo A, Goodman K. Isolation and genotyping of Helicobacter pylori from untreated municipal wastewater. *Appl Environ Microbiol.* 2002; **68(3):** 1436 – 1439.
35. Atherton JC, Cao P, Peek RM Jr, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific vacA types with cytotoxin production and peptic ulceration. *J Biol Chem.* 1995; **270(30):** 17771 – 17777.
36. Lundstrom AM, Sundaeus V, Bolin I. The 26-kilodalton, AhpC homologue, of Helicobacter pylori is also produced by other Helicobacter species. *Helicobacter.* 2001; **6(1):** 44 – 54.
37. Nikbakht Brujeni G, Jalali SAH, Koohi MK. Development of DNA-designed avian IgY antibodies for quantitative determination of bovine interferon-gamma. *Appl Biochem Biotechnol.* 2011; **163(3):** 338 – 345.
38. Barbieri E, Potenza L, Rossi I, Sisti D, Giomaro G, Rossetti S, et al. Phylogenetic characterization and in situ detection of a Cytophaga-Flexibacter-Bacteroides phylogroup bacterium in Tuber borchii Vittad. ectomycorrhizal mycelium. *Appl Environ Microbiol.* 2000; **66(11):** 5035 – 5042.
39. SchÜßler A, Kluge M. Geosiphon pyriforme, an endocytosymbiosis between fungus and cyanobacteria, and its meaning as a model system for arbuscular mycorrhizal research. In: Hock B, ed. *The Mycota IX*. Berlin: Springer Verlag; 2001: 151 – 161.
40. Bertaux J, Schmid M, Prevost-Boure NC, Churin JL, Hartmann A, Garbaye J, et al. In situ identification of intracellular bacteria related to Paenibacillus spp. in the mycelium of the ectomycorrhizal fungus Laccaria bicolor S238N. *Appl Environ Microbiol.* 2003; **69(7):** 4243 – 4248.
41. Michel R, Burghardt H, Bergmann H. Acanthamoeba, naturally intracellularly infected with Pseudomonas aeruginosa, after their isolation from a microbiologically contaminated drinking water system in a hospital. *Zentralblatt fur Hygiene und Umweltmedizin= Int J Hyg Environ Med.* 1995; **196(6):** 532.
42. Laochumroonvorapong P, Paul S, Manca C, Freedman VH, Kaplan G. Mycobacterial growth and sensitivity to H2O2 killing in human monocytes in vitro. *Infect Immun.* 1997; **65(11):** 4850 – 4857.
43. Marolda CL, Hauröder B, John MA, Michel R, Valvano MA. Intracellular survival and saprophytic growth of isolates from the Burkholderia cepacia complex in free-living amoebae. *Microbiol.* 1999; **145(7):** 1509 – 1517.
44. Saini LS, Galsworthy SB, John MA, Valvano MA. Intracellular survival of Burkholderia cepacia complex isolates in the presence of macrophage cell activation. *Microbiol.* 1999; **145(12):** 3465 – 3475.
45. Martin DW, Mohr CD. Invasion and intracellular survival of Burkholderia cepacia. *Infect Immun.* 2000; **68(1):** 24 – 29.
46. Keig PM, Ingham E, Kerr KG. Invasion of human type II pneumocytes by Burkholderia cepacia. *Microb Pathogenesis.* 2001; **30(3):** 167 – 170.
47. Simon L, Bousquet J, Lévesque RC, Lalonde M. Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. *Nature*. 1993; **363:** 67 – 69.
48. Bianciotto V, Lumini E, Lanfranco L, Minerdi D, Bonfante P, Perotto S. Detection and identification of bacterial endosymbionts in arbuscular mycorrhizal fungi belonging to the family Gigasporaceae. *Appl Environ Microbiol.* 2000; **66(10):** 4503 – 4509.
49. Berk SG, Ting RS, Turner GW, Ashburn RJ. Production of respirable vesicles containing live Legionella pneumophila cells by two Acanthamoeba spp. *Appl Environ Microbiol.* 1998; **64(1):** 279 – 286.
50. Kovacs-Nolan J, Mine Y. Egg yolk antibodies for passive immunity. *Ann Rev Food Sci Technol.* 2012; **3:** 163 – 182.
51. Yan J, Kumagai T, Ohnishi M, Ueno I, Ota H. Immune response to a 26-kDa protein, alkyl hydroperoxide reductase, in Helicobacter pylori-infected Mongolian gerbil model. *Helicobacter.* 2001; **6(4):** 274 – 282.
52. Shin JH, Nam SW, Kim JT, Yoon JB, Bang WG, Roe IH. Identification of immunodominant Helicobacter pylori proteins with reactivity to H. pylori-specific egg-yolk immunoglobulin. *J Med Microbiol* 2003; **52(3):** 217 – 222.
53. Reynders MB, Miendje Deyi VY, Dahma H, Scheper T, Hanke M, Decolvenaer M, et al. Performance of individual Helicobacter pylori antigens in the immunoblot-based detection of H. pylori infection. *FEMS Immunol Med Microbiol.* 2012; **64(3):** 352 – 363.
54. Kanižaj TF, Katičić M, Presečki V, Gašparov S, Cvrlje VC, Kolarić B, et al. Serum antibodies positivity to 12 Helicobacter pylori virulence antigens in patients with benign or malignant gastroduodenal diseases: cross-sectional study. *Croatian Med J.* 2009; **50(2):** 124 – 132.
55. Ye D, Willhite DC, Blanke SR. Identification of the minimal intracellular vacuolating domain of the Helicobacter pylori vacuolating toxin. *J Biol Chem.* 1999; **274(14):** 9277 – 9282.
56. Torres VJ, Ivie SE, McClain MS, Cover TL. Functional properties of the p33 and p55 domains of the Helicobacter pylori vacuolating cytotoxin. *J Biol Chem* 2005; **280(22):** 21107 – 21114.
57. van Doorn LJ, Figueiredo C, Sanna R, Pena S, Midolo P, Ng EK, et al. Expanding allelic diversity of Helicobacter pylori vacA. *J Clin Microbiol.* 1998; **36(9):** 2597 – 2603.
58. Figura N. Helicobacter pylori exotoxins and gastroduodenal diseases associated with cytotoxic strain infection. *Aliment Pharmacol Ther.* 1996; **10(1):** 79 – 96.
59. Cover TL, Blanke SR. Helicobacter pylori VacA, a paradigm for toxin multifunctionality. *Nat Rev Microbiol.* 2005; **3(4):** 320 – 332.
60. Torres VJ, McClain MS, Cover TL. Interactions between p-33 and p-55 domains of the Helicobacter pylori vacuolating cytotoxin (VacA). *J Biol Chem.* 2004; **279(3):** 2324 – 2331.
61. Terebiznik MR, Vazquez CL, Torbicki K, Banks D, Wang T, Hong W, et al. Helicobacter pylori VacA toxin promotes bacterial intracellular survival in gastric epithelial cells. *Infect Immun.* 2006; **74(12):** 6599 – 6614.
62. Johansson M, Lehto M, Tanhuanpaa K, Cover TL, Olkkonen VM. The oxysterol-binding protein homologue ORP1L interacts with Rab7 and alters functional properties of late endocytic compartments. *Mol Biol Cell.* 2005; **16(12):** 5480 – 5492.
63. 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 Helicobacter pylori infection. *Clin Diagn Lab Immunol.* 2002; **9(5):** 1061 – 1066.
64. Schwartz JT, Allen LA. Role of urease in megasome formation and Helicobacter pylori survival in macrophages. *J Leukoc Biol.* 2006; **79(6):** 1214 – 1225.
65. Ricci V, Sommi P, Fiocca R, Romano M, Solcia E, Ventura U. Helicobacter pylori vacuolating toxin accumulates within the endosomal-vacuolar compartment of cultured gastric cells and potentiates the vacuolating activity of ammonia. *J Pathol.* 1997; **183(4):** 453 – 459.
66. Zheng PY, Jones NL. Helicobacter pylori strains expressing the vacuolating cytotoxin interrupt phagosome maturation in macrophages by recruiting and retaining TACO (coronin 1) protein. *Cell Microbiol.* 2003; **5(1):** 25 – 40.
67. Tombola F, Morbiato L, Del Giudice G, Rappuoli R, Zoratti M, Papini E. The Helicobacter pylori VacA toxin is a urea permease that promotes urea diffusion across epithelia. *J Clin Invest.* 2001; **108(6):** 929 – 937.
68. Chae HZ, Robison K, Poole LB, Church G, Storz G, Rhee SG. Cloning and sequencing of thiol-specific antioxidant from mammalian brain: alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. *Proc Natl Acad Sci U S A.* 1994; **91(15):** 7017 – 7021.
69. O'Toole PW, Logan SM, Kostrzynska M, Wadstrom T, Trust TJ. Isolation and biochemical and molecular analyses of a species-specific protein antigen from the gastric pathogen Helicobacter pylori. *J Bacteriol.* 1991; **173(2):** 505 – 513.
70. Olczak AA, Seyler RW Jr, Olson JW, Maier RJ. Association of Helicobacter pylori antioxidant activities with host colonization proficiency. *Infect Immun.* 2003; **71(1):** 580 – 583.
71. Mohammadian T, Doosti M, Paknejad M, Siavoshi F, Massarrat S, Soukhtanloo M. Production of polyclonal antibody against alkyl hydroperoxide reductase of Helicobacter pylori and its antigenicity. *Hybridoma (Larchmt).* 2008; **27(6):** 481 – 485.
72. Chae HZ, Rhee SG. A thiol-specific antioxidant and sequence homology to various proteins of unknown function. *Biofactors.* 1994; **4(3 – 4):** 177 – 180.
73. Mauel J. Mechanisms of intracellular microbicide. *Bull Eur Physiopathol Respir.* 1983; **19(2):** 115 – 122.
74. Fleet GH. Yeasts in dairy products. *J Appl Microbiol.* 1990; **68(3):** 199 – 211.
75. Klionsky DJ, Herman PK, Emr SD. The fungal vacuole: composition, function, and biogenesis. *Microbiol Rev.* 1990; **54(3):** 266.
76. Kane PM. The where, when, and how of organelle acidification by the yeast vacuolar H+-ATPase. *Microbiol Molecul Biol Rev.* 2006; **70(1):** 177 – 191.
77. Veses V, Richards A, Gow NAR. Vacuoles and fungal biology. *Curr Opinion Microbiol.* 2008; **11(6):** 503 – 510.
78. Moodley Y, Linz B, Bond RP, Nieuwoudt M, Soodyall H, Schlebusch CM, et al. Age of the association between Helicobacter pylori and man. *PLoS Pathogens.* 2012; **8(5):** e1002693.

Archive of SID

www.SID.ir