Virulence Factors and Treatment of *Helicobacter pylori* infections

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(With a summary in English)

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Chapter 1

General Introduction

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Overview

The human pathogen *Helicobacter pylori* (*H. pylori*) is a gram negative, S-curved, microaerophilic bacterial species. *H. pylori* measures approximately 1 µm in width and 2 to 4 µm in length and is highly motile through its 2 to 6 uni-polar, 3 µm long sheathed flagella. Upon colonization of the gastric mucosa *H. pylori* causes a chronic inflammation and thereby induces a wide range of gastroduodenal disorders (1). The human stomach seems to be the main niche of infection, and transmission is thought to be predominantly due to person-to-person contact (1). *H. pylori* produces large amounts of the enzyme urease on which the bacterium depends for its survival in the highly acidic gastric lumen (2). The spiral morphology and its flagella make the bacterium highly motile and allow it to penetrate in the viscous mucus layer of the stomach where the pH is less acidic and more nutrients from the gastric mucosa are available, thus facilitating the growth of *H. pylori*. The clinical implication of *H. pylori* infections were recognized in 1983 when Marshall and Warren identified, and subsequently cultured this gastric bacterium (3). It was initially referred to as "*Campylobacter pyloridis*", but later it was reclassified as *Helicobacter pylori* (4). Until then, it was commonly believed that no bacteria could colonize in the stomach, because of the harsh acidic conditions present in this environment. This concept radically changed after the famous self-ingestion experiments by Marshall and Warren in 1983. With this experiment, they showed that *H. pylori* is able to infect and colonize the mucosal surface of human stomach (5). This has convinced many researchers to take a closer look at this putative pathogen and soon it became clear that *H. pylori* is not only capable of colonizing the gastric mucosa, but by doing so it is also inducing a wide range of gastroduodenal disorders (2, 6). It is now generally accepted that within a few weeks after the initial infection by *H. pylori* a superficial gastritis will occur (7). Unless treated, the infection

will last for life and thus result in a chronic gastritis which may ultimately progress in ulceration and possibly even gastric adenocarcinoma (7). *H. pylori* infections occur worldwide and there is an estimated 3.5 billion individuals currently infected with this bacterium (3, 8-11). Moreover, it is estimated that three quarters of the all gastric cancer cases and 90% of duodenal ulcer can be attributed to an *H. pylori* infection (2, 12), but major regional differences exist in infection rates and *H. pylori* related pathology (6). Soon after *H. pylori* was recognized as a gastric pathogen, it was shown that successful eradication of the infection significantly decreased the risk of ulcer formation and gastric malignancy (13). The latter prompted the World Health Organization (WHO) to categorize *H. pylori* as the first bacterial agent that functions a class-I carcinogen (14). After this statement by WHO, many researchers involved in gastroenterology, studied *H. pylori* trying to explain the mechanisms of bacterial virulence association with these gastroduodenal disorders (15-18). While this has resulted in a greatly improved understanding of how *H. pylori* induces chronic infection and the infection associated clinical consequences, there are still many unanswered questions.

Virulence of *H. pylori*

In spite of the major research efforts worldwide, there is still a lack of knowledge on the specific virulence factors of *H. pylori* which determine the pathogenesis of this microbe. In part this is due to i) humans being the only known host of *H. pylori* and thus animal models are mostly useless, and ii) the association of specific virulence factors with gastroduodenal diseases being often only of regional value and/or being only assessed for small local populations and thus might not be of general value. Although also sometimes the duodenum can become infected, the predominant natural niche of *H. pylori* is the mucus layer of the human stomach. For many centuries it was thought that the stomach is sterile. This dogma was abandoned when it became

clear that *H. pylori* can survive there for decades. In order to establish a successful *H. pylori* infection, the bacterium needs to first passage through the highly acidic lumen of the stomach (2). It is thought that flagellar movement of the bacterium is crucial for both a rapid passage of the lumen and the subsequent entry of the mucosal layer. Thus, adhesion to the mucosal layer or the epithelial cells alone is not enough to fulfil the requirement of a successful colonization. The bacterium needs to constantly reattach to the newly formed mucus and epithelial cells in order to avoid shedding into the gastric lumen. While less acidic than the lumen, the pH of the mucus layer is still very low and thus *H. pylori* has also to overcome these acidic conditions. It does so by the production of the enzyme urease. The urease enzymes can sometimes comprise up to 10% of the total bacterial protein content which reflects the importance of this enzyme for this bacterium (15). The low pH can also be of advantage to this bacterium as it has an adverse effect on other competitive colonizers of the stomach. In addition, it has an adverse effect on the stability of the antimicrobials used to eradicate *H. pylori* infection. In some cases, persistent colonization after treatment with antimicrobials is not the result of the antimicrobial resistance but *H. pylori* may occasionally enter the epithelial cells or Candida spp present in the stomach, and in this way being protected from the action of some antimicrobials (27, 28, 29). Another mechanism whereby the bacterium can potentially escape from antimicrobial therapy is that *Helicobacter pylori* can transform from its normal helical to the coccoid shape (19). In normal biopsy samples from patients as well as during prolonged culture of this bacterium coccoid forms have been observed. Some believe that these coccoid forms play a crucial role in helping the bacteria to survive in harsh acidic environment in stomach or during transmission (3, 19); others believe it is the morphological manifestation of dead bacteria. If indeed the coccoid forms represent a dormant form, their metabolism will be relatively low and thus they may become

relatively resistant to some antimicrobials. In addition to the aforementioned flagella, CagA (cytotoxin-associated gene A), vacA (vacuolating cytotoxin A), and urease production there are many other determinants that have been claimed as *H. pylori* virulence factors involved in the colonization of the host (1). These include factors that modify the host immune response and/or induce host cell death. This will be discussed in more details below and in the various chapters of this thesis.

Pathogenesis of *H. pylori*

The human gastric pathogen *H. pylori* is able to establish a successful infection in such hostile environment of stomach. For several *H. pylori* virulence factors it has now unambiguously been established that they are involved in bacterial pathogenesis (23). Also it is accepted that the clinical outcome of an *H. pylori* infection is dependent on the complex scenario of interaction between these bacterial factors and host determinants (24). These established virulence factors include the vacuolating toxin (*vacA*), bacterial enzymes (urease, proteases and phospholipases), and the immunogenic protein CagA (25).The *cagA* gene encodes a 120-145-kDa protein, and is a marker for the *cag* pathogenicity island (*cag* PAI). Patients infected with $CagA^+$ strains usually have more gastric inflammation and are significantly more at risk for developing peptic ulcer or gastric cancer. The *cag* PAI encodes 18 proteins that serve as the building blocks of a syringe-like structure (type IV secretion apparatus) which facilitates the translocation of CagA, peptidoglycan, and possibly other bacterial factors into host cells (26). Once delivered inside the cell, the CagA interacts with a range of host signaling molecules, which results in morphological changes and the induction of pro-inflammatory cytokines in epithelial cells (3). Approximately 50% of all *H. pylori* strains secrete VacA, a highly immunogenic 95-kDa toxin that binds to host cells and after being internalized induces massive vacuolization in epithelial cells *in vitro* (27). In

addition VacA also induces disruption of endosomal and lysosomal activity, and it interferes with cytoskeleton-dependent cell functions, and it induces apoptosis of the host cells (1, 25, 27). Although all *H. pylori* strains carry a *vacA* gene, there is considerable variation in vacuolating activities among strains due to the sequence heterogeneity within the *vacA* gene (28). Especially the *vacA s1/m1* genotypes are associated with an increased risk for peptic ulceration and gastric carcinoma. The urease enzyme is the main component of *H. pylori* acid resistance as it converts urea into ammonia and carbamate (3). Urease activity is present in all *H. pylori* isolates, but there is levels of urease activity differ significantly between strains and are also dependent on the growth conditions (16). The ammonia produced by the degradation of urea by this enzyme not only increases the pH, but also has a cytotoxic effect on gastric epithelial cells (3, 15, 29). While for the classical virulence factors like CagA, VacA, and urease the role in the pathogenicity is fairly well established, for others this is not the case. Although the clinical outcome of the infection is thought to be determined by host, bacterial and environmental factors (23), the exact mechanisms that determine the clinical outcome of the infection are not clear. On the host site the immune status and host acid secretion seem critical determinants in this process (1, 6). On the bacterial side virulence factors like *dupA*, *babA*$_2$, *oipA*, *homB, cagA, vacA* and *iceA* have been shown to affect the development of post-infection disorders (3).

Transmission of *H. pylori*

Even though there is no evidence for a zoonotic transmission of *H. pylori*, it is believed that the ancestral *H. pylori* strain was living in gastric epithelium of vertebrates (3, 29, 30). Several studies have investigated the possible role of *H. pylori* in the oral cavity and dental plaque but they mostly just confirmed the presence of bacterium in oral secretions and not actual colonisation of the oral cavity. While it is still not known exactly how *H. pylori* can be

transmitted, it is thought to occur by direct contact (human-to-human) (29, 30). The initial acquisition of *H. pylori* is thought mostly to occur during the early childhood and most children are infected by their parents (31). In spite of the fact that the majority of new infections is during childhood there is also an increase in incidence with increasing age in adults. In the recent past there was a high percentage of *H. pylori* infection that was probably due to lesser socioeconomic status and reduced hygiene.

Treatment of *H. pylori*

H. pylori infection is highly prevalent worldwide and considered as a key pathogen in diverse gastric pathology. Successful eradication therapy reduces *H. pylori*-associated disorders and even cure peptic ulceration (16). Therefore it is advised that all infected patients are being treated (34, 35). Antimicrobial resistance is the main cause of treatment failure which is strongly associated with a sharp decline in eradication rates (16, 17, 20). It is common for the first line therapy to treat with a combination of a proton pump inhibitor, clarithromycin and either amoxicillin or metronidazole. Furthermore, ciprofloxacin can be used in second or third line therapeutic regimens to increase their efficacy rates. The choice of the preferred combination of antimicrobials is predominantly dependent on local differences in the prevalence of antimicrobial resistance. Treating all positive individuals is probably not an option because the high prevalence of *H. pylori* would certainly result in high rates of antimicrobial resistance and treatment failures. Additionally, for pharmacological reasons, there are only few antimicrobials that can be used to treat *H. pylori* and resistance to these antimicrobials is already high. In addition one needs to combine two or three antimicrobials and an antacid in order to achieve successful *H. pylori* eradication. Although *H. pylori* resistance rates is no longer as rapidly increasing as it was in the past, it still remains very high (21). Having up to date information regarding *H. pylori* resistance

rate among the different regional populations is thus mandatory to provide a guideline for selecting the best local therapeutic regimens against *H. pylori*.

Antibiotic therapy against *H. pylori*

Having a treatment protocol with of a single drug sounds attractive and indeed for most bacterial infections monotherapies provides an efficient way of dealing with these infections. For *H. pylori* infections the efficacy of various drugs in monotherapy has been investigated. Only for clarithromycin some reasonable results have been obtained with monotherapy, but these were not sufficient to eradicate *H. pylori* (16). Since usage of a single antimicrobial for eradicating *H. pylori* was not successful, combinations with different antimicrobial formulas have been initiated (18, 30). Eventually, numerous different regimens against *H. pylori* infection have been tested and efficacy differs due to local resistance status (22, 32, 33). Obviously, the selection of antimicrobials for the optimal therapeutic regimen is further complicated by factors such as patient compliance, cost, drug side effects, and unknown status of antimicrobial resistance. Below the use of different combination regimens (dual, triple, and quadruple therapy) against *H. pylori* infections in Iran are described.

***Helicobacter pylori* Infection in Iran**

The prevalence of *H. pylori* infections can differ drastically both within and between countries and Iran is not an exception to this. *H pylori* is highly prevalent in the developing countries and is common in 57%-91% of Iranian population (Figure 1) (34). Thus in Iran the prevalence of *H. pylori* is higher than western countries (30-45%) and almost equal to eastern countries (80-95%) (35-38). With approximately 76 million inhabitants Iran has one of the largest populations in the Mid-East. Its relative high ulcer and gastric cancer rate has been a major trigger to perform local

studies on *H. pylori* pathogenesis, treatment, and prevalence. Mikaeli *et al* (39) claimed that the prevalence of *H. pylori* in Yazd and Ardebil are 30% and 47%, respectively and that there is a significant association between occurrence of gastric cancer and high prevalence of *H. pylori*. Local differences in the infection rate seem to exist that cannot be explained by methodological differences. Most likely the observed differences reflect better diagnostic/therapeutic approaches and improvements in hygiene and life style (40).

Figure 1. High percentage of conducted research at the small region in north of Iran

Table 1. Overview of studies evaluating the prevalence of *H. pylori* in Iran

Authors	**State/Province**	**Prevalence of *H. pylori* [%]**	**Year**	**Reference**
Mikaeli *et al*	Yazd	30	1999	(39)
Mikaeli *et al*	Ardebil	47	1999	(39)
Moghaddam	Mashhad	85	2010	(41)
Moghaddam *et al*	Mashhad	62	2005	(42)
Mansour-Gh *et al*	Rasht	40	2009	(43)
Metanat *et al*	Zahedan	34	2010	(44)
Nasrolahi *et al*	Mazandaran	46	2008	(45)
Talebi *et al*	Mazandaran	88	2011	(46)
Talebi *et al*	Mazandaran	93	2012	(47)
Milani *et al*	Tabriz	28	2012	(48)
Khameneh *et al*	Urmia	47	2011	(49)
Rasmi *et al*	Urmia	65	2011	(50)

The highest infection rates for *H. pylori* are reported in the North of Iran [State of Mazandaran] where in a recent paper it was estimated to be 93% (Table 1). The prevalence of *H. pylori* is much less in southern Iran (e.g. 34% Zahedan and 30 % in Yazd) (44). In the Mazandaran province the infection rate exceeds 90% (51) (Figure 1) and there is a high number of patients with gastrointestinal complaints. This high *H. pylori* prevalence facilitates the collection of a large number of strains from a well defined, small geographical region that facilitates to perform comprehensive studies on the putative association between the association of bacterial virulence factors and clinical outcome of *H. pylori* infection.

Treatment in Iran

It has been shown that successful eradication of *H. pylori* infection prevents the recurrence of duodenal ulcer and other disorders but it is still under debate if eradication will also reduce the *de novo* genesis of gastric carcinoma (52). In spite of all the efforts to create an optimal treatment protocol the normal cure rate of *H. pylori* infections is still much lower than those for most other bacterial infections. Due to increasing levels of antimicrobial resistance there even seems to be a global decline in the rate of successful treatment over the last years (53). The existing guidelines suggest to only use therapeutic regimens that result in an *H. pylori* infection eradication rate up to 80-90%, but mostly only efficacy data for western countries are available (16, 54). Most of these therapeutic failures are reported in the first-line treatment against *H. pylori*. Treatment usually consists of a so-called triple therapy, a combination of two antimicrobials and a proton pump inhibitor (52). Often empirical/standard regimes are used that have not been optimized to the antimicrobial resistance of the infecting strains. In addition, due to the severe side effect of these combination therapies, patients are often not motivated to complete the course (55). The resulting poor compliance not only results in therapy failure, it also is a major factor in the induction of antimicrobial resistance (54, 56). Given the high level of antimicrobial resistance against metronidazole, and clarithromycin in Iran (20) the generic western therapeutic protocols might need some tweaking, but to our knowledge no optimal therapeutic regimen for Iran has been reported. Below, the various generic treatment options for *H. pylori* will be discussed and while these are mostly based on data from Western countries we will wherever possible elaborate on the local situation in Iran. The combination of a proton pump inhibitor with one or two antimicrobials (clarithromycin and amoxicillin) has been suggested as the best first line therapy to eradicate *H. pylori* infections (52, 57). The efficacy for dual therapy in Iran was tested in two

studies (58). Both studies found that while the eradication rates were not acceptable (<50%) there was a significant reduction of the clinical symptoms. In the past, also metronidazole was frequently used in dual therapy, but currently the use of metronidazole in anti-*H. pylori* therapy is significantly reduced as a result of the high incidence of *H. pylori* strains that are resistant to this drug. It is to be expected however that due to the increase in clarithromycin usage, and hence the expected induction of resistance against clarithromycin, the efficacy of this regime will rapidly decrease. In addition the efficacy rate of dual therapy is ~30% less than triple therapy among the symptomatic and asymptomatic Iranian populations (33, 59). The first findings with triple therapy were satisfactory, although some small differences were observed between these studies (60). Proton pump inhibitor-based triple therapy (with eradication rate >80% in European countries) only showed 50% efficacy rate among the Iranian population (58). Globally, the combination of proton pump inhibitor with amoxicillin and clarithromycin is the most commonly prescribed triple therapy and this combination also seemed to work well in Iran (32). However, recent studies on triple therapy indicate a drastic decrease in eradication rate when compared to older studies (61). As with all therapies the efficacy of the therapy is highly dependent on the primary resistance of the strains present in the patients treated. It was shown that eradication rates are relatively high (88%) if strains are clarithromycin sensitive but decrease significantly (down to 19%) if strains were clarithromycin resistant (62). Another important contributor to the efficacy of the therapy is the optimal duration of treatment. In a meta-analysis from Calvet *et al* it was shown that two weeks therapy resulted in a 10% better eradication than a 7 days therapy (63). While longer regimens are more beneficial from the eradication perspective, they also result in more pronounced side effects for the patients treated. Also studies in Iran showed that 2 weeks of treatment is more effective for *H. pylori* eradication than shorter treatment periods (61).

In a study by Najafi *et al* (64) furazolidone plus amoxicillin with a proton-pump inhibitor showed high eradication rate among Iranian children. Especially with children special care should be taken to keep the treatment as short as possible since with children the compliance is highly dependent on the length of the therapy. The length of the treatment is often enhancing the putative adverse effects for the patient, resulting in premature termination of the therapy and as a consequence induction of resistance of the infecting *H. pylori* strain. Thus shortening the duration of the therapy is of utmost importance in order to avoid over exposure and to minimize the induction of resistance. Quadruple therapy regimens consist of a H_2 receptor blocker, or a proton pump inhibitor, combined with three antimicrobial agents (38) rendering it more effective than triple therapy. Most Iranian studies that examined the effect of quadruple therapy used a two week treatment regime, and they reached a high efficacy rate (>90%) (65). Fakheri *et al* reported the quadruple therapy with 80% efficacy rate, which can increase with dose, duration and better administration, accordingly (66). A recent study in Iran showed that a 2-week quadruple therapy containing high-dose furazolidone had been as successful as a clarithromycin-containing sequential therapy (85%) (67). A problem with the complex composition and dosing of quadruple therapy is that patient compliance is often lower than with dual and triple therapies (68). Recently several simplified quadruple therapies with twice-daily regimens have been tested in Iran. The compositions of these regimens were bismuth subcitrate plus amoxicillin or tetracycline, and metronidazole or furazolidone, and clarithromycin or azithromycin and both studies show that these simplified quadruple therapies have better patient compliance without losing their effectiveness.

Outline of this thesis

This thesis describes the major clinical concerns of *H. pylori* strains among the Iranian population. The first part of this thesis (chapter 2-7) describes the frequency of different virulence factor of *H. pylori* isolated from an ethnic population in Northern Iran. Furthermore, it describes the association between various virulence factors and disease outcomes. The second part of this thesis (chapters 8 and 9) describes the prevalence of antimicrobial resistance of *H. pylori* among the dyspeptic patients in Iran. Established antimicrobials as well as newly introduced drugs like a moxifloxacine are evaluated. Finally in chapter 10, we will provide a general discussion of the findings in this thesis and proper outline for *H. pylori* research in future.

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Chapter 2

Low Frequency of *cagA*-Positive *Helicobacter pylori* Strains Isolated from Iranian Patients with MALT Lymphoma

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Low frequency of *cagA*-positive *Helicobacter pylori* strains isolated from Iranian patients with MALT lymphoma

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Abstract *Helicobacter pylori* is predominantly involved in the etiology of digestive diseases. The aim of our study is to determine the relationship of *cagA* frequency with less investigated gastroduodenal disorders such as MALT (mucosal associated lymphoid tissue) lymphoma and gastric cancer. One hundred-twenty eight *H. pylori*-positive patients including: gastritis ($n = 74$), gastric cancer ($n = 26$) and MALT lymphoma ($n = 28$) were entered in our study. Antral biopsy specimen transport, bacterial culture and *cagA* detection were performed based on standard protocols. In brief, biopsies from positive *H. pylori* patients were investigated for presence of *cagA* gene by polymerase chain reactions (PCR) method. Of 128 consecutive Iranian patients with gastroduodenal disorders examined in our study, we identified 84 (65.6%) *cagA*-positive strains. However, six patients were excluded because of negative culture for identification of *H. pylori*. Prevalence of *cagA* in each categorized groups are following: 63/74 (85.1%) of gastritis patients, 16/28 (57.1%) and 5/26 gastric cancer (19.2%) of MALT lymphoma, respectively. Current findings reveal that the presence of *cagA* is not a reliable marker for prediction of digestive disorders caused by *H. pylori* infection. All our patients with gastric cancer were diagnosed as adenocarcinoma. The low rate of *cagA* among gastric cancer and MALT lymphoma groups was not statistically significant, possibly due to the small number of patients enrolled in the study. We suggest that a study with a high number of patients is needed for making more definitive assessment of the correlation between *cagA*-positive *H. pylori* and gastric cancer and MALT lymphoma.

Keywords *Helicobacter pylori* · *cagA* · Gastric cancer · Iran

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Introduction

Helicobacter pylori (*H. pylori*) is a gram negative, microaerophilic bacterium that can cause a diverse range of digestive disorders in infected patients [1]. *H. pylori* isolates may colonize more than 50% of the world's human population [1]. The majority of infected individuals show an asymptomatic chronic gastritis [2], despite the fact that the pathology of the gastric biopsy specimens may show signs of inflammation in gastric epithelial mucosa [3]. In fact, among the 5% of patients infected with *H. pylori*, gastroduodenal diseases will progress to malignant aspects, while almost 40–50% can develop peptic ulcer diseases [4]. *H. pylori* virulence factors, environmental determinants and population genetics are three major factors that may determine the outcome of infection [4]. Investigators have shown that *H. pylori*-specific virulence factors may have a significant relationship with the development of digestive diseases in infected patients [5–7]. Among the bacterial pathogenesis determinants, the cytotoxin-associated gene (*cagA*) that resides on a pathogenicity island (PAI) is the most important factor that has been recognized to this point [4, 8]. It has been proposed that the *cagA*-PAI is a 40 kb region that is responsible for encoding a type IV secretion system (T4SS), a complex apparatus that mediates the translocation of bacterial virulence factors into the gastric

epithelial cells [9, 10]. The *cag-PAI* gene positive strains have been shown to be active in induction of pro-inflammatory cytokines released by gastric cells [11], a critical fact that can explain why infected patients with *cagA* harboring strains tend to have more severe clinical presentations [10]. In the case of *cagA*, studies suggest that strains harboring the *cagA* gene are more virulent, and are strongly associated with peptic ulcer diseases and gastritis [4, 12, 13]. It has been observed that a significant correlation exists between *cagA*-positive isolates and the severity of clinical outcomes after infection [12–14]. Meanwhile, the MALT lymphoma is correlated with *H. pylori* colonization [4, 15, 16].To our knowledge, the prevalence of *cagA*(+) *H. pylori* among the Iranian MALT lymphoma patients has not been investigated. It has been established that the genetic diversity of *H. pylori* strains isolated from individuals in diverse geographical regions are quite variable [6, 7, 17]. The aim of this study is to determine the association of *cagA* among the less investigated disorders such as MALT lymphoma and gastric cancer.

Methods

Patients

A total of 134 patients were enrolled between November 2009 and October 2010 at Tooba Medical Center, Sari, Iran. Seventy-four patients presented with gastritis, 28 with gastric cancer and 26 with MALT lymphoma. None of the patients had taken antibiotics or anti-secretory drugs for the prior 4 months. Patients less than 14 years of age with negative results for *H. pylori* on culture, or who underwent prior gastrointestinal surgery were excluded from our investigation. All participants signed informed consents before endoscopic procedures were undertaken. Our study was approved by the ethics committee of Tarbiat Modares University, Tehran, Iran.

Bacterial culture and PCR assay for *cagA*

Two antral biopsies were taken from each patient, and placed in a sterile thioglycolate broth (Merck, Germany) and sent to diagnostic clinical microbiology laboratory at 4°C temperature. For culture purpose, biopsy specimens were ground and 100 µl of homogenate was streaked onto Colombia agar (Merck, Germany) plates supplemented with 10% fetal calf serum (FCS), 7% sheep blood and selective antibiotics (MAST, UK). Plates were incubated at 37°C, under 7% CO_2 and high humidity for 9 days [4]. After the incubation period, *H. pylori* was identified by colony morphology, gram's staining, and were additionally subjected to routine biochemical tests including catalase, urease and oxidase. In this study, *H. pylori* ATCC 43504 reference strain was used as a positive control. Bacterial genomic DNA was extracted by a commercially available kit (Roche, Germany) according to manufacturer's instructions with minor changes. Extracted DNA was stored in −20°C for further genotyping analysis. Primer sequences, sizes, and conditions of PCR amplifications for detection and confirmation of *H. pylori* were designed as published data [4, 6, 7]. Presence of expected PCR product bands was considered as criteria for regarding a positive strain for *cagA* gene. To confirm the validity of our genotyping results, we blindly repeated PCR testing for 30 of our isolates.

Statistical analysis

SPSS version 15.0 software was used for statistical analysis. Fisher exact and χ^2 tests were used for finding any statistically significant relationship, while a P value less than 0.05 was considered as significant.

Results

Confirmation of *H. pylori* and *cagA* identity

In the current study, we performed a PCR assay for *glmM* with specific primers that yielded a sharp 294 bp product for validation of our strains and for *cagA* detection between our samples. All of our *cagA*-positive strains showed a sharp band after electrophoresis on 2% gel agarose (Sinagen, Iran).

Detection of *cagA*

134 patients with digestive disorders were admitted to Tooba Medical Center in Sari, Iran, and were investigated for the possible presence of *H. pylori*. Of the 134 patients tested, 6 tested negative, and were excluded. The remaining 128 patients were categorized into three disease groups: gastritis ($n = 74$), gastric cancer ($n = 28$) and MALT lymphoma ($n = 26$) based on the pathology examination of gastric biopsies. Overall, our findings indicate that 84/128 (65.6%) of all isolates were *cagA* positive, while 63/74 (85.1%) of gastritis patients, 16/28 (57.1%) of gastric cancer and 5/26 (19.2%) of MALT lymphoma patients were scored as being infected with *cagA*-positive strains (Table 1).

The prevalence of *cagA* was the highest in gastritis patients (85.1%) and decreased in MALT lymphoma patients (19.2%), and those with gastric cancer 16/28 (57.1%) (Table 1). In our study, no difference between sex

Table 1 Prevalence of *cagA* genotype of *H. pylori* in different diseases groups with demographic data

Disease groups	Age group	Number	*cagA* positive (%)	*cagA* positivity		*P* value
				Women	Men	
Gastritis	15–17	14	12 (85.7)	5	7	0.01
	18–20	19	15 (78.9)	8	7	0.04
	21–23	19	16 (84.2)	8	8	0.002
	24–25	22	20 (90.2)	12	8	0.0009
MALT lymphoma	15–17	7	2 (28.5)	1	1	0.56
	18–20	9	1 (11.1)	1	0	0.14
	21–23	6	2 (33.3)	2	0	0.01
	24–25	4	0 (0)	0	0	0.87
Gastric cancer	<25	2	0	0	0	ND
	26–35	7	3 (33.3)	1	2	0.32
	36–45	1	0	0	0	ND
	46–55	12	9 (75)	4	5	0.41
	56–65	6	4 (66)	2	2	0.5

ratio and *cagA* positivity were observed ($P > 0.05$) (Table 1), although our sample size in non-gastritis groups (MALT lymphoma and gastric cancer) were not adequate to perform a significant statistical analysis (Table 1). Based on pathologic findings, all of the gastric cancer patients were categorized as adenocarcinoma subjects. In MALT lymphoma patients, prevalence of *cagA* was not significantly associated with disease ($P > 0.14$) with the exception of the 21- to 23-year-old patients ($P = 0.01$). Prevalence of *cagA* in the 24- to 25-year-old group (Table 1) was the highest (90.2%) in our study, although other age groups among the gastritis patients had a prevalence higher than 78.9% (Table 1). In the gastric cancer and MALT lymphoma groups, a low rate for *cagA* genes was noted, while in the 46–55 age group of gastric cancer patients 75% of strains were *cagA* positive ($P = 0.41$) (Table 1).

Discussion

In the current study, the frequency of *cagA* gene in *H. pylori* strains isolated from Iranian patients with diverse gastrointestinal disorders was investigated. Prevalence of *cagA* in different geographical area is still under debate. Additionally, it has been suggested that presence of *cagA* is strongly associated with different gastroduodenal diseases [14, 18]. Previous findings indicate a specific pattern of *cagA* distribution in different populations of five continents [18–21]. Herein, we determined the prevalence of *cagA* among individuals originating from the northern side of our country who developed the more severe gastroduodenal complications such as gastric cancer [22, 23]. In our investigation, we collected the largest sample of biopsy specimens for determination of gastritis ($n = 74$) and MALT lymphoma ($n = 28$) in Iran, which enabled us to determine an evaluation of the frequency of *cagA* among MALT lymphoma and gastritis patients. In our study, all of gastric cancer cases were diagnosed as adenocarcinoma. In accordance with a previous study of 128 patients in Iran [24], we detected a total of 65% (84/128) of patients harboring *H. pylori* who were *cagA* positive without

Table 2 Distribution of *cagA* in *Helicobacter pylori* isolated from different area of world in comparison with our study

Authors	Country	Year of study	(%) *cagA*	Reference
Current study	Iran[a]	2010	65	–
Bazargani et al.	Iran	2005	59	[25]
Yamazaki et al.	Japan	2005	90	[13]
Tiwari et al.	India	2005	92	[26]
Safaei et al.	Iran	2008	50	[27]
Erzin et al.	Turkey	2006	50	[28]
Douraghi et al.	Iran	2009	77	[29]
Oleastro et al.	Portugal	2003	58.5	[33]
Chomvarin et al.	Thailand	2008	96	[41]
Paniagua et al.	Mexico	2009	39.2	[32]
Dabiri et al.	Iran	2009	73	[31]
Jafarzadeh et al.	Iran	2007	67	[24]
Talebkhan et al.	Iran	2008	89	[36]
Douraghi et al.	Iran	2008	84	[30]
Torres et al.	Cuba	2009	73	[34]
Yakoob et al.	China	2002	75	[37]
Lin et al.	Taiwan	2004	83	[38]
Warburton et al.	England	1998	68	[35]
Jafari et al.	Iran	2008	76	[39]

[a] Different studies from Iran indicating various results

considering subgroup categories of the patients studied. Table 2 shows the results of previous investigations of the distribution of the *cagA* gene among gastroduodenal patients surveyed from various geographical locations around the world [24–41]. With regard to *cagA* status in our *H. pylori* strains, the 65% frequency of this putative virulence gene was moderate as compared to other Iranian studies [24, 25]; none of the previously published Iranian studies report more than 85.1% prevalence of *cagA* among *H. pylori* infected gastritis patients, which represents a new finding observed in our study [24, 25]. In the MALT lymphoma patients group, we observed a low rate for *cagA* among *H. pylori* strains, a finding also noted in the gastric cancer group (Table 1). These findings suggest that in our population, *cagA* cannot be a predominant virulence factor among the MALT lymphoma and gastric cancer patients in contrast with an earlier study [18]. No differences were observed between the status of *cagA* positivity and gender ($P > 0.05$). Among the gastritis patients, we have detected a high frequency of *cagA* between our patients (Table 1). All age groups in gastritis patients display a significant association with the presence of *cagA*, but in the 24- to 25-years-old patients, we observe a strong presence of *cagA* ($P = 0.0009$). Studies from other countries, showed diverse results between *cagA* positivity between different geographic populations; for example, the prevalence in India [26], Iran [27] and Turkey [28] were 92, 50 and 50%, respectively. The distribution, however, of *cagA* in Thailand (96%) [41] and Turkey (50%) [28] shows a diverse range in Asia regarding the *cagA* prevalence (Table 2). However, the total rate for *cagA* in Western countries varies between 50 and 70% [4, 28, 33, 35]; while these rates ranged from 60 to 97% among the Asian countries [13, 28, 37, 38, 41].The presence of a significant number of gastric cancer and MALT lymphoma patients identified with *cagA*-positive *H. pylori* strains was an unsuspected finding in a worldwide investigation [2, 4, 40]. In a study conducted by Malekzadeh et al. [22], findings suggest that in the northern provinces of Iran, including Sari, Mazandaran, where we undertook our study there is a high rate of gastric malignancy. To our knowledge, no former study was carried out for the determination of the prevalence of *cagA*-positive *H. pylori* between MALT lymphoma and gastric cancer patients in Iran. Moreover, studies from outside of Iran are not frequent. Since only low numbers of MALT lymphoma patients are available, scientists were not inclined to investigate the virulent genotypes of *H. pylori* in patients from this special group. It has been reported that no association was observed between *cagA* genotype and gastroduodenal disorders in Iran [27]. Our results show that there is no correlation between *cagA* genotypes isolated from *H. pylori* recovered from MALT lymphoma patients.

Conclusion

In conclusion, our data show a low prevalence of *cagA* among MALT lymphoma and gastric cancer patients, a data inverse to what occurs in our gastritis patients. Further studies with a larger number of participants might enable a clearer assessment concerning the association between *cagA*-positive *H. pylori* genotypes with different gastroduodenal disorders such as MALT lymphoma.

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Conflict of interest None.

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Chapter 3

Infection with *Helicobacter pylori* Strains Lacking *dupA* is Associated with an Increased Risk of Gastric Ulcer and Gastric Cancer Development

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Infection with *Helicobacter pylori* strains lacking *dupA* is associated with an increased risk of gastric ulcer and gastric cancer development

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Recently, *dupA* was reported as a new virulence factor in *Helicobacter pylori*, but its association with gastroduodenal disorders and its mode of action are still unclear. Here, an association of the *dupA* status with different disease groups was determined and a biological explanation for the observed associations was tested. In total, 216 *H. pylori* isolates were obtained from 232 presumed *H. pylori*-infected patients. A positive association was observed between the occurrence of duodenal ulcer (DU) and the presence of *dupA* [odds ratio (OR) 24.2; 95 % confidence interval (CI) 10.6–54.8]. In addition, an inverse association between the occurrence of gastric cancer (GC) [OR 0.16; 95 % CI 0.05–0.47] and gastric ulcer (GU) [OR 0.34; 95 % CI 0.16–0.68] with the presence of *dupA* was observed. A putative explanation for the observed associations might be a more corpus-located infection (pan-gastritis) by the *dupA*-positive strains due to their increased acid resistance. Indeed, a strong association between *dupA*-positive *H. pylori* isolated from gastritis patients and *in vitro* acid resistance was observed ($P<0.05$). The observed higher acid resistance of the *dupA*-positive strains suggests that these strains are adapted to a stomach with high gastric acid output. This may in part explain the observed associations, as an increased gastric acid output is thought to be typical for an antrum-predominant *H. pylori* infection and, whilst this is associated with an increased risk of DU formation, it also decreases the risk for the genesis of GUs and GC.

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INTRODUCTION

Helicobacter pylori colonization in humans is considered to play a critical role in the genesis of a wide array of gastroduodenal diseases including gastritis, peptic ulcers and gastric cancer (GC) (Kusters *et al.*, 2006). Although the clinical outcome of the infection is thought to be determined by host, bacterial and environmental factors (Mobley, 1997; Yamaoka, 2010), the exact mechanisms that determine the clinical outcome of the infection are still unknown. On the host side, immune status (Robinson *et al.*, 2007) and host acid secretion seem to be critical determinants in this process (Sobala *et al.*, 1991; Kuipers *et al.*, 1995). On the bacterial side, virulence factors such as $babA_2$, *oipA*, *cagA* (a marker of the pathogenicity island) and *iceA* have been shown to affect the development of post-infection disorders (Covacci *et al.*, 1999; Atherton, 2006). Recently, duodenal ulcer-promoting gene (*dupA*) has been proposed as a novel *H. pylori* virulence factor associated with an increased rate of occurrence of duodenal ulcer (DU) and a decreased risk for GC (Lu *et al.*, 2005). The DupA protein is a homologue of the VirB4 ATPase (Lu *et al.*, 2005; Gomes *et al.*, 2008) and probably represents an outer-membrane protein of a type IV secretion system.

In addition to the *cagA* pathogenicity island-encoded VirB4 homologue (Hp0544), the total genomic sequence of *H. pylori* strain 26695 revealed the presence of three additional VirB4 homologues (HP0017, HP0441 and HP0459) (Tomb *et al.*, 1997). The sequenced genome of strain J99 revealed the presence of an additional copy of a VirB4 homologue

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Abbreviations: CI, confidence interval; DU, duodenal ulcer; G, gastritis; GC, gastric cancer; GU, gastric ulcer; OR, odds ratio.

(jhp0917/0918) that is not present in strain 26695. Whilst originally designated two separate genes (*jhp0917* and *jhp0918*), they were subsequently shown to form one continuous ORF that is present in a substantial fraction of the tested *H. pylori* strains (Lu *et al.*, 2005). Due to its putative association with DU formation, the gene was named *dupA* (Lu *et al.*, 2005). The biological function of DupA has thus far not been identified, but *in vitro* studies indicate that DupA enhances survival rates at low pH and induces interleukin-8 production (Lu *et al.*, 2005). Recently, the ability of *dupA*-positive strains to induce *in vitro* interleukin-8 production has been disputed (Schmidt *et al.*, 2009). Whilst *dupA* is associated with disease outcome in various populations, this relationship is inconsistent among studies (Arachchi *et al.*, 2007; Argent *et al.*, 2007; Douraghi *et al.*, 2008; Zhang *et al.*, 2008; Nguyen *et al.*, 2010). Nevertheless, a recent systematic review suggested that these inconsistencies might in part be due to the small numbers of isolates that were included in the individual studies, although alternatively they might be due to regional and ethnic differences (Hussein, 2010).

Iran is a location with a high prevalence of *H. pylori* infection (Massarrat *et al.*, 1995; Talebi Bezmin Abadi *et al.*, 2009), especially in the Mazandaran province (state of Sari) where the infection rate exceeds 90 % (Talebi Bezmin Abadi *et al.*, 2009, 2010). The high prevalence of infection allowed us to collect a large number of *H. pylori* strains from a narrow geographical region in order to perform a comprehensive study of the putative association between *dupA* status and the clinical outcome of *H. pylori* infection.

METHODS

Patients. All patients with gastroduodenal complaints who visited the medical centres at Sari, Mazandaran province, Iran, between May 2007 and March 2010 for an endoscopic evaluation of putative *H. pylori* infection were invited to participate in this study. The study was approved by the ethical review board of the Tarbiat Modares University, Tehran, Iran. Endoscopic findings and gastric histopathological examinations were used as criteria for the determination of DU, gastric ulcer (GU), gastritis only (G) or GC, as described previously (Dixon *et al.*, 1996; Kusters *et al.*, 2006). Patients who had received antibiotic treatment up to 4 months prior to this study or who lacked histopathological analysis, or where no antral biopsy sample was available for *H. pylori* culture, were excluded from the study. In total, 232 patients (median age 44 years, range 17–73, 58.7 % male) were analysed (Fig. 1).

Bacterial culture and identification. Antral biopsy specimens were used for bacterial culture. Briefly, biopsies were collected in 1 ml

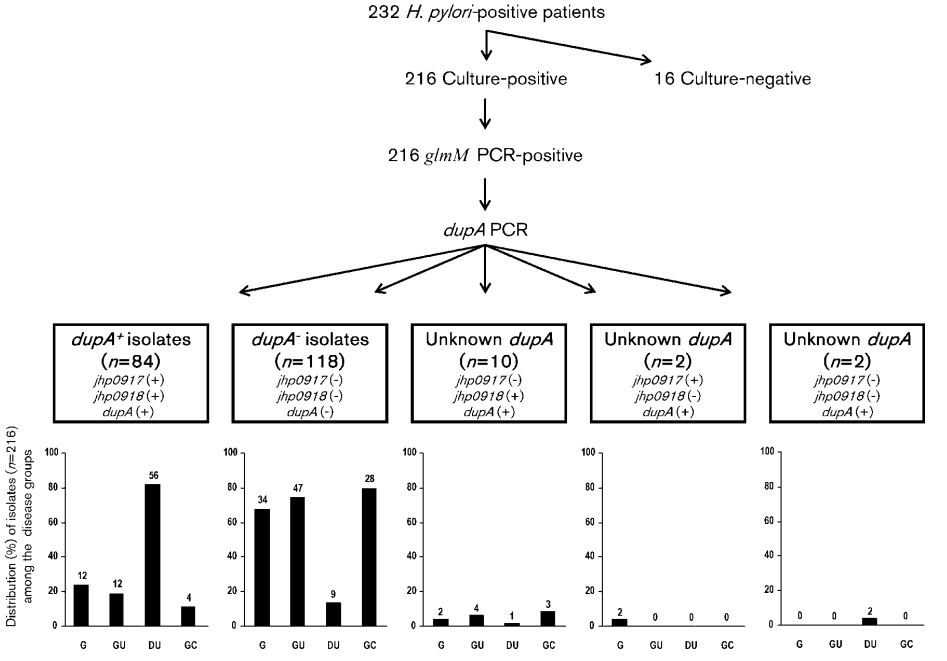

Fig. 1. Schematic representation of the patients included in the study. The graphs in the lower half show the distribution of isolates among the various disease types. The bars represent the percentage of isolates for each disease type, whilst the numbers above the bars show the absolute number in each group.

sterile thioglycolate broth (Merck). Immediately after biopsy collection, samples were homogenized in the sample medium with a sterile syringe. The homogenate was subsequently plated onto Colombia agar (CA) plates (Merck) containing 7 % defibrinated sheep blood (Jihad Daneshgahi), 7 % fetal calf serum (Gibco) and antibiotics (amphotericin B, polymyxin and vancomycin; Mast). The CA plates were incubated for 7 days under microaerophilic conditions (5 % O_2, 10 % CO_2 and 85 % N_2; Binder-USA) at 37 °C and in a water-saturated atmosphere. Suspected colonies were identified as *H. pylori* based on their colony morphology, their shape as determined by Gram staining and positive biochemical tests for catalase, oxidase and urease activity. A single colony per patient was selected at random from the primary culture plate, multiplied by culture on CA plates and stored at −80 °C in 20 % glycerol for future testing.

DNA extraction and PCR analysis of *glmM* and *dupA*. Bacteria from the −80 °C stocks were freshly grown on CA plates and chromosomal DNA was extracted using a commercially available DNA isolation kit (Roche). PCR assays for the *glmM* (encoding phosphoglucosamine mutase; Labigne *et al.*, 1991), *cagA* (Hamlet *et al.*, 1999) and *dupA* (Lu *et al.*, 2005; Nguyen *et al.*, 2010) genes were performed as described previously; the primers and PCR conditions are summarized in Table 1. All isolates were tested by the *H. pylori*-specific *glmM* PCR to validate the quality of the isolated DNA and to obtain independent confirmation of the *H. pylori* nature of the isolates. The correct size of the amplified genes was tested by running the PCR product on a 1.5 % agarose gel (Sinagene). To exclude the possibility that the absence of *dupA* amplicons was the result of inhibition of PCR rather than the absence of the gene, all *dupA*-negative PCRs were tested using an internal control, i.e. by the addition of the *glmM* primers to the *dupA* PCR mix. Only if there was a PCR product for the ubiquitously present *glmM* allele in the absence of the *dupA* product was the reaction scored as a true *dupA*-negative. In each PCR experiment, *H. pylori* strains J99 and 26695 were included as controls. Strains that gave no visible product for the *jhp0917* and *jhp0918* alleles were scored as *dupA*-negative, and strains that gave PCR products for both the *jhp0917* and *jhp0918* alleles were scored as *dupA*-positive. In this study, to obtain well-validated results on the presence of *dupA*, we repeated our experiments with an independent set of *dupA* primers (Table 1). To maximize the chance of including true *dupA*-positives, only the 202 strains that resulted in unambiguous PCR profiles for the *jhp0917*, *jhp0918* and *dupA* gene primers (see Fig. 1) were included in our analysis.

Acid resistance testing. To evaluate the acid resistance of the isolated strains, we tested their ability to grow on a set of CA plates adjusted to different pH values in a range of pH 3.0–7.0 (in steps of 1 pH unit), as described previously (Bijlsma *et al.*, 2000). Briefly, a random selection of *dupA*-negative (n=20) and all *dupA*-positive (n=12) *H. pylori* strains recovered from G patients were grown under normal conditions on regular CA plates for 5 days. Colonies were then suspended in 600 µl *Brucella* broth. Suspensions were adjusted to an OD_{600} of 1. From this standardized inoculum, tenfold dilutions were prepared (10^{-1}, 10^{-2} and 10^{-3}) in *Brucella* broth and 100 µl of each dilution was plated on pH-adjusted CA plates. Successful growth resulted in ~10–10 000 individual colonies on a single plate, depending on the dilution. Bacterial growth was assessed after 5 days. To avoid interference between closely spaced colonies, only dilutions that resulted in 20–200 colonies on the pH 7.0 plates were included in our analysis.

Statistical analysis. Depending on the dataset, Fisher's exact test or Student's *t*-test was used for analysis. A two-sided P value of <0.05 was considered statistically significant. The effect of the presence of *dupA* and *cagA* on the risk of developing specific gastric pathology was expressed as an odds ratio (OR) with a 95 % confidence interval (CI). All statistical analyses were conducted using SPSS 15.0.

RESULTS

Association between the presence of *dupA* and gastroduodenal disorders

Of the 232 putative *H. pylori*-positive patients willing to participate in our study, 216 (mean age 44, range 17–73 years, 58.7 % male) had a positive culture result for a single colony of *H. pylori*. In order to obtain a maximum likelihood of correct identification of strains with a functional *dupA* gene, we used three sets of PCR primers; thus, *dupA* genotyping was based both on the combined data of the PCR primer sets as described by Lu *et al.* (2005) and a third primer set designed by Nguyen *et al.* (2010). Twelve patients carried isolates that were positive for only a single PCR (*jhp0917*, *jhp0918* or *dupA*) but not for all three alleles, and these were excluded from our association study (Fig. 1). Much to our surprise, only two discrepant results were observed when comparing the two *dupA* genotyping methods, i.e. two of the strains that were negative

Table 1. PCR primers for amplification of *glmM*, *jhp0917*, *jhp0918*, *cagA* and *dupA* sequences

Target	Primer sequence (5′→3′)	Product size (bp)	PCR conditions	Reference
glmM	AAGCTTTTAGGGGTGTTAGGGGTTT AAGCTTACTTTCTAACACTAACGC	294	93 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min (32 cycles)	Labigne *et al.* (1991)
jhp0917	TGGTTTCTACTGACAGAGCGC AACACGCTGACAGGACAATCTCCC	307	94 °C, 30 s; 59 °C 30 s; 72 °C, 1 min (35 cycles)	Zhang *et al.* (2008)
jhp0918	AAGCTGAAGCGTTTGTAACG CCTATATCGCTAACGCGCTCG	276	94 °C, 2 min; 58 °C 30 s; 72 °C, 1 min (35 cycles)	Zhang *et al.* (2008)
dupA	TAAGCGTGATCAATATGGATT GGAACGCCGCATTCTATTA	350	92 °C, 45 s; 56.5 °C 51 s; 72 °C, 56 s (32 cycles)	Nguyen *et al.* (2010)
cagA	ATAATGCTAAATTAGACAACTTGAGCGA TTAGAATAATCAACAAACATCACGCCAT	298	94 °C 60 s; 60 °C 60 s; 72 °C 55 s (35 cycles)	Hamlet *et al.* (1999)

according to the criteria defined by Lu *et al.* (2005) (negative for both the *jhp0917* and *jhp0918* alleles) were positive using the primer set introduced by Nguyen *et al.* (2010) (Table 1). In total, 14 of the 216 (6 %) strains generated non-concordant results with the three primer pairs, and these strains were excluded from our analysis (Fig. 1). Detailed data relating to the histopathological findings, age of patients, and the *cagA* and *dupA* status of the remaining 202 patients are presented in Table 2. Whilst the various age subgroups were too small to allow any significant associations (data not shown), there was a clear trend for GC and DU to present at a higher age. Also, a trend for more severe atrophy was observed in the DU and GC group, but again, due to the small subgroup sizes, these differences did not reach statistical significance. When the *cagA* status of the various patient groups was analysed, no significant association between *cagA* and the G, GU or DU group could be observed (Table 3). There was, however, a weak association between *cagA* status and the GC group (OR 2.4, 95 % CI 1.1–5.1; Table 3). This was in striking contrast to *dupA* status where a significant statistical correlation was observed for all gastroduodenal disorders (Table 3). In our population, a strong positive correlation (OR 24.2, 95 % CI 10.6–54.8) was found between the occurrence of DU and the presence of a *dupA*-positive genotype (Table 3). Interestingly, the *dupA* gene showed a tendency to protect against the development of GU (OR 0.34, 95 % CI 0.16–0.68) and GC (OR 0.16, 95 % CI 0.05–0.47) (Table 3). We did not observe a statistical association between the *cagA* and *dupA* status, and a multivariate analysis did not show a significant contribution of the *cagA* status towards the observed associations with the *dupA* status (data not shown).

Acid resistance of G-derived *H. pylori* isolates

The presence of *dupA* has previously been associated with an increased resistance to acid shock. As this might in part explain the observed associations with gastric pathology, we wanted to expand on this observation and compare the ability of *dupA*-positive or -negative strains to grow at low pH. To carry out this test, we plated dilutions of freshly grown bacteria directly onto plates adjusted to pH values ranging from 7.0 to 3.0. In an attempt to reduce the risk of acid-resistance adaptations of the infecting strains to disease-specific pH conditions, only isolates from G patients were selected. At pH 7, 6 and 5, there were no obvious growth differences between *dupA*-positive and -negative strains (Fig. 2). However, at pH 4.0, only *dupA*-positive isolates were able to form normal-sized colonies. All 12 *dupA*-positive strains from the G patients in our study grew at pH 4.0 and 4/12 isolates (33 %) grew at pH 3.0. In contrast, only 8/20 (40 %) and 2/20 (10 %) of the *dupA*-negative isolates from G patients were able to grow on pH 4 and pH 3 plates, respectively (Fig. 2). This resulted in a strong association between *dupA*-positive *H. pylori* isolated from G patients and acid resistance (P=0.003) (detailed data not shown).

DISCUSSION

H. pylori is highly adapted to the hostile environment of the human stomach (Cover & Blaser, 2009), and chronic infection with this bacterium results in a wide range of gastrointestinal disease symptoms (Salama *et al.*, 2000). CagA is probably the best-studied disease biomarker in *H. pylori*. Many other disease-associated virulence factors have now been identified, but unfortunately most seem linked to the *cagA* status and hence probably do not represent independent risk factors (Kusters *et al.*, 2006). In this study, *cagA* was found at a similar level in all of the gastrointestinal disorders, which was somewhat unexpected considering a recent report that failed to find a significant link between *cagA* and *H. pylori* infection-associated disease types among Iranian patients (Khodaii *et al.*, 2011). In the current study, *cagA* and *dupA* were not co-linked to a specific disease type and thus they were independent predictors for the clinical outcome of *H. pylori* infection, as has been suggested previously (Argent *et al.*, 2007; Zhang *et al.*, 2008). The apparently unique mode of action of *dupA* merits further studies into the disease associations and biological functions of *dupA*. Here, the association between the presence of the *dupA* gene in the infecting strain and gastroduodenal disease outcome was tested and a putative link with acid resistance of *dupA*-positive strains was confirmed, thus providing a putative biological explanation for a positive association with the development of DU and a negative association with GC and GU.

Lu *et al.* (2005) were the first to report the association of the presence of the *dupA* gene with an increased risk of DU. Subsequently, several other studies have been carried out to test for a putative association between *dupA* status and different digestive disorders in various geographical areas (Argent *et al.*, 2007; Hussein *et al.*, 2008; Nguyen *et al.*, 2010).

In this study, the presence of *dupA* was observed in 56/65 (86.1 %) of the patients with DU and in 26.1, 20.3 and 12.5 % of the patients with G, GU and GC, respectively (Tables 2 and 3). Thus, the relative risk of having DU when infected with a *dupA*-positive strain compared with any of the other three diseases was 4.22, with a sensitivity of 83.6 % and a specificity of 79.6 %. Whilst the presence of the *dupA* gene was associated with an increased risk of developing DU (OR 24.2, 95 % CI 10.6–54.8), a strong association for the inverse situation was seen in GC patients where the infecting *H. pylori* strains seemed to be preferentially *dupA*-negative (OR 0.16, 95 % CI 0.05–0.47). A similar trend towards a protective effect was observed for GU formation, as a significant relationship between GU patients and *dupA*-negative strains was observed, but this did not reach statistical significance (OR 0.34, 95 % CI 0.16–0.68). Thus, our data suggested that the absence of *dupA* protects against GC and GU (Table 3). One has to bear in mind that a study like this will probably generate biased results, as it is based on patients who visit the

Table 2. Patient characteristics

Disease type	Sample size	Median age (range)	Gender (% male)	*cagA*$^+$ (%)	No. of *dupA*$^+$ (%)	Histopathology		Distribution over age cohorts (no. of *dupA*$^+$)			
						Atrophy*	No. of patients (no. *dupA*$^+$)	<20 years	20–39 years	40–59 years	⩾60 years
Gastritis (G)	46	29 (20–58)	63	56.5	12 (26.1)	Mild	4/46 (2)	2/46 (1)	2/46 (1)	0	0
						Moderate	37/46 (9)	20/46 (4)	5/46 (2)	12/46 (3)	0
						Severe	5/46 (1)	3/46 (1)	2/46 (0)	0	0
Gastric ulcer (GU)	59	24 (17–32)	64.4	42.6	12 (20.3)	Mild	14/59 (0)	5/59 (0)	9/59 (0)	0	0
						Moderate	34/59 (5)	3/59 (2)	31/59 (3)	0	0
						Severe	11/59 (7)	5/59 (2)	6/59 (5)	0	0
Duodenal ulcer (DU)	65	33 (22–71)	51.3	55.3	56 (86.1)	Mild	5/65 (2)	0	2/65 (1)	1/65 (0)	2/65 (1)
						Moderate	49/65 (46)	0	29/65 (27)	12/65 (11)	8/65 (8)
						Severe	11/65 (8)	0	5/65 (4)	3/65 (2)	3/65 (2)
Gastric cancer (GC)	32	34 (21–73)	62.5	43.7	4 (12.5)	Mild	3/32 (1)	1/32 (0)	1/32 (1)	0	1/32 (0)
						Moderate	26/32 (1)	3/32 (1)	22/32 (0)	1/32 (0)	0
						Severe	3/32 (2)	1/32 (1)	1/32 (1)	1/32 (0)	0
Total	202	44 (17–73)	60.3	50.0	84 (41.58)						

*Atrophy scores according to updated Sidney criteria (Dixon *et al.*, 1996).

Table 3. Association of *dupA* and *cagA* genotypes with disease types in the 202 patients included in the study group

Disease type	Sample size	*cagA*		*dupA*	
		Positive (%)	OR (95 % CI)	Positive (%)	OR (95 % CI)
G	46	20 (43)	0.71 (0.36–1.4)	12 (26.1)	0.41 (0.19–0.85)
GU	59	25 (42)	1.5 (0.83–2.8)	12 (20.3)	0.34 (0.16–0.68)
DU	65	29 (43)	1.6 (0.85–3.0)	56 (86.1)	24.2 (10.6–54.8)
GC	32	18 (56)	2.4 (1.1–5.1)	4 (12.5)	0.16 (0.05–0.47)

clinician with their first serious upper gastrointestinal symptoms. This might generate a strong selection bias, as *dupA*-positive-infected patients developing from G to DU are probably those developing complaints and presenting to a gastroenterologist with a desire to be treated. Thus, if there is a protective effect of the absence of *dupA* on GC, these patients will not develop complaints and thus are less likely to present themselves to a gastroenterologist, and this will result in a skewing of this patient group. To our knowledge, a putative protective effect of *dupA* against GC has not been reported before. Possibly, this is due to the above-mentioned bias that selects against finding such an association, and/or the association may have been missed due to the limited number of GC patients included in other *dupA* studies (Lu *et al.*, 2005; Arachchi *et al.*, 2007; Argent *et al.*, 2007; Douraghi *et al.*, 2008; Zhang *et al.*, 2008; Nguyen *et al.*, 2010). This study included 59 GU and 32 GC patients (Table 2) and to our knowledge represents the largest population of GU and GC patients analysed for an association with the *dupA* gene. A trend for such an association has, however, been observed in a recent systematic review (Hussein, 2010) and in a meta-analysis by Shiota *et al.* (2010).

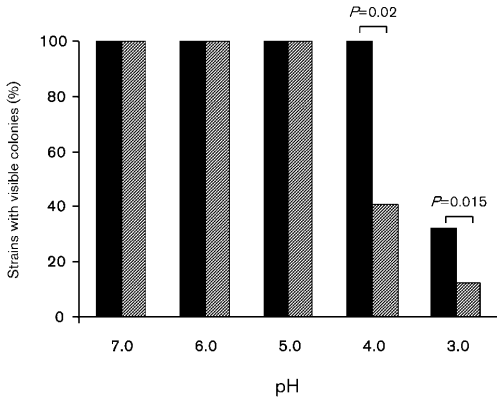

Fig. 2. Comparison of the growth of *dupA*-positive versus -negative *H. pylori* isolates at various pH values. Dilutions of fresh *H. pylori* cultures were plated on CA plates with the pH ranging from 7 to 3. The plates were inspected after 5 days of incubation. Filled bars, *dupA*-positive isolates (n=12); hatched bars, *dupA*-negative isolates (n=15). Only when a significant difference between *dupA*-positive and *dupA*-negative isolates at a given pH was present are *P* values provided.

The lack of a functional DupA test prohibits defining true-positives and true-negatives, so the analytical sensitivity and specificity of the two PCR-based genotyping tests used cannot be calculated. Interestingly, there were no significant ($P<0.05$) differences between the results obtained with the *dupA* primer sets, indicating that both tests performed equally well. Thus, in spite of a gold standard for determining true positives/negatives, these data suggest that the specificity of either test is >95 %. Based on the common belief that non-functional genes are more prone to genetic drift resulting in a non-productive PCR due to primer mismatch, isolates with non-concordant *dupA* typing data were excluded from our analysis to maximize the chance of correct identification of the *dupA* status. Strictly adhering to this concordance rule resulted in the elimination of 14/216 (6 %) of the isolates (Fig. 1).

In the present study, the total rate of *dupA*-positive *H. pylori* strains was 41.6 % (84/202), a rate that was slightly less than that observed in a previously published study performed in Iran (Douraghi *et al.*, 2008). Whilst the differences in *dupA* prevalence were only minor and can probably be explained by the sample size of the study populations, our result is more in line with most other reports (Lu *et al.*, 2005; Hussein *et al.*, 2008; Schmidt *et al.*, 2009; Nguyen *et al.*, 2010). Interestingly, the *dupA* frequency seems to vary widely among studies and ranges from 7.1 % in an Indian population (Schmidt *et al.*, 2009) to 89.5 % in a Brazilian study (Gomes *et al.*, 2008). Similarly, reports from Iran (49.7 %; Douraghi *et al.*, 2008), India (31.3 %; Arachchi *et al.*, 2007), China (27.6 and 65.3 %; Argent *et al.*, 2007; Zhang *et al.*, 2008), the USA (45.5 %; Argent *et al.*, 2007) and Japan (21.3 % and 28.8 %; Lu *et al.*, 2005; Nguyen *et al.*, 2010) indicate a wide range for the prevalence of *dupA*. Collectively, it has been assumed that these differences in *dupA* prevalence probably reflect geographical differences (Hussein, 2010). Nevertheless, they may also be the result of bias in patient selection and/or the relatively small sample numbers present in most studies. The high prevalence of *H. pylori* infection in northern Iran allowed us to collect a large number of *H. pylori* strains from a narrow geographical region, thereby largely ruling out putative bias due to

geographical distribution of the *dupA* gene. Whilst a recent study in Iran (Douraghi *et al.*, 2008) reported a *dupA* incidence of 49 % in DU patients, in the current study an incidence of 86.1 % was observed. This is probably due to differences among the study populations: Douraghi *et al.* (2008) focused mainly on cases in Tehran (the densely populated capital of Iran), whilst the subjects in the current study were from the more rural northern areas of Iran where there is a different incidence of *H. pylori*-related symptoms (Malekzadeh *et al.*, 2009). Also, when comparing the GC patients from our study with those of others, it was noted that the mean age of the GC patients in our study was relatively low (Table 2). Whilst we have no explanation for this, others have also noted that in this area there is a relative high incidence of *H. pylori*, and patients may already be suffering from *H. pylori*-induced GC at a relative young age (Malekzadeh *et al.*, 2009; Talebi Bezmin Abadi *et al.*, 2009).

In spite of the differences in *dupA* prevalence among individual reports, most studies confirm the association between the presence of *dupA* and an increased risk of DU and protection against GC as originally reported (Lu *et al.*, 2005), or observe at least the same significant trend towards such an association. Two studies, both from Brazil, however, revealed no significant relationship between clinical outcomes including DU, GC, G and GU (Gomes *et al.*, 2008; Pacheco *et al.*, 2008). In addition to the reported observations, this study showed that there was a significant correlation between the absence of *dupA* and GU (OR 0.34, 95 % CI 0.16–0.68). This result suggests a strong protective effect of *dupA* against GU formation, a finding that to our knowledge has not been reported before.

Given that *dupA*-positive patients are more prone to developing DU (Table 3) and the common dogma that there is a cascade of events leading to *H. pylori*-induced GC (Atherton, 2006; Kusters *et al.*, 2006), it is safe to assume that patients infected with a *dupA*-positive isolate are more likely to visit a clinician with clinical signs of an infection. Thus, *dupA*-positive isolates are more likely to be detected and treated, and hence the long-term infection effects of an *H. pylori* infection (i.e. GC) are more likely to be observed with *dupA*-negative isolates. If so, the negative association of *dupA* with GC might in part result from this selection bias. More probably, the negative association of *dupA* with GC can be explained by the higher acid shock tolerance of *dupA*-positive strains, as initially reported by Lu *et al.* (2005). Here, we extended their findings by analysing the ability of *dupA*-positive and -negative strains to grow at low pH (Fig. 2). Growth not only requires survival of the initial acid shock (as tested by Lu *et al.*, 2005) but also the ability for long-term adaptation and replication at low pH. In order to minimize the possibility that the infecting strains had lost their acid resistance due to adaptation to a potentially less acidic environment resulting from atrophy of the gastric mucosa, only strains from G patients were selected for this test. A strong association (P=0.02) between the presence of *dupA* and the ability to grow at low pH was observed (Fig. 2). The ability of *dupA*-positive strains to grow at low pH might form an explanation for the positive association of these strains with DU, because, if correct, *dupA*-positive strains would be more prone to colonize the more acidic locations of the stomach (i.e. the antrum) than *dupA*-negative strains. The observed higher acid resistance of the *dupA*-positive strains suggests that these strains have been adapted to a high gastric acid output. Dixon *et al.* (1996) postulated that the type of gastric pathology that develops following an *H. pylori* infection is dependent on the location of chronic gastritis, and thus on the predominant colonization site of the infecting strain. A high gastric acid output is believed to be typical for an antrum-predominant *H. pylori* infection and, whilst associated with an increased risk of DU formation, it lowers the risk for the genesis of GU and GC. This is in line with our finding that *dupA* seems to protect against GC and perhaps also against GU. The trend for a negative association with both GU and GC is an indication that the selection bias hypothesis for GC as outlined above may not play a major role in the observed negative association between *dupA* and GC. Further analysis of the role of the *H. pylori dupA* gene in bacterial physiology and pathogenesis may lead to new options to prevent *H. pylori*-induced DU and GC.

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Chapter 4

Helicobacter pylori homB, but not cagA, Is Associated with gastric cancer in Iran

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Helicobacter pylori homB, but Not *cagA*, Is Associated with Gastric Cancer in Iran▽†

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While several distinct virulence factors of *Helicobacter pylori* have been shown to be associated with different clinical outcomes, there is still much to learn about the role of different bacterial factors in gastric carcinogenesis. This study looked at the distribution of the *cagA*, *homA*, and *homB* genes in strains isolated from patients suffering from gastroduodenal diseases in Iran and assessed if there was any association between disease state and the presence of the aforementioned virulence factors. Genomic DNA from 138 *H. pylori* strains was isolated and genotyped via PCR. Strains were obtained from dyspeptic patients (35 from gastritis patients, 62 from peptic ulcer patients, and 41 from gastric cancer patients) at the Teaching Touba Clinic and Imam Hospital of the Mazandaran University of Medical Sciences in Sari, Iran. The overall prevalence rates of *cagA*, *homA*, and *homB* were 58%, 54%, and 43%, respectively. Stratification of patients showed a significant difference in the prevalence of *H. pylori* virulence genes across the disease states. The frequency of *homB* was statistically significantly higher in gastric cancer patients (78%) than in patients suffering from peptic ulcers (20%) or gastritis (43%) ($P < 0.0001$). The presence of *homB* was also associated with the presence of *cagA* ($r = 0.243$). These data suggest that in this population the presence of *homB* may be a predictor of more virulent strains of *H. pylori* and influence the severity of disease manifestation.

Helicobacter pylori is a Gram-negative, spiral-shaped, microaerophilic bacterium that infects more than 50% of the world's population (2, 30, 31). Colonization and long-term persistence of *H. pylori* can induce a complex immune response that can potentiate severe mucosal damage, including atrophy, intestinal metaplasia, and dysplasia, thereby making *H. pylori* the etiologic agent of acute and chronic gastritis, peptic ulcer disease (75% of gastric ulcers and 90% of duodenal ulcers), and two forms of gastric cancer (mucosa-associated lymphoid tissue lymphoma and gastric adenocarcinoma) (8, 15, 41, 42, 51). The association with the development of two forms of cancer led to the classification of *H. pylori* by the World Health Organization as the only bacterial class I carcinogen (24).

H. pylori's association with cancer, combined with the fact that gastric cancer is the second most common cause of cancer-associated death (33), has led to numerous studies designed to elucidate the bacterial factors responsible for progression to this disease. However, understanding why some individuals develop severe disease and others develop only mild disease has not been a straightforward task. This is due to the complex nature of interactions between the bacterium and the host. Important variables include variation in genetic content across bacterial strains and the role of host factors, such as host genetics, dietary intake, and other environmental factors in the disease process. Among the bacterial factors that have been implicated to impact disease development are a number of virulence factors that show heterogeneity across strains. Included among these are the toxins CagA and VacA, the IceA (for induced by contact with epithelium) protein, the proinflammatory outer membrane protein OipA, and the *Helicobacter* outer membrane proteins HomA and HomB (4, 11, 21, 37, 38, 43, 46, 53). The virulence factor that has emerged to be one of the major determinants of severe disease manifestations is *cagA*, and carriage of the gene is associated with peptic ulcers, atrophic gastritis, and adenocarcinoma (9, 17). In fact, *H. pylori*-infected gastric cancer patients are at least twice as likely to be infected with *cagA*-positive strains (9, 18). *cagA* is the last gene on the *cag* pathogenicity island (PAI), the majority of which encodes a type IV secretion apparatus that is used to directly inject CagA into host cells (10). Once injected, CagA is phosphorylated and forms a complex with SHP-2 (Src homology region 2-containing phosphatase 2 [22]), thereby altering multiple host signaling pathways (20–22, 32, 47, 52).

The *Helicobacter* outer membrane (Hom) adhesion molecules constitute a small paralogous family of proteins that contain alternating hydrophobic motifs and signal sequences in the C terminus, which are typical of other outer membrane proteins (1). *homA* and *homB* are the best-studied members of the Hom family, and *H. pylori* contains two possible genomic locations that can carry these two adhesion molecules (38).

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TABLE 1. Primer sequences

Primer name	Sequence	Gene(s) amplified	Amplicon size (bp)	Reference
glmM forward	5′-AAGCTTTTAGGGGTGTTAGGGGTTT-3′	*glmM*	294	28
glmM reverse	5′-AAGCTTACTTTCTAACACTAACGC-3′			
F1-jph0870/jhp0649	5′-AGAGGGTFTTTFAAACGCTCAATA-3′	*homA*	128	38
R1-jhp0870/jhp0649	5′-GGTGAATTCTTCTGCGGTTTG-3′	*homB*	161	
D008	5′-ATAATCGTAAATTAGACAACTTGAGCGA-3′	*cagA*	298	19
R008	5′-TTAGAATAATCAACAAACATCACGCCAT-3′			

The *homA* and *homB* genes are 90% identical, and the variability between these genes is contained in the central domain (1). Adding to the complexity, six distinct different allelic variants within a 300-bp region within this central domain have been identified. Three of these variants are exclusively found in *homB*-positive strains, while only one of these variants is found exclusively in *homA*-positive strains (35, 36). There are also geographic differences in the sequences at both the 5′ and 3′ ends of the *homB* gene, and these differences can be divided among East Asian and Western strains (35). Strains can have a single *homA* or *homB* gene, in which the other locus remains empty; two copies of *homA* (*homA* and *homA*) or *homB* (*homB* and *homB*); a single copy of each gene (*homA* and *homB*); or neither of these genes (34). Studies have suggested that the number of *homB* genes affects the number of bacteria adhering to host cells and that the presence of *homB* is associated with secretion of the proinflammatory cytokine interleukin-8 (IL-8) (34). Studies to address whether the presence of *homB* is associated with increased disease severity have shown that the presence of *homB* is associated with peptic ulcer disease but not gastritis (34). More recently, the presence of *homB* has been suggested to be a discriminative factor between development of gastric cancer and duodenal ulcer (25).

Residents in the northern regions of Iran are at high risk for development of gastric cancer. Specifically, gastric cancer clusters exist within the Mazandaran region (29). Given the complexity of *H. pylori* virulence factors, the rather poorly understood distribution of these factors in Iran, and the high incidence of gastric cancer in this region, the prevalence of the virulence genes *cagA*, *homA*, and *homB* in patients with gastroduodenal disorders within a population at risk for gastric cancer from the northern section of Iran was assessed (29). In this population, we found that while *cagA* presence had no significant effect on disease type, there was a strong association between the presence of *homB* and the progression to gastric cancer. Additionally, there was an association between the presence of the *homB* and *cagA* genes. These data suggest that within this population, *homB* may serve as a better predictor of severe disease development than *cagA*.

MATERIALS AND METHODS

Study participants. Biopsy samples were obtained from patients undergoing endoscopic procedures for dyspepsia at the Teaching Touba Clinic and Imam Hospital of Mazandaran University of Medical Sciences, Sari, Iran. Exclusion criteria for the study included previous gastric surgery; *H. pylori* eradication treatment; and previous use of antibiotics, nonsteroid anti-inflammatory drugs, proton pump inhibitors, or H2-receptor blockers in the previous 30 days. Additionally, patients who had received antisecretory drugs, bismuth salts, or sucralfate within the 2 weeks prior to the endoscopy procedure were excluded. Three biopsy specimens were obtained from the gastric antrum of each patient and were used for pathological examination, urease test (*H. pylori* Quick Test; Biohit Diagnostics Helsinki, Finland), and microbial culture, respectively. Patients were included in the study upon a positive urease test, and written informed consent was obtained from all participants. The study protocol was approved by the Medical Research Ethics Committee of Mazandaran University of Medical Sciences. Diagnosis of gastric disorders was based on clinical presentation, endoscopic findings, and histopathologic confirmation (14, 26). A total of 138 *H. pylori*-positive patients were enrolled in the study.

Isolation of *H. pylori* isolates. Biopsy specimens were placed in sterile thioglycolate broth (Merck, Germany) and transferred to the microbiology laboratory for further processing. The specimens were dissected and then plated on Columbia agar (Mast, United Kingdom) plates containing 7% fetal calf serum (Gibco), 10% defibrinated sheep blood (Jihad Daneshgahi, Iran), and *H. pylori*-selective antibiotic tablets (Mast, United Kingdom). These plates were incubated under microaerobic conditions at 37°C for a maximum of 7 days. *H. pylori* colonies were confirmed via morphology, Gram stain, and positive oxidase, catalase, and urease activity tests, as well as by successful amplification of the housekeeping gene *glmM* (13). PCR amplification of the *glmM* gene for the detection of *H. pylori* isolates was performed with the *glmM* forward and reverse primers described by Lu et al. (28).

***homA*, *homB*, and *cagA* genotyping.** Genomic DNA was extracted from a single colony per patient using an AccuPrep genomic DNA extraction kit (Bioneer, South Korea) according to the manufacturer's directions. All primers used in this study are listed in Table 1. The presence of *cagA* was indicated by a 298-bp amplicon when the gene was amplified with primers D008 and R008 using the conditions described previously (19) (Fig. 1). Identification of the presence of the *homA* and/or *homB* gene was accomplished through a single PCR using the F1-jhp0870/jhp0649 and R1-jhp0870/jhp0649 primers, which yielded a 128-bp product denoting the presence of the *homA* gene and a 161-bp product denoting the presence of the *homB* gene using conditions described previously (38) (Fig. 1). These primers amplify an internal region of both genes that lies approximately 505 to 530 bp from the start of the genes. This region contains variations between the *homA* gene and the *homB* gene, but the conserved nature of the primers allows amplification of all *homA* and *homB* variants currently identified. Reference strain 26695 was used as a positive control for *homA*, and strain J99 was used as positive control for *homA* and *homB* (38).

Data analysis. Isolates were assessed for the presence of the *cagA*, *homA*, and *homB* genes, according to disease state, age, and gender. Comparisons of continuous and discontinuous variables were performed with the Student *t* test, analysis of variance, the Fisher exact test, or the chi-square test. Logistic regression analysis was used for multivariate analysis, and log linear modeling was used to assess any higher-order associations, fitting a saturated model using categorical variables representing *cagA*, *homA*, *homB*, disease state, gender, and age. Higher-order associations were identified using a backward-selection algorithm with statistical significance assessed at the 5% level. Data were analyzed using SPSS (version 16) software (SPSS Inc., Chicago, IL).

RESULTS

Sample acquisition and virulence gene genotyping. A complete list of isolates and epidemiological data (age and gender), disease state, and virulence factor status is available in Table S1 in the supplemental material. *H. pylori* was successfully cultured from 138 patients suffering from gastroduodenal disorders from northern Iran. The presence of *H. pylori* was ver-

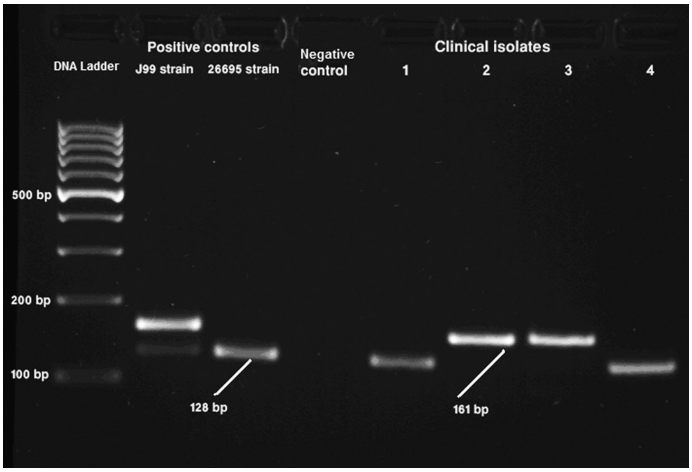

FIG. 1. Genotyping of *homA* and *homB* in *H. pylori* isolates. The image is from a representative gel electrophoresis of PCR amplification products of *homA* (128 bp) and *homB* (161 bp) genes from clinical isolates and J99 and 26695 as positive-control strains for both *homA* and *homB* and only *homA*, respectively. The negative-control lane represents a PCR performed with no template DNA.

ified via morphology, Gram stain, and positive oxidase, catalase, and urease activity tests and further confirmed by successful amplification of the housekeeping gene *glmM*. The mean age of the overall population was 42.9 ± 13.5 years. The population was fairly evenly distributed across gender, with 63 (45.7%) being female and 75 (54.3%) being male. Moreover, the *H. pylori*-positive samples were distributed across the different disease states: 35 (25.4%) from gastritis patients, 62 (44.9%) from peptic ulcer disease patients, and 41 (29.7%) from gastric cancer patients. The complete breakdown of the virulence gene status and epidemiological factors across the different disease states is shown in Table 2. There were no significant differences in mean age ($P = 0.151$) or gender ($P = 0.500$) across the disease spectrum.

Virulence factors. (i) Cytotoxin-associated gene A, *cagA*. The presence of *cagA* has been linked to the development of more severe disease states (9, 27, 40) and therefore is an important virulence factor to assess within this population. A majority of the strains (80 [58%]) were found to be *cagA* positive, while 58 (42%) were *cagA* negative. Of the 80 *cagA*-positive strains, 39 (48.75%) were from female patients and 41 (51.25%) were from male patients. Eighteen isolates were from gastritis patients, 34 were from peptic ulcer disease patients, and 28 were from patients presenting with gastric cancer. Of the 58 *cagA*-negative strains, 24 (41.4%) were from female patients and 34 (58.6%) were from male patients. Seventeen of these isolates were from gastritis patients, 28 isolates were from peptic ulcer disease patients, and 13 isolates were from gastric cancer patients (Table 2).

The presence or absence of the *cagA* gene was not statistically significantly linked to gender ($P = 0.4888$). Even though the *cagA*-positive strains were more prevalent in patients suffering from gastric cancer (68.3%; Table 2 and Fig. 2A), *cagA* status did not have a statistically significant effect on disease state in this population ($P = 0.2654$). In fact, *cagA* status did not statistically impact any breakdown of disease states: cancer versus gastritis and peptic ulcer disease ($P = 0.3291$), cancer versus peptic ulcer disease ($P = 0.2185$), cancer versus gastritis ($P = 0.1618$), peptic ulcer disease versus gastritis and gastric cancer ($P = 0.6033$), peptic ulcer disease versus gastritis ($P =$

TABLE 2. Breakdown of epidemiological and virulence factors of a north Iranian population

Characteristic	No. of patients with the following disease state[a]:			
	Total	Gastritis	Peptic ulcers	Gastric cancer
Overall population				
Total	138	35	62	41
Female	63	15	26	22
Male	75	20	36	19
cagA status				
Positive	80	18	34	28
Female	39	7	16	16
Male	41	11	18	12
Negative	58	17	28	13
Female	24	8	10	6
Male	34	9	18	9
homA status				
Positive	75	20	49	6
Female	35	8	22	5
Male	40	12	27	1
Negative	63	15	13	35
Female	28	7	4	17
Male	35	8	9	18
homB status				
Positive	60	15	13	32
Female	26	7	4	15
Male	34	8	9	17
Negative	78	20	49	9
Female	37	8	22	7
Male	41	12	27	2

[a] The mean ages ± 1 standard deviation are 42.9 ± 13.5, 43.6 ± 14.7, 44.7 ± 12.9, and 39.5 ± 13.2 years for all patients and patients with gastritis, peptic ulcers, and gastric cancer, respectively.

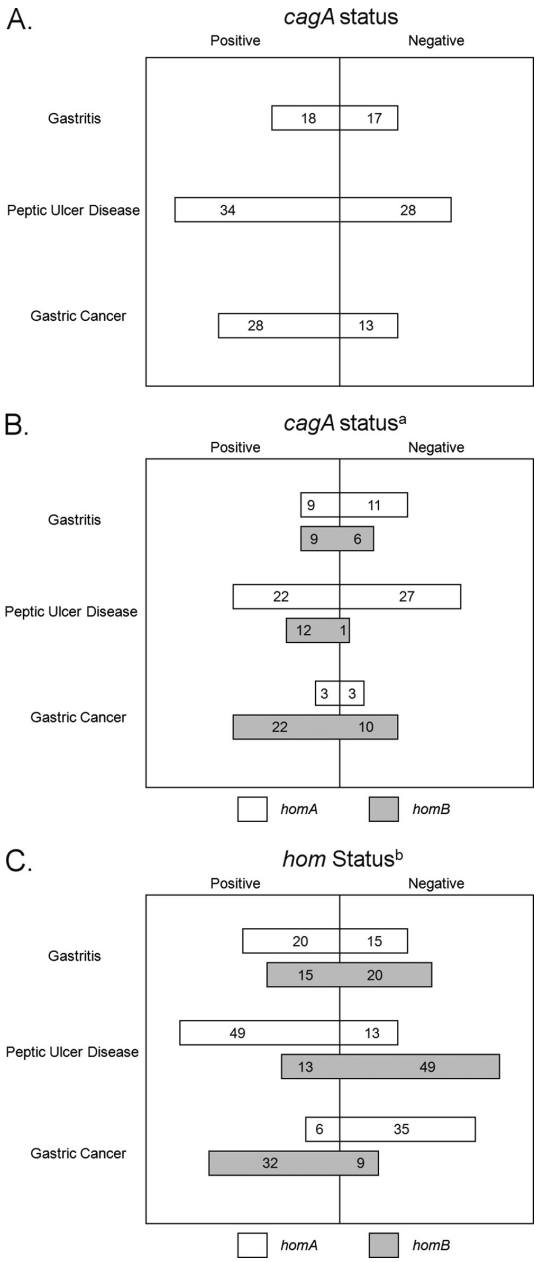

FIG. 2. Schematic depiction of the distribution of the different virulence factors stratified by disease state within the Iranian population of this study. (A) The distribution of the *cagA* gene stratified by disease state is depicted. (B) The distribution of *cagA*, *homA*, and *homB* stratified by disease state is depicted. Only *homA*- or *homB*-positive strains are depicted among the *cagA*-positive or -negative isolates. [a], positive correlation between the presence of the *cagA* gene and the *homB* gene ($r = 0.243$) and an inverse correlation between the presence of the *cagA* gene and the *homA* gene ($r = -0.279$). (C) The distribution of the different *hom* genes stratified by disease state is depicted. [b], a significant association exists between the distribution of the *homA* gene and disease state ($P < 0.0001$) and the distribution of the *homB* gene and disease state ($P < 0.0001$), and the progression to gastric cancer is influenced by the presence of the *homB* gene.

TABLE 3. Distribution of virulence factors across the different disease states

Gene and groups compared	*P* value[a]
Distribution of *cagA*	
Gastric cancer vs peptic ulcer disease and gastritis	0.3291
Gastric cancer vs peptic ulcer disease	0.2185
Gastric cancer vs gastritis	0.1618
Peptic ulcer disease vs gastric cancer and gastritis	0.6033
Peptic ulcer disease vs gastritis	0.8331
Gastritis vs gastric cancer and peptic ulcer disease	0.4293
Distribution of *homA*	
Gastric cancer vs peptic ulcer disease and gastritis	<0.0001
Gastric cancer vs peptic ulcer disease	<0.0001
Gastric cancer vs gastritis	0.0001
Peptic ulcer disease vs gastric cancer and gastritis	<0.0001
Peptic ulcer disease vs gastritis	0.0348
Gastritis vs gastric cancer and peptic ulcer disease	0.8445
Distribution of *homB*	
Gastric cancer vs peptic ulcer disease and gastritis	<0.0001
Gastric cancer vs peptic ulcer disease	<0.0001
Gastric cancer vs gastritis	0.0022
Peptic ulcer disease vs gastric cancer and gastritis	<0.0001
Peptic ulcer disease vs gastritis	0.0348
Gastritis vs gastric cancer and peptic ulcer disease	1

[a] Shading indicates *P* values that are statistically significant, as defined in the Materials and Methods.

0.8331), or gastritis versus gastric cancer and peptic ulcer disease ($P = 0.4293$) (Table 3). However, the presence of the *cagA* gene was significantly linked to the distribution of both *homA* ($P = 0.0017$) and *homB* ($P = 0.0054$) (Fig. 2B).

(ii) *Helicobacter* outer membrane family members *homA* and *homB*. In a population where the presence of *cagA* is not a good indicator of progression to more severe disease manifestations, other bacterial factors must play a more important role in disease progression. Recently, the presence of different outer membrane proteins has been linked to more severe disease manifestations (25, 34, 38). In fact, the presence of the *Helicobacter* outer membrane protein B (*homB*) has been linked to progression to gastric cancer (25). In order to assess if this correlation existed within this population, the presence of either *homA* or *homB* was assessed. The presence of *homA* was identified by the presence of a 128-bp amplicon, whereas the presence of *homB* was indicated by the presence of a 161-bp amplicon, as previously described (38) (Fig. 1). Either *homA* or *homB* was present in 135 (97.8%) of all strains; 3 gastric cancer strains were negative for both the *homA* and *homB* genes. However, all three *homA*- and *homB*-negative strains carried *cagA*. Seventy five isolates (54.3%) were *homA* positive and 60 isolates (43.5%) were positive for *homB*. Of note, with the exception of the three *homA*- and *homB*-negative strains, strains carried just one or the other *hom* gene, and no strains carried both *homA* and *homB*.

(a) homA status. Of the 75 *homA*-positive strains, 35 (46.7%) were from female patients and 40 (53.3%) were from male patients. Twenty isolates were from gastritis patients, 49 isolates were from peptic ulcer disease patients, and 6 were from patients diagnosed with gastric cancer. Of the 63 *homA*-negative strains, 28 (44.4%) were isolated from females and 35 (55.6%) were isolated from males. Fifteen isolates were from gastritis patients, 13 isolates were from peptic ulcer disease patients, and 35 were from gastric cancer patients (Table 2 and Fig. 2C).

The presence or absence of the *homA* gene was not statistically significantly linked to gender ($P = 0.8643$). However, the distribution of *homA* had a statistically significant effect on disease state ($P < 0.0001$; Fig. 2): cancer versus gastritis and peptic ulcer disease ($P < 0.0001$), cancer versus peptic ulcer disease ($P < 0.0001$), cancer versus gastritis ($P = 0.0001$), peptic ulcer disease versus gastritis and gastric cancer ($P < 0.0001$), and peptic ulcer disease versus gastritis ($P = 0.0348$). However, the presence of *homA* did not statistically significantly impact gastritis versus peptic ulcer disease and gastric cancer ($P = 0.8445$). The presence of the *homA* gene was significantly linked to the distribution of both *cagA* ($P = 0.0017$) and *homB* ($P < 0.0001$). The association with *homB* is due to the fact that, with the exception of the three *homA*-negative and *homB*-negative strains, strains that carry *homA* do not carry *homB* and vice versa.

(b) homB status. Of the 60 *homB*-positive strains, 26 (43.3%) were from female patients and 34 (56.7%) were from male patients. Fifteen isolates were from gastritis patients, 13 isolates were from peptic ulcer disease patients, and 32 were from gastric cancer patients. Of the 78 *homB*-negative strains, 37 (47.4%) were from female patients and 41 (52.6%) were from male patients. Twenty isolates were from gastritis patients, 49 isolates were from peptic ulcer disease patients, and 9 were from patients diagnosed with gastric cancer (Table 2).

The presence or absence of the *homB* gene was not statistically significantly linked to gender ($P = 0.7306$). However, the status of *homB* had a statistically significant effect on disease state ($P < 0.0001$). In fact the presence or absence of *homB* status statistically significantly impacted most of the disease states (Fig. 2C and Table 3): cancer versus gastritis and peptic ulcer disease ($P < 0.0001$), cancer versus peptic ulcer disease ($P < 0.0001$), cancer versus gastritis ($P = 0.0022$), peptic ulcer disease versus gastritis and gastric cancer ($P < 0.0001$), and peptic ulcer disease versus gastritis ($P = 0.0348$). However, the presence of *homB* did not statistically significantly impact gastritis versus peptic ulcer disease and gastric cancer ($P = 1.0000$). Indeed, the majority of isolates from gastric cancer patients (78%) carried *homB.* The presence of the *homB* gene was significantly linked to the distribution of both *cagA* ($P = 0.0054$) and *homA* ($P < 0.0001$). Again, the association with *homA* is due to the fact that, with the exception of the three *homA*- and *homB*-negative strains, strains that carry *homA* do not carry *homB* and vice versa.

***cagA*, *homA*, and *homB* genotypes.** Next, we wanted to examine the association between *cagA* and either of the *hom* genes. Through statistical analysis, a significant inverse correlation between the presence of the *cagA* and *homA* genes was identified ($r = -0.279$, $P = 0.001$). This means that *homA* positivity was more frequently found in strains lacking the *cagA* gene (Table 3). Moreover, a statistically significant positive correlation was observed between *cagA* positivity and *homB* status ($r = 0.243$, $P = 0.004$). This positive correlation indicates that there is an association between being positive for *homB* and being positive for *cagA*. Indeed, the majority of *homB*-positive strains were *cagA* positive (71.7%), whereas among *homB*-negative strains, the *cagA* status was evenly distributed: 47.4% were *cagA* positive, and 52.6% were *cagA* negative.

Since both the *homA* and *homB* genes showed a direct association with disease and *cagA*, we wanted to know if there was any association between *homA*, *cagA*, and disease state or *homB*, *cagA*, and disease state. However, neither of these associations reached statistical significance ($P = 0.178$ and $P = 0.073$, respectively). Next we wanted to know if either *homA* or *homB* was a predictor of gastric cancer independent of *cagA*. Logistic regression analysis revealed that the *homB*-positive genotype was a predictor of gastric cancer independent of *cagA* (odds ratio = 8.453, $P < 0.001$). Unfortunately, due to the almost inverse relationship of *homA* and *homB* status, we were unable to assess the association between these two genes.

DISCUSSION

Gastric cancer is the fourth most common malignancy and is responsible for over 700,000 deaths per year (16), and infection with *H. pylori* has been attributed to over 63% of all cases of gastric cancer worldwide (39). Additionally, *H. pylori* is responsible for 75% of all gastric ulcers and 90% of all duodenal ulcers (8), making *H. pylori* a medically important bacterium. Given only this evidence, eradication of this gastric intruder seems to be the most logical course of action. However, recent epidemiological studies have identified protective correlates between *H. pylori* colonization and different diseases, specifically, active tuberculosis, which is a worldwide health concern (44); asthma (45), which is on the rise in developed countries; and esophageal cancer, which is also on the rise (7). Thus, scientists are attempting to determine what makes some strains more virulent than other strains. If bacterial factors that are responsible for increased virulence are identified, then perhaps highly virulent strains can be eradicated without losing the protective effects generated through colonization with less virulent strains.

The bacterial factor that has emerged to be the major bacterial determinant for progression to more severe disease is *cagA* (9, 27, 40). In fact, *cagA*-positive *H. pylori* strains have been associated with the severe mucosal inflammation that underlies peptic ulcer, atrophic gastritis, and gastric carcinoma (9, 27). Even though the rate of gastric cancer is high in Iran, the prevalence of infection with *cagA*-positive *H. pylori* strains varies from 68% (48) to 76% (23). In our study, only 58% of isolates were *cagA* positive, and the distribution of the *cagA* gene was not statistically significantly linked to disease. While they are surprising, our results are congruent with those of another study that showed that *cagA* presence was not significantly associated with peptic ulcer disease in Iran (23). Indeed, Iran seems to represent a gastric cancer enigma in terms of *H. pylori* virulence factors; several studies have shown that the presence of *cagA* does not influence progression to peptic ulcers or gastric cancer (6, 12, 23, 29, 49, 50). However, we do note that though not significant in our population, the majority

(68.3%) of isolates from gastric cancer patients did carry the *cagA* gene and the three gastric cancer isolates that were *homA* and *homB* negative also carried the *cagA* gene. These findings suggest that in this population, *cagA* alone is not an adequate predictor of disease and suggest that other virulence genes are important. Indeed, this finding presents the opportunity to assess the impact of other virulence factors on severe disease progression.

Outer membrane proteins are very important during infection and can influence the levels of bacterial colonization. Additionally, an increased number of *H. pylori* cells adherent to the gastric epithelium could induce greater changes in host cell signaling pathways. *H. pylori* carries two paralogous outer membrane proteins, HomA and HomB, which have recently been suggested to be important determinants of disease severity (25). While the *homA* and *homB* genes are 90% identical (1), the differences between *homA* and *homB* translate to different impacts on disease manifestations; *homB* presence is associated with gastric cancer (25).

In this population, the presence of *homB* was associated with progression to gastric cancer; *homB* was present in 78% of all isolates from gastric cancer patients (Table 2). In fact, logistic regression revealed that *homB* was a predictor of gastric cancer independent of *cagA*. However, we did note a positive correlation between the presence of *cagA* and *homB* (Table 2). This suggests that *cagA* and *homB* may interact in some fashion. Of note, *homB* has been shown to be prevalent in the majority of East Asian strains, while the presence of *homA* within these strains is rare (37): East Asian strains typically carry the most virulent form of CagA (5, 21). Despite these correlations, a three-way association between *homB*, *cagA*, and disease state was not identified within this population. If *homB* was influencing only adherence of the bacteria to the gastric epithelium, we would expect that gastric cancer development would still be linked to *cagA*, that there would be a three-way interaction, or that *homB* is not an independent contributor to the progression to gastric cancer. However, data from this population suggest that the presence of *homB* does more than just influence bacterial adherence. Interestingly, it has been demonstrated that the presence of *homB* increases secretion of the proinflammatory cytokine IL-8 (34), and increased inflammation is indicative of more severe disease presentations, such as gastric ulcers and gastric cancer (3). Thus, perhaps HomB promotes more severe inflammation, which in turn affects gastric cancer progression. Since the identification of allelic variants within the central domain of *homB* is recent (35), the impact of these alleles within both Western and East Asian populations still needs to be elucidated. For instance, it would be interesting to determine if different *homB* variants/mutants with point mutations are more prevalent among gastric cancer strains than noncancer strains. If this was the case, then isogenic strains that vary only in the *homB* variant carried could be created and then progression to gastric cancer could be monitored in an animal model. Clearly, the exact mechanistic role of the *homB* gene product in gastric pathology, particularly corpus inflammation, mucosal atrophy, and metaplasia, still needs to be defined.

While it is clear that *H. pylori*-induced disease pathology is influenced by multiple host, dietary, environmental, and bacterial factors, it is clear from this study that the presence of *homB* correlates with severe disease progression. We have shown that there is a statistically significant association between development of gastric cancer and the presence of *homB*. Currently, the reason for this correlation is unclear, and further studies are required to elucidate the molecular role that *homB* plays in cancer development. This study also demonstrates the importance of *H. pylori* virulence factor polymorphism in disease progression, since there is a functional discrepancy between two highly similar genes.

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Chapter 5

High correlation of *babA*$_2$-Positive Strains of *Helicobacter pylori* with Presence of Gastric Cancer

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High correlation of *babA$_2$*-positive strains of *Helicobacter pylori* with the presence of gastric cancer

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Abstract *Helicobacter pylori (H. pylori)* is a Gram-negative bacterium that is well known in the involvement of chronic inflammation in the gastric mucosa of the human stomach. Several studies have investigated the possible role of *H. pylori* presence in different gastroduodenal disorders with conflicting results. This study aimed to further investigate such a field. *Helicobacter pylori* strains were cultured from 160 patients (mean age of 42 years; range 15–75; 90 were male, and 70 were female) [40 gastric cancer (GC), 55 duodenal ulcer (DU) and 65 non-ulcer dyspepsia (NUD)]. In this study, allelic variants of *iceA$_1$*, *iceA$_2$* and *babA$_2$* were identified by polymerase chain reaction. The overall prevalence of *babA$_2$* gene was 40.6% (65/160). The prevalence of *babA$_2$* gene was 95% with gastric cancer, 18.1% with duodenal ulcer and 26.1% with non ulcer dyspepsia, respectively. The prevalence of *babA$_2$* in GC patients was significantly higher as compared to either NUD or UD patients ($P = 0.0004$), while no statistical significance was found between the latter two patient groups. Our study finds that *babA$_2$* and *iceA$_1$* genes are more prevalent in GC compared to either NUD or DU patients in Iran.

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Keywords *Helicobacter pylori* · *babA$_2$* · *iceA$_1$* · *iceA$_2$* · Iran

Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative, urease and catalase positive bacterium that may colonize gastric mucosa in more than 50% of the world population [1]. The infection is generally acquired during childhood, and it continues until death [2]. Different factors affect the clinical outcomes of infection [3], but there are some unknown points. Bacterial factors were the focus of clinical research for determination of possible roles in gastroduodenal disorders [3, 4]. Indeed, *H. pylori* is well characterized for having high level of genetic variation enabling it to have a high adaptation to the host gastric epithelium [4]. The *babA$_2$*, *iceA$_1$* and *iceA$_2$* are major genes contributing to *H. pylori* adherence in the stomach [5–7]. The blood group antigen-binding adhesin gene (babA) is proposed as an active gene in the binding activity between Lewis-b blood group antigens on gastric epithelial cells and bacterial surface. Although three bab alleles have been discovered (*babB*, *babA$_1$*, *babA$_2$*), only the *babA$_2$* gene product is needed for Lewis-b binding activity. Some studies show a significance between *babA$_2$* positive genotypes and occurrence of peptic ulcer diseases [8, 9], but others do not confirm these claims [3, 4]. In the case of *iceA*, there is not enough data to enable investigation of its correlation with gastroduodenal patients. Some studies find a role for *iceA* alleles in development of gastroduodenal diseases [10, 11], while contradictory data are also reported [12–14]. Therefore, we designed the present study to assess the role of *H. pylori* adhesions in clinical outcomes of infection.

Materials and method

Patients

In this study, 160 single colonies of *H. pylori* isolates were consecutively obtained from culture of 160 patients who had undergone upper gastric endoscopy at Tooba Medical Center, Sari, Iran for upper gastrointestinal symptoms. The mean age of the patients was 41 years (range 15–75; M/F = 90/70) from spring of 2009 to spring of 2010. Prior to endoscopy, informed consent was obtained from each patient. This study was approved by the ethics committee of Tarbiat Modares University, Tehran, Iran.

Exclusion criteria were: (1) age < 15 years old, (2) consumption of antibiotics or anti-secretory drugs during the last 4 months. In this study, diagnosis of disease was based on endoscopic examination and pathology laboratory findings [15].

Bacterial culture

For culture purposes, antral biopsy specimens were placed in sterile thioglycolate broth (Merck, Germany), and then quickly shipped in a cold flask within 3 h to a diagnostic laboratory. In our laboratory, 100 µl of suspension from homogenized biopsy were added onto the surface of Colombia agar plates (MAST, UK), containing 10% defibrinated sheep blood, 8% FCS (Fetal Calf Serum) (Gibco, USA) and *H. pylori* selective supplement (Oxoid-SR147E). Finally, agar plates were incubated in 10% CO_2 provided by anaerobic jar, for up to 10 days. In this study, we aimed to use single colonies from each clinical sample of *H. pylori*. Briefly, primary suspected *H. pylori* colonies were selected for secondary culture in new plates with the former conditions in order to get a single colony of *H. pylori*. Phenotypically, *H. pylori* identification was performed by routine biochemical tests including urease, catalase and oxidase. Examination of colony morphology and Gram's staining were applied for further phonotypical tests. We used PCR assay for *glmM*, which is confirmatory for suspected strains of *H. pylori*. Notably, we just select a strain as positive for entry in our genotyping when it was both positive in phenotypic, and genetic confirmations.

PCR assay and genotyping

Briefly, chromosomal DNA from each single colony *H. pylori* isolate was extracted from the bacterial suspension by use of the protease/phenol–chloroform method, suspended in TE buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA) [17]. We stored our extracted DNA at −70°C for further experiments. Primers used in the current study are listed in Table 1, additionally, conditions of each PCR are shown in detail; however, we made a little modification for each PCR reaction. In this study, we have used *26695* and *J99* as reference strains in our examinations.

Statistical analysis

We used SPSS version 16.0 software for statistical analysis. Fisher exact and χ^2 tests were applied to establish any statistically significant associations. A P value <0.05 were considered as significant.

Results

We isolated *H. pylori* from 40 patients with gastric cancer (GC), 55 with duodenal ulcer (DU), and 65 with non-ulcer dyspepsia (NUD). The presence of expected PCR bands for each of the investigated genes was considered after electrophoresis, as shown in Figs. 1 and 2. Overall, prevalence of $babA_2$ gene was 40.6% (65/160) and it was significantly higher in GC (95%) as compared to either DU (18.1%) or NUD (26.1%) patients, while no statistically significant differences emerged between the last two patient groups. Similarly, the $iceA_1$ allele was most prevalent in gastric cancer patients as compared to the other groups. The $iceA_2$ allele was equally frequent in the three patient groups (GC, DU and NUD: 30, 16.3 and 20%, respectively).

Table 1 Primers sequence and conditions of PCR applied in this study

Primers	Sequences	Size	Condition	Reference
glmM	AAGCTTTTAGGGGTGTTAGGGGTTT AAGCTTACTTTCTAACACTAACGC	294 bp	93°C, 57 s; 56°C, 50s; 72°C, 53 s (35 cycles)	[21]
babA2	CCAAACGAAACAAAAAGCGT GCTTGTGTAAAAGCCGTCGT	271 bp	94°C, 45 s; 44°C, 40 s; 72°C, 54 s (28 cycles)	[21]
iceA1	GTGTTTTTAACCAAAGTATC CTATAGCCATTATCTTTGCA	247 bp	95°C, 55 s; 57°C, 40 s; 72°C, 55 s (33 cycles)	[21]
iceA2	GTTGGGTATATCACAATTTAT TTTCCCTATTTTCTAGTAGGT	229 bp	95°C, 55 s; 57°C, 56 s; 72°C, 55 s (32 cycles)	[21]

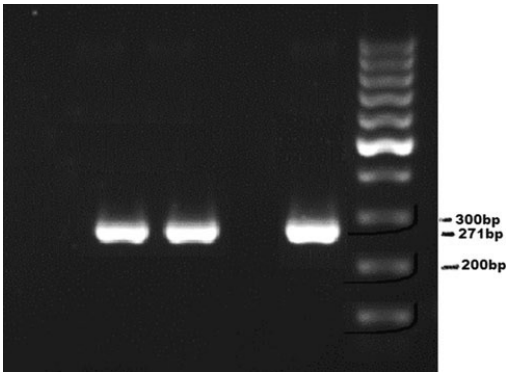

Fig. 1 PCR amplification, performed with $babA_2$ primers, from *right* to *left*, (*lane 2*: positive control), (*lane 3*: negative control) (*lanes 4–6*: clinical positive and negative strains), (*lane 1*: 100–1,000 bp DNA ladder)

Discussion

It has been suggested that presence of certain *H. pylori* adhesions can be related to the occurrence of different digestive disorders. Interestingly, certain genes are involved to modulate genes contributing in *H. pylori* colonization [5]. In detail, virulence factors including *vacA* and *cagA* are reported as important determinants contributing to gastroduodenal disorders [3]. In addition, it has been suggested that *H. pylori* adherence factors may play a role in gastroduodenal disease development, but the data are still controversial [3, 4]. Indeed, it has been reported that peptic ulcer diseases can be associated with *iceA* alleles [18], in some studies [10, 19] but not in others. It has been suggested that $iceA_1$ is associated with the development of peptic ulcers, because of the $iceA_1$-positive isolates can produce more of the pro-inflammatory factor such as IL-8 than strains without $iceA_1$ [19]. In this study, the prevalence of $iceA_1$ (36.8%) was higher than $iceA_2$ (21.2%). Studies from Japan and Korea report [21] similar results, but Miehlike et al. [21] report that the prevalence of $iceA_2$ is greater than $iceA_1$. In fact, the rate of $iceA_1$ is higher than $iceA_2$ in all diseases groups (GC and DU) with the exception of NUD patients, although the differences are not significant ($P > 0.05$). In contrast with some studies [10, 11], we failed to find any association between $iceA_1$ and duodenal ulcer. Furthermore, studies from Colombia, Japan and Korea disclose an unreliable association between $iceA_1$ and any kind of peptic ulcer disease such as duodenal or gastric ulcer [21, 22]. Not considering NUD and duodenal ulcer patients, we have observed a significant association between the presence of $iceA_1$ and gastric cancer ($P = 0.001$) (Table 2). In a study from Thailand [20], the prevalence of $iceA_1$ and $iceA_2$ are 45.5 and 33.1%, respectively. In this study, we report a rate for $iceA_1$ allele (36.8%), different in each of the disease groups (Table 2). We observed that $iceA_1$ was higher in our population, a data that has been reported before [11, 21–24]. Surprisingly $iceA_2$ is dominant in populations from USA and Brazil [18, 25]. There are no methodological differences between the abovementioned studies, but we suggest geographical discrepancy as a possible explanation for these diverse results. The most recent publications describe a variation of $babA_2$ prevalence among different countries around the world [8, 9, 26–29]. Contrary to former studies from Asian countries [24, 26, 27] that report a rate of $babA_2$ close to 100% in Taiwan [26], our result, of $babA_2$ (40.6%) was close to a Brazilian study [30]. Torres et al. [31] show 71.9% of their strains as $babA_2$ positive that we consider exceptional. Eshaghi et al. [32] report 71.6% for $babA_2$ during the first study in Iran. In this study, we have demonstrated that 65/160 (40.6%) of isolates were $babA_2$ positive, a lower rate than Eshaghi et al. [32]. Variation

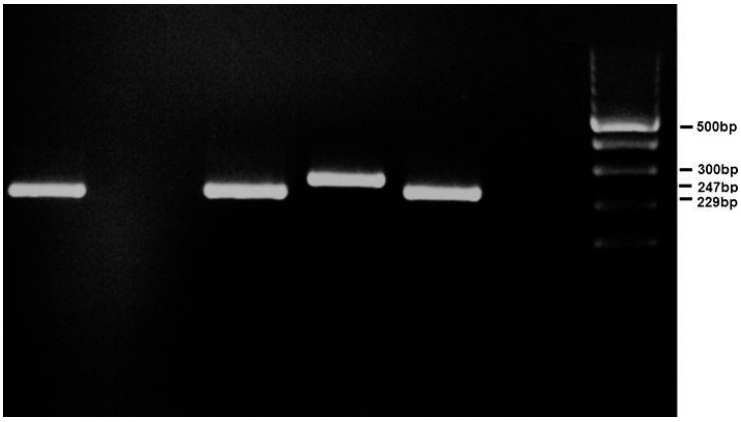

Fig. 2 Gel electrophoresis after PCR amplification of the *H. pylori* $iceA_1$ and $iceA_2$ primers, from *right* to *left*, (*lane 2*: negative control), (*lane 3*: positive control), (*lanes 4–7*: clinical positive and negative strains), (*lane 1*: 100-1,000 bp DNA ladder)

Table 2 Prevalence of $iceA_1$, $iceA_2$ and $babA_2$ among different diseases groups

Genotypes	Diseases					
	Gastric cancer	*P* value	Duodenal ulcer	*P* value	Non-ulcer dyspepsia	*P* value
$iceA_1$	36/90 (90)	0.001	13/55 (23.6)	0.51	10/65 (15.3)	0.507
$iceA_2$	12/40 (30)	0.26	9/55 (16.3)	0.35	13/65 (20)	0.45
$babA_2$	38/40 (95)	0.0004	10/55 (18.1)	0.41	17/65 (26.1)	0.625

Numbers in parenthesis are percentage of those genes

A *P* value <0.05 considered as significant

between our results and Eshaghi et al. [32] may again be explained by geographic differences between the two studies (our location: Sari in the north of Iran, their location: Isfahan at mid Iran), data which are confirmed by antibiotic resistance status in Iran as well [33, 34]. The inconsistency found between different researches [9, 31] may be due to inefficacy of primers in the study by Gerhard et al. [9] The inconsistent research makes it impossible to accurately know the total world distribution. Not only is there not enough data to estimate the total prevalence of $babA_2$ in world, but there is also a scarcity of data in Iran. However, our findings strongly support that in gastric cancer patient's $iceA_1$ and $babA_2$ alleles are more frequent than other studied groups (Table 2).

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Conflict of interest None.

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Chapter 6

Clinical relevance of *cagA*, *tnpA* and *tnpB* genes in *Helicobacter pylori*

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Clinical relevance of the *cagA*, *tnpA* and *tnpB* genes in *Helicobacter pylori*

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Abstract:

Background: Numerous proteins have been proposed as virulence factors for the gram negative gastric bacterium *Helicobacter pylori* but only for a few this has unequivocally been demonstrated. The aim of the current study was to evaluate the association of the putative virulence factors *tnpA* and *tnpB (no cagA)* with *H. pylori* associated gastroduodenal diseases.

Materials and Methods: A PCR based assay was used to determine the presence of the *tnpA* and *tnpB* genes, as well as of *cagA*, in 360 *H. pylori* strains isolated from *H. pylori* infected patients.

Results: Of 360 *H. pylori* culture positive patients (196 men, 164 women; average age 42.1 years (range 17-73), 95 had gastritis, 92 had gastric ulcers, 108 had duodenal ulcers, and 65 had gastric cancer. Using the gastritis group as a reference a significantly aberrant gene distribution was observed for the *tnpA,* the *cagA*, but not the *tnpB* gene in the gastric cancer group.

Conclusion: The increased incidence of the *tnpA* gene in gastric cancer patients suggests a role of the *tnpA* gene in the development of *H. pylori* induced gastric cancer.

Introduction

Helicobacter pylori is the most prevalent pathogenic microorganism colonizing the gastric mucosa of humans. Infection rates range between 85-95% in developing countries and 30-50% in developed countries [1]. Colonization always results in acute gastritis and chronic gastritis when left untreated [2]. Additional complications such as gastric ulcers (GU), duodenal ulcers (DU), or gastric cancer (GC) may develop in some of these *H. pylori* infected patients [3]. The outcome of the infection is determined by both the duration of infection and environmental, host, and bacterial factors [4]. *H. pylori* strains display extensive genetic variability with considerable variation in the presence of virulence factors, which is thought to cause the many different clinical presentations of *H. pylori* infections [5-7]. The CagA protein is a commonly accepted virulence factor and the *cagA* gene is often used as a marker for the presence of the *cag* (cytotoxin-associated gene) pathogenicity island (*cag*PAI) [4]. Patients infected with *H. pylori* strains that carry *cagA* have a higher risk for developing peptic ulcer and gastric cancer [8]. Other virulence determinants located on the *cag*PAI such as *cagE, cagG, cagH, cagI, cagL*, and *cagM* are required for *cag*PAI mediated NF-$_{\kappa}$B induction, and *cagT* and *cagY* are required for the formation of a needle-like structure that serves to inject *cagA* into the host cell [9]. Although these factors play a critical role in the pathogenesis of *H. pylori*, their association with specific disease outcomes is not as obvious as with *cagA*. It has been reported that in some *H. pylori* strains the *cag*PAI is split into two separate regions due to the integration of the IS*605* insertion sequence [10]. The putative IS*605* transposases (*tnpA* and *tnpB*) that can mediate this *cag*PAI disruption [10] might affect the virulence of *H. pylori* [11], but the exact biological role and clinical relevance of these two determinants is poorly studied. Iran is a developing country with a high prevalence of *H. pylori*, among both symptomatic and asymptomatic individuals, and with a prevalence as high as 95% in the northern part of the country [12, 13]. This high prevalence is coupled to an even higher rate of *H. pylori* induced peptic ulcer disease and gastric cancers [14]. This makes it an ideal geographically confined region to study the effect of genetic variation of this gastric pathogen on infection associated disorders. In this study we determined associations of the presence of *tnpA* and *tnpB* and clinical manifestations of *H. pylori* infections in patients from the North of Iran.

Materials and Methods:

Patients:

All patients suspect of a *H. pylori* infection that visited the Tooba Medical Center, in Sari, Iran for endoscopic examination between May 2008 and October 2010 were invited to participate in this study. Patients participating in this study underwent routine gastroscopic examination and the regular biopsy samples for patients' suspect of *Helicobacter* infection were obtained. One gastric biopsy sample was sent to the pathology lab and tested by routine histopathological techniques and evaluated by standard criteria [15]. The other routinely obtained biopsy samples were used for microbiological culture and Rapid Urease Test (RUT), as described below. Ages below 16 years, antibiotic use within four months prior to endoscopy, or use of anti-secretory drugs within one month before endoscopy were used as exclusion criteria. This study was approved by the local ethics committee of Tarbiat Modares University, as no extra biopsy samples were needed for this study and that the obtained data could not be traced back to the patient level.

Microbiological analysis:

One of the biopsy samples was routinely tested by the gastroenterologist by Rapid Urease Test; and if positive a second sample was obtained and placed in 200μl sterile thioglycolate (Merck, Germany) broth and then immediately shipped to the diagnostic laboratory for routine culture. Upon arrival in the microbiology lab this sample was immediately grinded and 100μl of the resultant homogenate was inoculated on a Colombia agar (Merck, Germany) plate supplemented with 7% defibrinated sheep blood (Jihad Daneshgahi, Tehran, Iran), 10% Fetal Calf Serum (FCS) and antibiotics (DENT, Supplement, Oxoid) [15]. Plates were incubated at 37^{o}C, in 10% CO_2 conditions provided by incubator (Binder, USA) and high humidity until typical *H. pylori* colonies appeared or for a maximum of 7 days if no suspect colonies were observed. Colony shape, morphology in microscopic examination, routine biochemical tests such as urease, catalase and oxidase tests were performed for identification of *H. pylori* strains.

DNA Extraction and PCR:

Bacterial DNA was extracted from single colonies of *H. pylori* using a commercially available kit (ExiPrep™ Bacteria Genomic DNA Kit, Bioneer, Daejeon, South Korea). Genotyping was performed by PCR, using specific primers for *cagA, tnpA* and *tnpB* as previously described (Table 1). In addition a *glmM* PCR (Table 1) was carried out [16], both as an additional control for *H. pylori* identification and quality check of the isolated DNA (positive PCR control). The PCR amplified fragments were size separated on 2% agarose gel (Sinagene, Tehran, Iran) and the ethidium bromide stained DNA was visualized using UV illumination.

Table 1. Primers used in this study

Primers	5'-3' Sequence	Reference
glmM	AAGCTTTTAGGGGTGTTAGGGGTTT AAGCTTACTTTCTAACACTAACGC	20
tnpA	ATCAGTCCAAAAAGTTTTTTCTTTCC TAAGGGGGTATATTTCAACCAACCG	13
tnpB	CGCTCTCCCTAAATTCAAAGAGGGC AGCTAGGGAAAAATCTGTCTATGCC	13
cagA	ATAATGCTAAATTAGACAACTTGAGCGA TTAGAATAATCAACAAACATCACGCCAT	5

Statistical analysis:

The chi-square and Fisher exact test was used to test for the association between patient demographics, *H. pylori* genotypes, and disease groups. A *P* value of less than 0.05 was accepted as statistically significant. Microsoft Excel 2010 was used to calculate the *P* values, odds ratio (OR) and 95% confidence interval (95% CI).

Results

376 patients suspect for *H. pylori* infection (positive RUT test) were enrolled, but *H. pylori* specific growth was not observed from the biopsy specimen in 16 of them. The remaining 360 patients that were *H. pylori* culture positive (96%) comprised 95 patients with gastritis (G), 92 with gastric ulcer (GU), 108 with duodenal ulcer, and 65 with gastric cancer (GC) (Table 2). The average age was 42.1 years (range 17 to 73 year) and there were slightly more men (n=196) than women (n=164). Detailed demographic data of dyspeptic patients according to age, disease symptoms, and histological findings are shown in Table 2. There were slightly more males with duodenal ulcers, and less with gastric ulcers, but there were no statistically significant associations between age, gender, histopathological findings, and *H. pylori* associated disease groups.

PCR screening of *tnpA*, *tnpB* and *cagA*

The overall prevalence of the *tnpA, tnpB,* and *cagA* genes were 47.5%, 13.1%, and 59.2%, respectively, and the prevalence of these genes in the four disease groups is listed in Table 3. No significant associations were observed between the presence of the *tnpA, tnpB* and *cagA* genes and histological findings. Statistical analysis did however reveal a significant association between the presence of the *cagA* gene and GC [Relative risk: 1.81; 95% CI 1.44-2.29], and a weak, but significant correlation was observed between the presence of the *cagA* gene and DU [Relative risk: 1.30; 95% CI 1.01-1.69] and the *tnpA* gene with GC [Relative risk: 1.45; 95% CI 1.04-1.93] (Table 3). No significant association was observed for *tnpB* and gastroduodenal diseases.

Table 2. Detailed demographic data of dyspeptic patients according to the age and pathologic findings

Disease type	Sample size	Male (%)	Pathology findings	Age range detailed data for each disease groups				
				<30	31-40	41-50	51-60	>60
G	**95**	**51 (53.6)**	Mild (n=14)	6	7	1	0	0
			Moderate (n=67)	33	26	6	2	0
			Atrophic (n=20)	8	5	6	1	0
GU	**92**	**38 (41.3)**	Mild (n=15)	2	3	4	4	2
			Moderate (n=84)	7	12	21	11	33
			Atrophic (n=13)	0	2	2	6	3
DU	**108**	**72 (66)**	Mild (n=23)	4	5	5	6	3
			Moderate (n=57)	6	18	13	12	8
			Atrophic (n=23)	6	7	7	6	4
GC	**65**	**35 (53.8)**	Mild (n=7)	0	0	1	2	4
			Moderate (n=47)	0	0	17	16	14
			Atrophic (n=11)	0	0	6	1	4

Table 3. Prevalence of the *tnpA*, *tnpB*, and *cagA* genes in the four patient groups

Disease groups	*tnpA*			*tnpB*			*cagA*		
	Positives	**Relative Risk**	**95% CI**	**Positives**	**Relative Risk**	**95% CI**	**Positives**	**Relative Risk**	**95% CI**
Gastritis (n=95) (Control group)	40 (42.1%)	Reference		16(16.8%)	Reference		45(47.4%)	Reference	
Gastric ulcer (n=92)	48 (52.2%)	1.23	0.97-1.61	10(10.9%)	0.64	0.30-1.34	45(48.9%)	1.03	0.76-1.39
Duodenal ulcer (n=108)	44 (40.7%)	0.96	0.69-1.34	11(10.2%)	0.59	0.29-1.22	67(62%)	1.30	1.01-1.69
Gastric cancer (n=65)	39 (60.0%)	1.45	1.04-1.93	10(15.4%)	0.91	0.44-1.88	56(86.2%)	1.81	1.44-2.29

Discussion

To our knowledge, this is the largest study (n=360) investigating the distribution of the *H. pylori* virulence *tnpA, tnpB* and *cagA* in dyspeptic patients. In the first study on *tnpA* and *tnpB* by Matter *et al* [11], 63% of 215 clinical *H. pylori* isolates were *tnpA* positive and 13.5% were positive for *tnpB*, with a statistically significant association between peptic ulcer disease (PUD) and *tnpA* positive strains. This association was not apparent for *tnpB*. In the current study there was a similar prevalence of *tnpA* and *tnpB* [171/360; 47.5% and 47/360; 13.1%, for *tnpA* and *tnpB* respectively], and a similar association between *cagA* and gastric cancer patients as observed in a preliminary study by Matter *et al* [11]. Unfortunately in their study the associations of *tnpA* and *tnpB* with *H. pylori* associated disease types were not determined. In a more recent but smaller study Matter *et al* investigated associations between presence of *tnpA* and *tnpB* and gastric cancer in Brazilian patients with gastric

cancer (n=34) and gastritis (n=34) [17]. The prevalence of *tnpA* and *tnpB* among gastric cancer and gastritis patients in the Iranian population included in the current study was 42.1% and 60.0% for *tnpA*, and 16.8% and 15.4% for *tnpB,* respectively, which, again, was comparable to the findings in the Brazilian population with gastric cancer or gastritis (29.4% and 73.5% for *tnpA*; and 2.9% and 5.9% for *tnpB,* respectively). Kersulyte *et al* [18] also reported a higher frequency of *tnpA* in Peruvian gastric cancer strains than in gastritis strains (9/14 (46%) versus 15/45 (33%), respectively). Although the observed associations between *tnpA* and gastric cancer are similar in the populations in Peru, Brazil and Iran [11, 18], there are striking differences for associations of *cagA* with disease status between these populations. We observed a clear association between the presence of *cagA* and gastric cancer in the Iranian population, while Matter *et al* [11] did not observe such an association in Brazil. While most studies report an association between the presence of *cagA* and gastric cancer some studies do not observe this association [19, 20]. In this particular case it may be due to the low number of patients included in their study (n=64; versus 160 in our study). After the recognition of *H. pylori* as an important gastric pathogen [20], many attempts have been made to identify *H. pylori* virulence factors predicting clinical outcome as this might assist physicians in prediction of disease progression [21]. When using the gastritis group as controls for gene distribution we observed an increased prevalence of the *tnpA* and *cagA* genes in the gastric cancer group. To our knowledge this study represents the largest cohort tested thus far for the prevalence of *tnpA* for an association with the various *H. pylori* infection associated disease groups. While it is tempting to conclude from the increased prevalence of *tnpA* and *cagA* in the gastric cancer group that these genes may serve as useful biomarkers for gastric cancer one cannot draw that conclusion from a cross-sectional study like ours. A large prospective cohort study would be required to establish reliable positive and negative predictive values of these putative biomarkers. Due to the long time between infection and cancer development such a study would require long follow-up times, and since only few infected individuals develop cancer a large study cohort would be required. In addition there are ethical issues with such a study as the hypothesis to be tested is that patients infected with *tnpA* positive *H. pylori* strains are more prone to developing gastric cancer than patients infected with *tnpA* negative strains. In order to

test this hypothesis one must establish the presence of the *tnpA*$^{+/-}$ *H. pylori* strain at the start of the study while refraining from eradication of these potentially carcinogenic strains for a long period of time. In spite of the shortcomings of our cross-sectional study it provides strong indications for the clinical relevance of the *tnpA* gene of *H. pylori* strains isolated from the Iranian population where the prevalence of *H. pylori* is relatively high [13] and this high prevalence is coupled to a high incidence of *H. pylori* induced peptic ulcer disease and gastric cancers [22]. In conclusion *tnpA* but not *tnpB* is clearly associated with a more severe disease outcome of *H. pylori* infections. As such *tnpA* could be a valuable novel biomarker but clearly further studies are required to confirm these results especially since at present no obvious biological explanation for a GC inducing function of this putative transposase can be provided.

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Chapter 7

Detection of the *Helicobacter pylori dupA* gene is strongly affected by the PCR design

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Detection of the *Helicobacter pylori dupA* gene is strongly affected by the PCR design

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Abstract:

The duodenal ulcer promoting gene (*dupA*) of *H. pylori* is a virulence factor whose presence has been associated with *H. pylori* induced duodenal ulcer formation, but some dispute this association. Here we postulate that technical limitations of the PCR assays used to detect the *dupA* might explain these discrepant results. An *in silico* evaluation of the *dupA* gene sequences from the public DNA databases revealed significant mismatches of the different *dupA* primers that have been used in published studies. To further provide experimental evidence for our hypothesis, we designed new primers located within the conserved regions of *dupA* and then compared these new and old primers for their potential to detect *dupA* in a collection of clinical isolates. Our newly designed PCR detected *dupA* in 253/394 (64.2%) of the tested strains, while the old primer sets each detected *dupA* at levels that ranged from 29.9-37.8%. Our data show that the various PCR assays that have been published differ in sensitivity to detect different *dupA* alleles. Thus rather than the *dupA* gene itself being associated with specific *H. pylori* induced disease symptoms the observed associations should be assigned to specific *dupA* alleles.

Introduction:

Following the first report on the identification of *Helicobacter pylori* it rapidly became clear that *H. pylori* is a major human pathogen and infection can result in gastroduodenal disorders; ranging from chronic gastritis to peptic ulcer and gastric cancer [1]. The exact mechanisms involved in the development of these diseases are still under debate but probably involves both host factors as well as environmental and bacterial factors [1, 2]. Lu *et al* described the duodenal ulcer promoter gene A (*dupA*) which was found to be associated with an increased risk of ulcer formation [3]. In the fully sequenced reference strain 26695 the *dupA* gene is a single open reading frame (ORF) while in the other sequenced reference strain J99 the *dupA* gene seems to consist of two ORFs (*jhp0917* and *jhp0918*) [3]. The exact function of the *dupA* is unclear [4], and it is unknown if for full biological function the *dupA* gene needs to be present as a single ORF. Shortly after the report by Lu *et al*, several reports appeared and although many of these confirm the association with duodenal ulcers and some even report a negative association with gastric cancer, others do not find these associations [5-10]. Local differences between patients and infecting strains have been proposed as the most likely explanation for these controversial findings. The fact that *H. pylori* is genetically highly variable, and the *dupA* gene is located in a variable area on the *H. pylori* genome, might lead to an alternative explanation. In most reports the absence/presence of the *dupA* gene is tested with a PCR assay, and the genetic differences between strains could explain the observed discrepancies [5-10]. The aim of the study was to determine the effect of an alternative PCR design on the detection of *H. pylori dupA* in isolates obtained from Dutch patients.

Materials and Methods

Strains selection:

H. pylori strains were selected from a historical collection that contained all cultured isolates from patients suspected of an *H. pylori* infection that had consulted the Zaans Medical Center, Zaandam, the Netherlands, between 2005 and 2007. Biopsy samples were obtained from these patients and routine diagnostic microbiological culture for detection of *H. pylori* was performed on Colombia agar plates supplemented with 7% defibrinated sheep blood, 10% Fetal Calf Serum (FCS) and antibiotics (DENT, Supplement, Oxoid). Plates were incubated at 37ºC and 7% CO_2, and high humidity for a maximum of 10 days, or until typical *H. pylori* colonies appeared. Colony morphology, bacterial morphology, routine biochemical testing for urease, catalase, and oxidase tests were performed to confirm that cultured bacteria represented *H. pylori*. All positive cultures were then routinely stored at -80ºC. *H. pylori* reference strain J99 and 26695 were used as controls.

DNA Extraction:

DNA-isolation from the bacterial strains was by standard automated DNA extraction using the MagNA Pure 96 DNA and Viral NA Small Volume Kit on a Roche Magna Pure 96 with the Viral NA Universal SV extraction protocol according to the manufacturer's instructions (Roche Diagnostics, Almere, the Netherlands). Isolated DNA was stored at -80^{o}C. The use of this strain collection was approved by the local ethics committee, as it was based on a stored collection of strains, no extra biopsy samples were needed for this study, and the obtained data could not be traced back to the patient level.

Primer design and PCR setup

We created an alignment of all 221 *dupA* gene sequences present in the NCBI DNA database on November 2011 with Clone Manager Professional Edition (version 9.2) [11]. Based on this alignment we designed a new set PCR primers and a probe (Table 1) that targeted a highly conserved area of the *dupA*

gene. PCR conditions for this Real Time PCR (*AR-dupA*) were as follows: 95ºC for 10 minutes (pre-incubation), 42 amplification cycles consisting of 95ºC for 20 seconds and 60ºC for 55 seconds. PCR amplification was performed on a Roche Light Cycler 480 II (Roche Diagnostics, Almere, the Netherlands). In addition we used three *dupA* primer sets [6] that have been used previously by others to establish the presence/absence of the *dupA* gene (Table 1) and PCR was performed according to the conditions as reported by the authors that originally designed these primers (See table 1).

Table 1: Oligonucleotide primer sequences and annealing temperature used in current study

Gene	Sequences (5-3)	Size of PCR fragment	Annealing temp (^{o}C)	Reference
jhp0917	TGGTTTCTACTGACAGAGCGC AACACGCTGACAGGACAATCTCCC	307bp	59	Lu *et al*
jhp0918	CCTATATCGCTAACGCGCGCTC AAGCTGAAGCGTTTGTAACG	276bp	*58*	Lu *et al*
dupA	TAAGCGTGATCAATATGGATT GGAACGCCGCATTCTATTA	350bp	*56*	Nguyen *et al*
AR-dupA	CATGGCGTTTCAAAAAATATCTCAA TTCATCAGTATCTTTTGTGGGGTA **FAM**-GGCAACCTTTCTCAAGTGATTATC-**BBQ**	112bp	60	Current study

Statistical analysis:

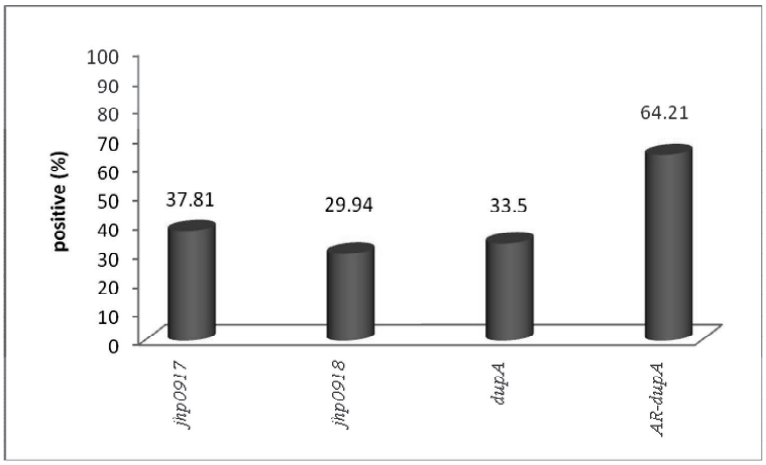
IBM SPSS Statistics version 21 was used for statistical analysis and a *p* value <0.05 considered as significant.

Results:

From the stored collection of frozen strains we selected the first 400 strains, i.e. all stored isolates from 2005 (n=44) and 2006 (n=141), and the first 215 isolates from 2007. From six of these strains we could not obtain DNA thus leaving 394 *H. pylori* strains for our analysis with the *dupA* PCR primers. Comparing the positivity rate of all four PCR assays showed significant differences between the different investigated primer sets (p<0.05; Figure 1). The *jhp0918* and *AR-dupA* showed the lowest and highest

detection rates (29.9% and 64.2%, respectively) of *dupA* in these clinical isolates (Figure 1). The newly designed *AR-dupA* PCR showed to be the most sensitive PCR to detect *dupA*. In fact for 30/394 (7.6%) of the strains only the *AR-dupA* PCR was positive. Sequencing the *AR-dupA* PCR products from the strains that were only positive in the *AR-dupA* PCR confirmed that these PCR products actually represented true *dupA* positives that were false negative in the PCR reactions based on any of the other three primer sets. The *jhp0917* PCR was positive in 149/394 (37.8%) of the samples and the *jhp0918* PCR in 118/394 *(*29.9%). When using the recommendation of the original designers of the *jhp0917* and *jhp0918* PCR to report the *dupA* status as positive (or negative) when both the *jhp0917* and *jhp0918* PCRs were positive (or negative), only for 254 of the 394 isolates (64.4%) a *dupA* status could be obtained as. Eliminating the 140 isolates with discordant *jhp0917* and *jhp0918 PCR data* resulted in a *dupA* positivity rate of 41/254 (16.1%).

Figure 1. Detection of *H. pylori dupA* with different primer sets



Discussion

H. pylori is genetically diverse and can easily change its genomic contents by obtaining or losing genes. Eight years after the first report by Lu *et al* on *dupA* as a putative virulence factor for *H. pylori* a lot of data has been generated that confirm their initial claim [3]. However, there have also been controversial findings regarding the possible association between specific disease outcome and the presence of *dupA* [8-10]. Most investigators agree that *dupA* is an independent virulence factor with no linkage to other classical virulence factors like *cagA*, *vacA*, and *iceA* and the prevalence of *dupA* varies depending on the geographical region [12]. However not all agree that *dupA* positive *H. pylori* strains are indeed more prevalent in patients with duodenal ulcers than in gastritis patients [8, 10]. This discrepancy has been attributed to differences in diet, smoking habits, socioeconomic conditions, and immune status of the infected patients [4]. Also local differences in the infecting *H. pylori* strains have been mentioned, e.g. with regard to *dupA* genes or the presence/absence of other virulence factors. As far as we know none has actually tested the technical limitations of the PCR assays used to detect the *dupA* status as potential reason for these discrepant results. *H. pylori* is a genetically variable bacterium and especially at the plasticity region where the *dupA* gene is located is known to display substantial genomic diversity [13, 14]. This prompted us to check the effect of the variability of this region on the various PCR reactions that have been used to detect the *dupA* gene. An *in silico* evaluation of the *dupA* gene sequences from the public DNA databases revealed significant mismatches of the different *dupA* primers that have been used in the published studies. Indeed the prevalence of *dupA* among the *H. pylori* strains was significantly higher when using a newly designed primer set that targeted conserved areas of the *dupA* gene, indicating that the *dupA* PCR assays that have been used in the published studies probably missed some *dupA* positive isolates. In addition all four primer sets seemed to detect a different subset of strains. Unfortunately, we do not have clinical data related to the strains from our collection, nor do we pose such a collection, and thus we cannot check the association of the various PCRs with specific disease outcome and the presence of *dupA*. Obviously, this needs to be examined in various geographical areas to further test the validity or our hypothesis. In conclusion, our findings suggest that previous results on an

association between the increased prevalence of *dupA* in patients suffering from *H. pylori* induced ulceration should be interpreted with caution. More likely these papers reported the association of a specific *dupA* allele instead of the actual presence of the *dupA* gene. Thus rather than disputing or confirming the association between the presence of *dupA* and different gastroduodenal disease these reports tested a relationship between a specific *dupA* allele and disease. Since various primer sets were used these different PCR assays probably tested for different *dupA* alleles and can thus not be compared to each other. It is commonly accepted that for the classical *H. pylori* virulence factor *vacA* there exist different alleles (s_1/s_2 m_1/m_2), and that it is not the presence of *vacA*, but the presence of a specific allele that is associated with a different risk on inducing *H. pylori* associated disease [15]. Our data indicate that also for *dupA* there exist different alleles that, as with *vacA,* might be associated with different risks for the induction *H. pylori* associated disease. The challenge would be to identify these disease specific *dupA* alleles and to design allele specific primers for their detection.

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Chapter 8

Primary resistance of *Helicobacter pylori* to Levofloxacin and Moxifloxacine in Iran

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Primary resistance of *Helicobacter pylori* to levofloxacin and moxifloxacine in Iran

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Abstract The increase in the prevalence of antibiotic resistance in *Helicobacter pylori* had a drastic effect on successful treatment. Up-to-date information on *H. pylori* antibiotic therapy in Iran is still limited. In this study, we aim to determine the prevalence of antibiotic resistance among the *H. pylori* strains. Furthermore, the possibility of using fluoroquinolones for antibiotic treatment was investigated. Antral biopsy specimens obtained from dyspeptic patients were investigated for *H. pylori*. Bacterial culture and susceptibility tests were done based on standard methods. *H. pylori* ATCC 43504 was used as a quality control. In the current study, 30 *H. pylori* strains were selected randomly and retested to confirm our susceptibility tests. Of 170 patients, 150 were identified as positive for *H. pylori* (88.2%). In this study, 150 single colonies of *H. pylori* strains [81 women (54%), 69 men (46%); mean age 38.6; aged 21–70 years] were collected. Primary resistance of *H. pylori* isolates were clarithromycin (34%), metronidazole (78.6%), tetracycline (9.3%), amoxicillin (10%), levofloxacin (5.3%) and moxifloxacine (4.6%). In conclusion, our results show that we are confronting a new generation of resistant strains of *H. pylori* in Iran. This alarming finding indicates an urgent need for introduction of new effective antibiotics in our country. Since the majority of clinicians prefer to continue with the ineffective antibiotics as therapeutic regimens, they must also be prepared to deal with treatment failures.

Keywords *Helicobacter pylori* · Treatment · Moxifloxacine · Iran

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Introduction

Helicobacter pylori can cause a chronic infection that infects more than 50% of the world population [1]. Depending on the individual country and its population, the prevalence of *H. pylori* infection varies widely; 20% in developed countries to 95% in third-world countries, such as Iran [2, 3]. In addition to the high prevalence of *H. pylori* infections in Iranian people, various studies conclude that a large proportion of symptomatic and asymptomatic patients are suffering from gastric ulcer, gastritis and gastric cancer [4–7].It has been shown that antibiotic resistance can reduce the eradication rates up to 70% [1]. Certainly, the low rate of treatment efficacy with current treatment regimens has disappointing results in Iran. Other studies have shown that gastric cancer is also common and highly common in northern Iran [2, 8, 9]. Different reports from this area of country disclose a high prevalence of *H. pylori* infections and antibiotic resistance [10, 11]. In the meantime, our findings strongly suggest that treatment failure is a major outcome of the antibiotic resistance and an incompetent monitoring system in Iran (data not published). In this situation, several studies show

that fluoroquinolones are active against *H. pylori* isolates [12–14]; so this class of drug can be used as a possible candidate for a rescue regimen designed for *H. pylori* eradication [15, 16]. Recently, the prevalence of fluoroquinolones resistance rates is reported as up to 32% in different studies [17–20]; in contrast to other authors who report a good efficacy of fluoroquinolone-based therapy for the eradication of *H. pylori* infections [21–23]. Our preliminary goals are to examine: (1) the exact profile of resistance rate to clarithromycin, metronidazole, tetracycline and amoxicillin, commonly used antibiotics in patients who have no prior treatment; (2) to determine the possibility of prescription for newly introduced antibiotics such as moxifloxacin for treatment of *H. pylori* in Iran.

Materials and methods

Patients

Antral biopsy specimens and corresponding single colony *H. pylori* strains were obtained from consecutive patients who had routine upper GI endoscopy during the period of October 2009 to July 2010 in Iran. Moreover, three biopsies were taken for histological examination and sent to pathology. Our exclusion criteria were patients receiving H_2-receptor blocking drugs or anti-*H. pylori* antibiotics. None of the patients had been treated with antibiotics against *H. pylori* before this study. For ethics limitations, patients under 15 years old were also excluded in our study. A written informed consent was obtained from all participants before enrollment. Our study protocol was approved by the ethics committee of Tarbiat Modares University, Tehran, Iran.

H. pylori strains: culture and identification

Two antral biopsy specimens per patient were taken during endoscopy, the first specimen was used for a rapid urease test (Shim-Anzim, Tehran, Iran) and the second biopsy specimen was placed in a sterile thioglycolate broth medium (Merck, Germany), and then sent to the diagnostic laboratory within 2 h [24, 25]. In the diagnostic laboratory, after homogenization with a grinder, 100 μl of homogenate was inoculated onto the surface of Columbia agar (Merck, Germany) plate, as previously described [25]. In this study, microaerophilic conditions were provided by a plastic jar and an anaerobic gas pack (Merck, Germany), the temperature was kept at 37°C during the incubation period of 7–8 days [25, 26]. For *H. pylori* culture, Colombia agar (Merck, Germany) supplemented with 7% defibrinated sheep blood (Bahar-Azma, Tehran, Iran), 7% fetal calf serum (FCS) (Gibco, CA, USA), were used; furthermore, antibiotics (amphotericin B, polymyxin B and vancomycin) (MAST, Mercy Side, UK) were added in order to prevent contamination [24]. The *H. pylori* identification was done by typical colony morphology, Gram's staining, microscopic observation and biochemical tests such as catalase, oxidase and urease [26]. In order to get accurate results, all of the antimicrobial susceptibility tests were performed after obtaining a single colony of all clinical isolates with use of subculture technique at least for 5 days after primary isolation [25]. Of the 150 *H. pylori* isolates, 30 isolates were selected randomly and retested to confirm our antimicrobial susceptibility tests.

Antimicrobial susceptibility

Resistance of *H. pylori* strains to eight antibiotics [amoxicillin (Sinagen, Tehran, Iran), levofloxacin (Sigma Co, MO, USA), metronidazole (Sinagen, Tehran, Iran), tetracycline (Sinagen, Tehran, Iran), clarithromycin (Sinagen, Tehran, Iran), moxifloxacin (Bayer Pharma Co., Germany)] was determined in this study based on agar dilution [27] and E-test (AB Biodisk, Solna, Sweden) methods. In this study, E-test method was used as the gold standard for determination of minimal inhibitory concentration (MIC) of *H. pylori* clinical isolates, and the results were confirmed with agar dilution [9, 27]. For the susceptibility test, an inoculum of *H. pylori* suspension was adjusted to a density corresponding to 2 McFarland's turbidity standard tube, and then 200 μL of bacterial suspension was inoculated on Muller-Hinton agar (Merck, Germany) plate, supplemented with 5% defibrinated sheep blood (Bahar-Azma, Tehran, Iran) [25]. The inoculated plates were left at room temperature for 10 min for drying, and then E-test strips were placed on the agar surface.

The plates were incubated at 37°C for 5 days with high humidity atmosphere. The resistance breakpoints for metronidazole, amoxicillin, clarithromycin, and tetracycline were $\geq$8, $\geq$0.5, $\geq$1 and $\geq$4 μg/mL, respectively [27]. Since there was no exact determined standard breakpoint for fluoroquinolones, we defined >1μg/mL for these antibiotics based on recent published data [6, 25, 27]. In this study, *H. pylori* ATCC 43504 was used as a quality control. All *H. pylori* single colony strains were stored in −80°C in Brucella broth (Merck, Germany) supplemented with 25% (FCS) (Gibco, CA, USA) and 20% glycerol for further analysis.

Statistical analysis

Our results were analyzed using SPSS (version 15.0) software, while Student's *t* and Chi-square tests were used for analysis. A *P* value less than 0.05 was considered statistically significant.

Results

Out of 170 patients with gastroduodenal complaints, 150 patients have been identified as positive for *H. pylori* (150/170: 88.2%), while all of 170 were RUT positive. Subsequently, we have isolated 150 single colony strains of *H. pylori* from antral biopsy specimens of 150 *H. pylori* positive patients who were admitted to Tooba medical center at Sari, north of Iran. Of the 170 patients examined in this study, 150 were positive for *H. pylori* [70 gastritis, 23 gastric cancer, 30 duodenal ulcer and 27 with gastric ulcer]. In this study, 150 consecutive single colony *H. pylori* strains isolated from antral biopsy specimens of patients [81 women (54%) and 69 men (46%); mean age 38.6; aged 21–70 years] were collected at Sari, Iran. No significant associations were observed between antibiotic resistance and age ($P = 0.87$), clinical presentation of patients ($P = 0.49$) and gender ($P = 0.34$). Our data shows a primary resistance of *H. pylori* isolates to clarithromycin 51/150 (34%), metronidazole 118/150 (78.6%), tetracycline 14/150 (9.3%), amoxicillin 15/150 (10%), levofloxacin 8/150 (5.3%) and moxifloxacine 7/150 (4.6%).

Our findings indicate that dual resistant strains (metronidazole–clarithromycin) were found in 39 (26%), while the resistance rate for triple resistant (metronidazole–clarithromycin–amoxicillin) and quadruple resistant (metronidazole–clarithromycin–amoxicillin–tetracycline) were 9 (6%) and 5 (3.3%), respectively. Surprisingly, of the 150 *H. pylori* strains, we did not identify strains with resistance to more than one fluoroquinolone antibiotic (Table 1). Reported resistance of *H. pylori* strains in a former study [9] in our area was compared with the current results in Fig. 1.

Table 1 Detailed frequency of resistance profile of *H. pylori* strains to investigated fluoroquinolones in current study

Strain number	LEV	MOX
H3	+	−
H9D	−	−
H24	−	−
H33C	+	−
H52	−	+
H75	+	−
H80M	−	+
H82A	+	−
H85	+	−
H88N	−	+
H90M	−	+
H93	+	−
H95M	−	+
H101	+	−
H110	+	−
H131B	−	−
H139	−	−
H140	−	+
H143M	−	−
H146A	−	+

(+) indicating on presence of resistant strain and (−) referred to susceptibility of mentioned strain

MOX moxifloxacine, *LEV* levofloxacine

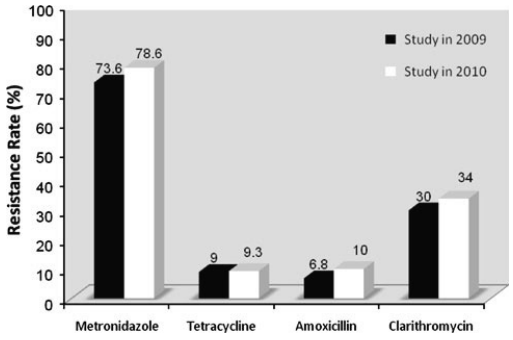

Fig. 1 Comparison between current prevalence of four antibiotics results with former study performed in our area at 2009

Discussion

Many studies identify a strong association between *H. pylori* infections and different gastroduodenal disorders among Iranian patients [4, 5, 7]. Recent reports showing treatment failure related to dual and triple therapies have shown a trend towards the usage of rescue therapy with different agents [6, 7]. After triple therapy, quadruple drug regimens have been introduced, which generally have a better eradication rate (>78%) against *H. pylori* infections [28]. Treatment failure suggests antibiotic resistance. Data indicate a growing tendency of antibiotic resistance between the *H. pylori* strains, which is a major concern around the world, especially in developing countries such as Iran [11, 12, 14, 22]. Fluoroquinolones are suggested as new possible candidates for *H. pylori* treatment, since numerous studies demonstrate the inefficacy of routine antibiotics such as metronidazole, clarithromycin, and tetracycline in our country [7, 9, 10]. In the current study, we aimed to increase our data regarding resistance status of commonly used antibiotics and fluoroquinolones family agents including moxifloxacine and levofloxacin.

Resistance to commonly used antibiotics

According to different publications, resistance rate of *H. pylori* to clarithromycin and metronidazole are increasing

quickly [9, 10]. Our survey showed an increasing trend of resistance to metronidazole (73.4% in 2009 to 78.6% in 2010) and clarithromycin (30% in 2009 to 34% in 2010) (Fig. 1). In agreement with other studies [10, 11], our results identified that constant usage of clarithromycin and metronidazole in therapeutic regimens can be strongly associated with high risk of treatment failure. Previous reports from the Middle East (58–95%) are consistent with our findings in this investigation [10, 20, 29]. Clarithromycin resistance in *H. pylori* is reported around 20% in developed countries [30, 31], a rate continuously found in Iran [9, 10, 32]; although some contradicting data are still available [33–35].

In European countries, most of the studies report a low level (10–15%) of resistance to clarithromycin [36–38], but recently, Agudo et al. [39] report a relatively high rate of resistance (35.6%) to clarithromycin among the *H. pylori* strains in Spain. In our study, the rate of resistance to tetracycline (9.3%) is not different from the last study in this area (9%) [9]. However, Rafeey et al. [29] from Tabriz, northwest of Iran, report a similar rate for tetracycline-resistant strains, a fact which correlates with other studies [37]. A study by Falsafi et al. [32] from Tehran, central Iran identifies more than 68% of isolates as tetracycline resistant. Two main explanations can be associated with these unexpected results, the first is different methodology (agar dilution in our study whereas they used disk diffusion method), and the second is differences between involved populations that contribute to genetic differences. In general, primary resistance to amoxicillin is low (10%), similar to the results in our former study in Iran (6.8%) [9]. Totally, the resistance rate of amoxicillin is not high in European countries, and it then can be prescribed continuously [6, 40]. Although, we detected an increased level of amoxicillin resistance over the last 3 years in our area (Fig. 1), we still utilize this antibiotic as a major member of therapeutic regimens in our area. Surprisingly, Rafeey et al. [29] detect a high resistance to amoxicillin among their isolates (59%), a fact which demonstrates the real value of local susceptibility tests for determining the best choice of treatment.

Fluoroquinolones: new era in *H. pylori* treatment

For the first time, we have identified the resistance profile of *H. pylori* isolates to fluoroquinolones in a single-center survey in Iran. After several studies showing the ineffectiveness of current antimicrobial agents such as metronidazole, clarithromycin and tetracycline for treatment, it was expected that new attempts would be made to develop alternative choices in order to update *H. pylori* eradication regimens. Our findings identified that primary resistance rates were 8/150 (5.3%) for levofloxacin and 7/150 (4.6%) for moxifloxacine. Surprisingly, no dual resistant strains to fluoroquinolones were detected among *H. pylori* strains (Table 1).Emergence of multiple drug resistant strains of *H. pylori* is a problem for treatment guidelines, because they decrease the efficacy of current regimens for the eradication of *H. pylori*. In summary, our study has shown the rate of resistance to fluoroquinolones agents to be less than 5.3%. Resistance rates to moxifloxacine, levofloxacin were between 4.6 and 5.3%. This new finding has led us to suggest this antibiotic for the eradication of *H. pylori* infections. To date, our findings are the first results supporting the usage of fluoroquinolones for the treatment of *H. pylori* in Iran. In contrast with other countries that report an increasing rate of fluoroquinolones resistant *H. pylori* strains, our results suggest that we are not yet confronting those resistant strains of *H. pylori* in Iran. Furthermore, there is a very low range of MIC for both susceptible/resistant *H. pylori* strains in our study, thus showing promise for the aim of designation as a rescue regimen of treatment in combination with the fluoroquinolones family of antibiotic such as moxifloxacine (detail data not shown). In summary, failed therapeutic regimens can be correlated with an increased probability of more severe outcomes for infected patients. Consequently, extra costs are incurred to cure the infection. Overall, scarcity of up-to-date data regarding fluoroquinolones efficacy, locally and globally, limited our accurate assessment for this family of antibiotics. We show new possible applications for two newly introduced fluoroquinolones such as moxifloxacine in our country.

Conclusion

Constant surveillance and monitoring are essential for having updated data on antibiotic resistance of *H. pylori* clinical strains. Since the majority of clinicians prefer to continue with the common standard of triple therapy, they must also be prepared to deal with treatment failures. We strongly recommend the utilization of levofloxacin and moxifloxacine as first-line treatment to obtain an effective and successful elimination rate of *H. pylori* infection.

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Conflict of interest None.

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Chapter 9

Antibiotic Resistance of *Helicobacter pylori* in Mazandaran, North of Iran

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Antibiotic Resistance of *Helicobacter pylori* in Mazandaran, North of Iran

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Keywords
Helicobacter pylori, antibiotic resistance, metronidazole, clarithromycin, Iran.

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Abstract

Background: The aim of this study was to investigate the prevalence of resistances in *Helicobacter pylori* against commonly used antibiotics including metronidazole, clarithromycin, amoxicillin, and tetracycline in Iranian patients.

Methods: *H. pylori* isolates were collected from gastric biopsies from patients referred for upper gastrointestinal endoscopy at Tooba Medical Center, Sari, Iran, from 2007 to 2010. None of them had been using antibiotics for at least 8 months. *H. pylori* was identified based on morphological shape and positive biochemical tests for catalase, oxidase, and urease activity. Antibiotic resistance for metronidazole, clarithromycin, amoxicillin, and tetracycline was investigated by using epsilometer test. Resistance was defined by minimal inhibitory concentration (MIC) > 0.5 mg/L for amoxicillin (AMX), >4 mg/L for tetracycline (TET), >8 mg/L for metronidazole (MTZ), and >1 mg/L for clarithromycin (CLR).

Results: Strains were collected from 132 patients, mean age 45.8 years, 52 (39%) were women. Patients had diverse diagnoses: gastritis 42 (31.8%), duodenal ulcer 45 (34%), gastric cancer 15 (11.3%), or gastric ulcer 30 (22.7%). The prevalences of resistance of *H. pylori* strains isolated from the patients were 73.4% for metronidazole, 30% for clarithromycin, 6.8% for amoxicillin, and 9% for tetracycline. Twenty-eight (21.2%) were double resistant to MTZ-CLR, 16 (12.1%) showed triple resistance to MTZ-CLR-AMX, and 8 (6%) were resistant to all four tested antibiotics (MTZ-CLR-AMX-TET). No associations were detected between multiple resistant strains and clinical manifestations ($p > .05$).

Conclusions: The prevalence of *H. pylori* antibiotic resistance to metronidazole and clarithromycin was high in Iran consistent with the reported low success rates for *H. pylori* treatment in this country.

Helicobacter pylori is a Gram-negative, microaerophilic bacterium recognized as etiologically involved in gastritis, peptic ulcer, duodenal ulcer, gastric cancer, and MALT lymphoma [1–3]. Successful eradication can cure some cases of dyspepsia and most cases of peptic ulcer [4–6]. Treatment failure has been increasingly experienced as resistant strains have emerged [7–10]. Easy access and also frequent, unprescribed use of antibiotics in developing countries such as Iran have complicated treatment in these countries. The prevalence of *H. pylori* in the North of Iran has been reported to be >85% [11]. Northern Iran is also a region of high risk for gastric cancer and other gastroduodenal disorders [12]. The aim of this study was to determine the antibiogram pattern of *H. pylori* isolates to commonly used antibiotics agents in Sari, Iran during 2007–2010.

Materials and Methods

Patients

Strains were collected from *H. pylori*-infected patients undergoing upper gastrointestinal endoscopy in Tooba

Medical Center, Sari, Iran. Informed consents were taken from all patients involved. The study protocol was approved by the ethics and research committee of Mazandaran Medical University. None of them reported any use of antibiotics for at least 8 months.

Biopsy Specimen and *H. pylori* Culture

One fresh antral biopsy was taken for culture and a second for rapid urea testing (Bahar Azma, Iran). Culture biopsy specimens were placed in thioglycolate broth as transport medium and shipped to diagnostic laboratory at cold temperature within 3 hours. After homogenization, the biopsies were smeared on Colombia agar plates containing 7% defibrinated sheep blood (Jihad Daneshgahi, Tehran, Iran), 7% Fetal Calf Serum (FCS) (Gibco, CA, USA), and antibiotics (amphotericin B, polymyxin and vancomycin) (MAST, Mersey Side, UK). The plates were incubated for 7–9 days under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) in an incubator (Binder, Tuttlingen, Germany) at 37 °C with 95% humidity [13]. We picked a single colony from each patient's culture plate and subcultured it for 3 days. *H. pylori* was identified based on morphological shape with Gram staining and positive biochemical tests including catalase, oxidase, and urease activity.

Susceptibility Tests

Resistance to four antimicrobial agents (metronidazole, clarithromycin, tetracycline, and amoxicillin) was investigated by epsilometer test (E-test) (AB, Biodisk, Sweden). Inocula of *H. pylori* were subcultured and adjusted to a defined density, corresponding to 2 McFarland turbidity standard units, then plated onto Colombia agar (Merck, Darmstadt, Germany) supplemented with 5% sheep blood. We placed strips on the plates after 10- minute drying. The plates were incubated for 6 days at 37 °C under microaerophilic conditions. Resistance was defined by minimal inhibitory concentration (MIC) >0.5 mg/L for amoxicillin, >4 mg/L for tetracycline, >8 mg/L for metronidazole, >1 mg/L for clarithromycin [14]. We used the prior NCCLS standards for reading the MIC breakpoints for tetracycline [15]. With some strains, agar dilution test [15,16] was performed to confirm MIC determined by E-Test.

Statistical Analysis

Data were analyzed using the SPSS (version 16.0) software. Chi-square test was used for analysis, and p values <.05 were considered statistically significant.

Results

Strains were collected from 132 patients, mean age 45.8 years, 80 (61%) were men, and 52 (39%) were women. Patients had diagnoses of gastritis (n = 42), duodenal ulcer (n = 45), gastric cancer (n = 15), or gastric ulcer (n = 30). No significant differences were observed between age and *H. pylori* resistance status ($p < .05$). The prevalences of resistance were 73.4% to metronidazole, 30% to clarithromycin, 6.8% to amoxicillin, and 9% to tetracycline (Fig. 1). The distribution of MIC values for the four tested antibiotics in clinical isolates is shown in Fig. 2–5. Data regarding distribution of *H. pylori*-resistant strains among different age groups and also gender status are shown in Table 1. We did not detect any significant association between resistance rate and demographic data such as age and gender ($p > .05$) (Table 1).

Because of the high rates of resistance found using E-test, we confirmed resistance by agar dilution. In the case of metronidazole and clarithromycin, we selected 10 resistant isolates and then checked for antibiogram pattern; all amoxicillin- and tetracycline-resistant strains were checked by agar dilution. The results were identical with the exception of two strains for

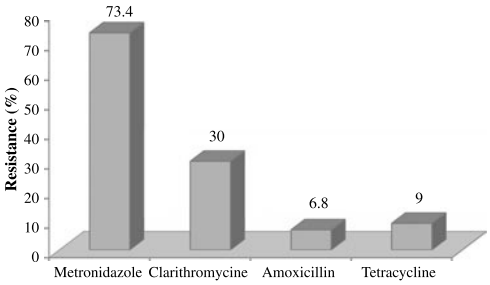

Figure 1 Prevalence of antibiotic resistance among four investigated antibiotics in this study.

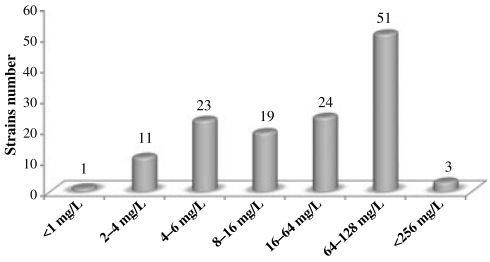

Figure 2 MIC distribution of metronidazole in *Helicobacter pylori* strains.

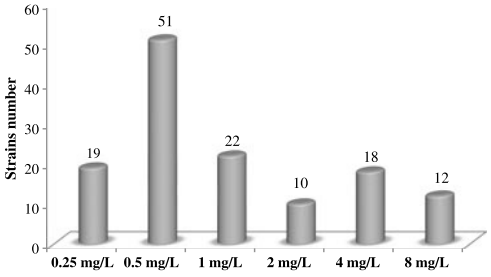

Figure 3 MIC distribution of clarithromycin in *Helicobacter pylori* strains.

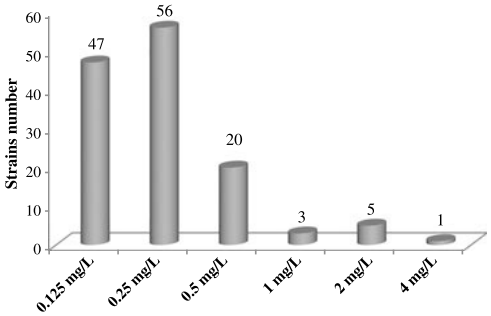

Figure 4 MIC distribution of Amoxicillin in *Helicobacter pylori* strains.

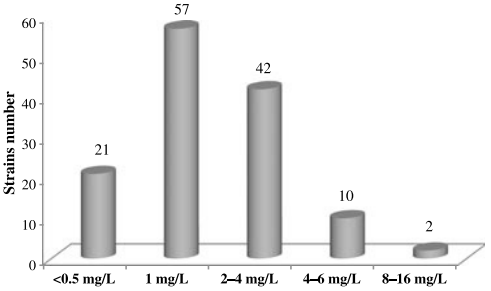

Figure 5 MIC distribution of Tetracycline in *Helicobacter pylori* strains.

amoxicillin, which changed the results to 7 of 132 (5.3%) resistant to amoxicillin.

No associations were detected between multiple-resistant strains and disease groups. We had 97 (73.4%) strains resistant to metronidazole and 40 (30%) were resistant to clarithromycin. We found that just 28 (21.2%) were resistant to both clarithromycin and metronidazole, while 16 (12.1%) isolates were resistant to three antibiotics (metronidazole-clarithromycin-amoxicillin) and 8 (6%) were resistant even to all four antibiotics investigated in this study. We did not observe any correlation between outcomes of infection and resistance of *H. pylori* isolates ($p < .05$).

Table 1 Distribution of *Helicobacter pylori*-resistant strains among different age groups

Age group	Number of patients	Resistance status to: Metronidazole r = 97 s = 35	Clarithromycin r = 40 s = 92	Amoxicillin r = 9 s = 123	Tetracycline r = 12 s = 120	*p* value
17–30	37	n = 27 *f* = 15 *m* = 12	n = 7 *f* = 0 *m* = 7	n = 2 *f* = 1 *m* = 1	n = 1 *f* = 0 *m* = 1	.2
31–44	42	n = 35 *f* = 7 *m* = 28	n = 5 *f* = 2 *m* = 5	n = 1 *f* = 0 *m* = 1	n = 2 *f* = 2 *m* = 0	.34
45–58	28	n = 18 *f* = 7 *m* = 11	n = 7 *f* = 2 *m* = 5	n = 1 *f* = 0 *m* = 1	n = 0 *f* = 0 *m* = 0	.12
59–72	19	n = 14 *f* = 11 *m* = 3	n = 10 *f* = 7 *m* = 3	n = 3 *f* = 1 *m* = 2	n = 4 *f* = 3 *m* = 1	.5
73–79	6	n = 3 *f* = 3 *m* = 3	n = 11 *f* = 5 *m* = 6	n = 2 *f* = 2 *m* = 0	n = 5 *f* = 3 *m* = 2	.2

r, resistant; *s*, sensitive; n, number of resistant strains in each age group; *f*, female patients; *m*, male patients.

Discussion

Indeed great progress has been achieved in gastroenterology after discovery of *H. pylori's* role in development of gastroduodenal diseases [17]. The patterns of resistance for *H. pylori* around the world have been shown to vary greatly [18–23]. We did not find any significant association between resistance and clinical outcomes of *H. pylori* infection in our study ($p > .05$). Resistance has proven to be the primary cause of treatment failure during antibiotic therapy in *H. pylori* infections [24]. We found that the prevalence of resistance to metronidazole in the North of Iran was extremely high (73.4%) compared with rates reported from the USA and European countries [25–31] and also higher than reported previously in different areas in Iran [21,32,33]. This high resistance rate might be attributed to frequent unauthorized use of antibiotics without prescription by physicians. Prevalence of resistance to metronidazole in the USA and European countries has varied between 20 and 40% [14,17,34]. Our results are similar to rates from developing countries including Saudi Arabia (78.5%), India (90%), and United Arabian Emirates (62.5%) [35–37].

Clarithromycin, Amoxicillin, and Tetracycline Resistance

In our study, the resistance rate for clarithromycin, amoxicillin, and tetracycline was 30, 6.8, and 9%, respectively. In agreement with data from developing countries, our determined resistance rate for clarithromycin fell in the reported range of between 25 and 50% [38]. The resistance rate for clarithromycin that we found was remarkably higher than in earlier studies in Iran [39,40]. It is unclear whether this reflects a geographic difference in antimicrobial use or continued widespread use of macrolides in Iran [21,39]. Overall, according to our results, neither clarithromycin nor metronidazole would be expected to provide good treatment success in Iran, and this expectation is also consistent with the clinical observations [33,41,42].

Tetracycline resistance is rare in most countries [24,25] but has been reported from China [43] and Italy [44]. In this study, we used from NCCLS standards for considering the MIC breakpoints for tetracycline as 4 mg/L [15] and found tetracycline resistance to be nearly 10%. Studies from different European countries and from the USA have shown that resistance to amoxicillin is also rare [23,25,29,30]. We found 6.8% of our strains to be resistant to amoxicillin by E-test or 5.3% by agar dilution. Prior studies in Iran by Khashei et al. [40] reported 2.5% resistance to amoxicillin, and Mohammadi et al. [21] reported 1.6%.

Conclusions

Routine monitoring of antibiotic resistance status is necessary for physicians to make up-to-date therapeutic decisions. Our results suggest that neither metronidazole nor clarithromycin should be used for anti-*H. pylori* in our region of Iran.

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Chapter 10

General Discussion

H. pylori; current status

H. pylori is a Gram-negative microaerophilic organism which, unless treated effectively, can cause a life-long colonization of the human stomach. The initial infection usually occurs during childhood as the stomach is less acidic in young children (1). Currently, more than 50% of the world population is colonized with *H. pylori* (2, 3), and this colonization significantly increases the peptic ulcer and gastric cancer risk of the infected individuals (4, 5). About 10-20 % of the colonized individuals will develop the more severe *H. pylori* infection associated disease such as peptic ulcers or gastric cancer (6-8). Not only is the prevalence of *H. pylori* in various regions of the world different, also the prevalence of *H. pylori* induced gastroduodenal disorders differs widely between different geographical regions (2, 7). Strikingly, there is no straightforward universal association between the prevalence of *H. pylori* and the *H. pylori*-induced gastric cancer prevalence. That is in some regions such as a Japan there is a relatively low rate of *H. pylori* infections but a high prevalence of *H. pylori* induced gastric cancer cases while in other regions like Africa there is a high infection rate but only a low rate of gastric cancer. In Mid-Eastern regions, such as the north of Iran, the prevalence of both gastric cancer and *H. pylori* infection are relatively high (9, 10). One potential explanation for these differences between infection rates and disease outcomes could be in that there are differences in the virulence of the infecting *H. pylori* strains. *H. pylori* is a high heterogeneous species with ~1600 genes, but these are not all functionally characterized (11, 12). Of these genes ~5% is unique to *H. pylori* and ~15% of the genes are highly variable or sometimes lacking completely between the different isolates (11, 13). Many of these unique and variable genes are clustered in so called plasticity regions (11). Plasticity regions represent small area's within the genome that are distinct from the rest of the genome in four aspects; i) they have a different GC content than the rest of the genome, ii) their gene content varies considerably between different isolates, iii) they do not seem to contain any essential (house keeping) genes, but instead carry genes that are potentially involved in the bacterial communication with the outside world such as type IV secretion factors. Thus in many aspects these plasticity regions are very similar to the 'pathogenicity islands' of other bacterial pathogens. It is

thus tempting to speculate that these plasticity regions contain *H. pylori* specific virulence factors that are present in some, but lacking in other strains thereby explaining the diverse clinical outcomes of an *H. pylori* infection. If the absence/presence of specific genes in these plasticity regions shows an association with the outcome of an *H. pylori* infection, these genes might represent good biomarkers to predict the chance for a certain clinical outcome of an *H. pylori* infection. Accepting that not all *H. pylori* strains are equal in their disease potential raises the question if the treatment procedure should be the same for all *H. pylori* infections. Some strains might even not require treatment at all, but it is currently not regarded acceptable to deny treatment to a patient that is infected with a potentially cancer inducing bacterium. Being able to reliably predict the chance of development of serious clinical disease would allow us to differentiate between the harmful and more innocent *H. pylori* and thus perhaps provide a rationale for a negative treatment advice in those patients infected with a low risk *H. pylori* strain. Due to the high prevalence of *H. pylori* globally, the wide spectrum of gastroduodenal diseases attributed to this bacterium, and the unknown additional factors with influence on outcome it will probably not be easy to define simple generally applicable criteria for defining such high/low risk groups that should (or should not) receive treatment. In order to do so one would require answers for unsolved issues such as how strong is the association between presence/absence of virulence factors and the outcome of *H. pylori* colonization, and what is the role of host genetics and environmental factors (e.g. gut flora composition) on this outcome. In this thesis, we discuss some of these issues and try to provide an initial approach to the identification of bacterial biomarkers.

Goals of this thesis

The identification of the virulence mechanisms and the search for effective ways to eliminate an ongoing infection have been the two major research topics for those working in the field of *H. pylori* (14). In fact there is only scarce data on this, that is mostly derived from studies showing an association between the presence/absence of these genes and specific *H. pylori* induced disease symptoms. In this thesis we aim to assess the value of the presence/absence of *H. pylori* virulence factors on the prediction of the clinical manifestations of an infection. The main reason for investigating the potential use of these virulence factors as markers is not only out of academic curiosity, but also to aid clinicians to reliably predict the clinical outcome of the infection. Given that i) there are many infected patients, ii) there is only a very limited set of 4-5 antibiotics to effectively treat *H. pylori* patients, iii) that these few antibiotics are only truly effective if used in combination, and iv) resistance to these drugs is rapidly increasing, have triggered initiatives on selective treatment.

H. pylori is a typical example of a panmictic bacterial species, and it has often been claimed that every infected patient carries his/her own strain (13). One of the reasons for this diversity is that *H. pylori* is naturally competent and can take up environmental DNA, especially from other *H. pylori* isolates. Sequence analysis of various *H. pylori* isolates has revealed that DNA exchange is an effective mechanism to create novel variants representing potentially more virulent/resistant bacteria (15). In addition it is not uncommon to find different isolates of *H. pylori* within a single host (16), thus creating ideal conditions for the generation of new 'mixed' strains. Given the high prevalence of *H. pylori*, its high genetic variability, and the high rate at which this bacterium develops resistance against these drugs, one can easily predict that in a few years there will not be any effective therapy left (17). Unlike many other bacteria where resistance is based on the acquirement of a new resistance encoding gene, with *H. pylori* antimicrobial resistance is always based on single point mutations in house keeping genes (18). This *de novo* induction of resistance can rapidly occur in *H. pylori*. As there is only a limited choice of effective antimicrobials (17, 19), we are now rapidly running out of options for effective treatment. Knowing that

the majority of *H. pylori* infected individuals will only suffer from a symptomless chronic gastritis as the result of this infection (5, 7, 14), one could reduce the number of patients to treat by offering treatment only to those patients that are at risk for a more severe disease outcome. This would allow for restricted antimicrobial use that will consequently prevent further increase in antimicrobial resistance. Currently, there is no reliable algorithm available to predict who will develop more severe outcomes and thus require treatment and who can be left untreated. Except for the well-established *H. pylori* virulence factors *cagA* and *vacA*, there are no other well characterized virulence factors that show good associations with disease outcome. In the first part of this thesis, we selected some of less characterized virulence factors such as *babA*, *dupA*, *homB*, *iceA*, *tnpA*, and *tnpB* and assessed their association with the disease outcomes in an Iranian population. In the second part of this thesis, the presence of antimicrobial resistance among the *H. pylori* strains isolated from ethnic individuals in North of Iran is analyzed

Section 1: Virulence factors

Clinical relevance of *H. pylori*

H. pylori is the only known bacterial pathogen able to persistently colonize the human gastric mucus. The initial colonization by *H. pylori* usually is without symptoms but over time it will develop in an acute gastric inflammation. If left untreated most *H. pylori* strains will persist lifelong in the gastric mucosa. Although many infected patients will not develop clinical symptoms they will suffer from chronic gastritis and consequently develop atrophy of their gastric epithelium. Over time 10-15% of all *H. pylori* patients will develop severe gastroduodenal diseases (Figure 1). Initially most clinicians believed that the final outcome of a *H. pylori* infection could be explained solely by the differences in virulence factors of the infecting strains. Given the sheer number of studies which have been performed for finding these disease outcome determining virulence factors of *H. pylori*, and the fact that there is still no such disease determining virulence factor identified illustrates that this concept is to simple (20). Currently, it is believed that in addition to bacterial factors also environmental factors and host factors play an important role and it is the combined interplay of these factors that determines the clinical outcome in the *H. pylori* infected patient (21). In conclusion the prediction of the final outcome of an *H. pylori* infection based on only virulence factors may not be entirely possible, but it will provide a good starting point for the design of such an algorithm.

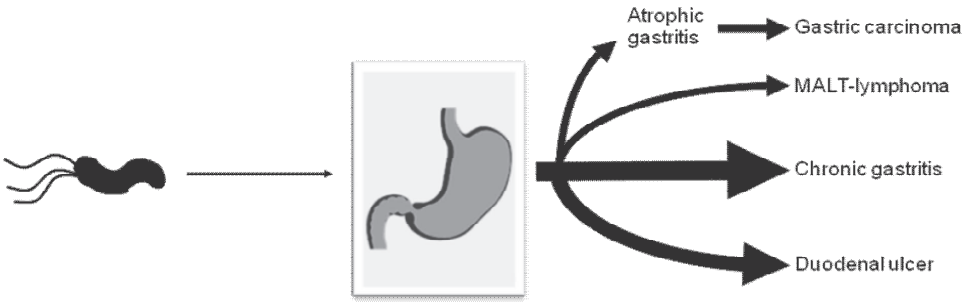

Figure 1. Distribution of different clinical outcomes after primary infection by *H. pylori*

Can virulence factors serve as an actual biomarker for disease outcome?

Finding the *H. pylori* virulence factors responsible for the clinical outcome of *H. pylori* has been the aim of several studies (22, 23). Close associations have been found between the presence of certain virulence factors and the occurrence of clinical gastrointestinal disorders (20, 24). To date, most studies have focused on classic, well established virulence factors such *cagA* and *vacA* which showed an association with the more severe diseases outcomes. The presence of the *cagA* gene can easily be detected by molecular methods (25, 26). As we showed in **chapter 2**, the low frequency of the *cagA* positive genotype among the *H. pylori* strains isolated from the patients with gastric cancer and MALT lymphoma in our study implies that there is not always a positive association between *cagA* and gastric cancer/MALT lymphoma. This shows that even for a well established virulence factor like *cagA* there is not always an unambiguous link between its presence and disease outcome. But there are more issues when trying to design a simple algorithm, as is exemplified by the other well established *H. pylori* virulence factor *vacA*. The *vacA* gene is present in all strains, hence its association with gastric pathology is not determined by its presence/absence but by specific allelic variants in both the signal and middle region of the *vacA* gene, with the *vacA* s_1m_1 allele being the most virulent allelic type (27, 28). Reliable detection of these allelic variants may not always be possible by a simple PCR reaction and the routine use of these *vacA* alleles for the pathotyping of *H. pylori* is currently not a viable option (29). It becomes even more complicated with both *vacA* and *cagA:* there is much variation in pathogenesis associated motifs and the simple absence/presence of these genes may not suffice as a biomarker for the prediction of disease outcome. In **chapter 3**, we looked for an association of *dupA* with different *H. pylori* induced digestive disorders with patients from Northern Iran. To our knowledge, our cohort included the largest gastric cancer patient collection ever tested for putative associations with *dupA*. This not only allowed us to confirm the association of *dupA* with duodenal ulcer induction, but we also observed a negative association between the presence of *dupA* and gastric cancer. In this regard, *dupA* is an interesting

example. When testing for an association between the presences of *dupA* and clinical manifestations we selected a PCR primer set that had been used by others. In addition we used more than one primer set in an attempt to avoid putative bias due to false negative as a result of lack of primer binding resulting from the high genetic variability of *H. pylori*. We initially believed (**chapter 3**) that the presence/absence of the *dupA* gene was indeed positively associated with duodenal ulcer formation, and negatively associated with gastric cancer formation, as had been described by others. Later (**chapter 7**) we reevaluated the primers that we used and realized that using primers for a variable region of the genome can easily lead to unreliable results. Indeed the percentage *dupA* positives ranged from 29%-64% depending on the primer set used. This shows that one has to be particularly careful when analyzing a genetically diverse organism as *H. pylori* as in this case almost eight years after describing the *dupA* as new virulence factor a lot of data has been generated that suggest towards *dupA* being a useful biomarker. However, this data is in part dependent on the PCR-design; i.e. the primers used may not bind to all the genetic variants of the genes tested, and thus there is a serious concern of false-negative PCR results. This makes that for some studies the conclusions have to be critically evaluated. Finding a much higher prevalence of *dupA* with our newly designed primers indicate that it was not the presence of the *dupA* gene that was tested by these PCR reactions, but more likely the presence of selected *dupA* allelic variants. Thus our conclusion that there is an association between the presence of *dupA* and duodenal ulcer formation should be rephrased into: "An association between duodenal ulcer formation and the specific *dupA* allele detected by the PCR used". The recent introduction of affordable whole genome sequencing techniques is a promising new technique to replace PCR based analysis that will avoid such artifacts in the near future. In search of alternative markers clinicians and researchers used the information from the many available genome sequences. These revealed the presence of some highly variable regions carrying genes which can potentially act as novel virulence factors (13). In **chapter 6**, we focused on two novel virulence factors that might serve as biomarkers: i.e. *tnpA* and *tnpB*. These genes are likely to encode for the transposases that can induce deletions in the *cag* pathogenicity island, and thereby might affect the virulence of this gastric pathogen

(30). Our findings indicate that the *tnpA* gene might serve as a biomarker to detect strains associated with higher risk to develop gastric cancer. Unfortunately the observed associations were not very strong, thus one can easily dispute the clinical relevance of our findings and further prospective studies would be needed to provide more definitive evidence. In **chapter 5**, we tested for an association between $babA_2$ and clinical manifestations. We observed that the prevalence of $babA_2$ in *H. pylori* strains is close to 40% which is in contrast to other studies where it sometimes was up to 90% (31). We observed that there is a correlation between $babA_2$ and gastric cancer. While $babA_2$ may serve as a potential biomarker that is associated with gastric cancer further experiments with larger numbers of samples in different geographical areas are needed to show the general validity of this. The same is true for the work described in **chapter 4**, where we selected a population from the North of Iran to investigate the proposed role of *homB* as a biomarker for detecting the presence or prognosis of gastric cancer in infected patients. Also here we performed a cross-sectional study which shows a distribution of this gene in the infected population at a single time-point, but to substantiate our claims we would need to perform a prospective study using asymptomatic patients only and study the distribution of these genes after a long time interval and estimate the level of association between presence/absence of a particular gene and the development of gastrointestinal symptoms in these patients. The same goes for many of the other factors that have been proposed as putative biomarkers. Hopefully in the near future large prospective studies will be performed determining the strength of the associations between these virulence-associated *H. pylori* genes and increased risk of specific disease outcomes. However, to define a good algorithm for the prediction of disease outcome, one cannot depend on bacterial factors alone. As mentioned above the disease risk not only dependents on *H. pylori* strain-specific components, but also on environmental factors and host factors. While some of these factors can still be objectively established retrospectively, others (like food intake during a patients childhood) requires going back in time by >50 years and is thus very much dependent on the perception of the patient. As a result most research went into finding links between stable genetic determinants of host factors such as immune polymorphisms. Here there is clear evidence

that polymorphisms in TNF-alpha, IL-8, IL-1 and IL-10 in *H. pylori* infected patients are associated with an increased risk of gastric cancer and other gastrointestinal disorders (32-35). Based on current data, it has been proposed that *H. pylori* are able to rapidly adapt to their hosts, thus effectively each individual is infected by their own specific strain (36). In other words, a successful bacterial infection depends on a well arranged communication between the bacterium and its host. The exact details of this process are not yet fully understood, questions that remain are e.g. what is the source of the newly introduced genes, what exactly is driving the selection of new variants, and is there a stable situation reached after a certain period of time or is the changes a continuous process. In spite of the many efforts made by us and others, there currently is no reliable algorithm to predict the specific *H. pylori* associated disease. Suppose that finally good and well validated biomarkers can be identified to predict the gastroduodenal disease caused by the *H. pylori* infection, how will these be used in a clinical setting, how strict can one be in the resulting advice on whether or not to eradicate the infecting strain, and are these decisions considered to be ethically correct? These issues will be complicated further by the fact that often a patient was exposed to variable environmental conditions and/or is infected by more than one strain. So then the question is if there is a dominant environment, strain/genotype with regard to disease development. Even if we can solve the issue of what is this dominance, there remains the issue of how often we have to rescreen our infected patients in order to allow for a reliable prediction as it is known that e.g. strains rapidly evolve and can change their genotype during the infection.

Section 2: Treatment

In vitro, *H. pylori* is susceptible to the most antimicrobials, but successful eradication of *H. pylori* in the patient can only be achieved by a handful of them with clarithromycin, amoxicillin, tetracycline, and metronidazole being used most frequently in routine practice (17, 19). This gap between *in vitro* and *in vivo* findings is further complicated by the finding that successful eradication can only be achieved if two or more of these few effective antimicrobials are combined. There are many papers on how to use the current antibiotic regimens in order to achieve optimal treatment (37-39), and how to limit the induction of antimicrobial resistance. There is a growing concern on how to effectively treat patients with primary resistance because the prevalence of antimicrobial resistance is still increasing. **Chapters 8 and 9** show that resistance in Iran is currently 78% and 34%, against the two most commonly used antibiotics (metronidazole and clarithromycin, respectively). Against the newly introduced antimicrobials moxifloxacine and levofloxacin, resistance in Iran was already 5% and 10%, respectively (**chapter 8**). It is expected that also these resistance levels will rapidly increase over time. Luckily in Iran there is a surveillance system for nationwide resistance rates in *H. pylori;* in many western countries there is no such surveillance for the resistance profiles of the locally circulating *H. pylori* isolates. In many countries there is even a trend to no longer perform susceptibility tests prior to starting *Helicobacter* treatment, and given the high levels of antimicrobial resistance this can easily result in the dual and triple therapy effectively only being monotherapy. This is a scenario for therapy failure and induction of resistance, thus completing the circle. This is especially alarming as it is not expected that any new effective antimicrobials will be available soon.

Having *H. pylori*: Good or bad?

To date the available eradication regimens look far from ideal. The fact that most *H. pylori* infected individuals lack any clinical symptoms supports the concept that we do not need to eradicate all *H. pylori*.

Thirty years ago most clinicians regarded *H. pylori* as part of the normal gram negative bacterial flora of the stomach which could be found in histopathological smear of the gastric epithelial cells nearly all patients. This view has drastically changed as with the discovery of *H. pylori* strong evidence became available for a direct association between an increased risk of gastric cancer development in *H. pylori* infected individuals. The situation is perhaps more complicated if we realize that next to the benign associations with duodenal ulcers and gastric cancer there are also indications of beneficial effects of *H. pylori* colonization. E.g. it was reported that there is a negative association between the presence of *H. pylori* colonization and Gastro-Esophageal Reflux Disease (GERD), Barrett's, and esophageal cancer (7, 40). Perhaps, in the case of *H. pylori*, we need to replace the word "pathogen" for specific strains with a new phrase which would be "symbiont". This poses the problem of choosing to eradicate the *H. pylori* infection in stomach and decrease the chance of Duodenal ulcer (DU) and Gastric cancer (GC) induction, or just leave it in the stomach and reduce the chance of BE and esophageal adenocarcinoma. Currently we are unable to distinguish which *H. pylori* strains should be considered as beneficial symbionts or true pathogens and this makes it difficult to decide how to deal with *H. pylori* infections. Given that the final outcome of *H. pylori* infection is the combined result of host, environmental, and bacterial factors, it is remarkable that there is often such a strong correlation between bacterial virulence factors and disease outcome. It implies that some virulence factors must have a very strong effect on the disease outcome. But algorithms that attribute the *H. pylori* induced disease symptoms only to specific virulence determinants are doomed to fail if they do not at some point also include host and environmental factors.

Conclusion

Three decades of intense research into *H. pylori* virulence factors have revealed many aspects of the relationships between this bacterium, the gastric mucosal surface, and the induction of disease. Strain-to-strain genetic variability in bacterial virulence factors not only affects the ability of the organism to colonize and cause disease but also affects inflammation and gastric acid output. In the continuous

interactions with the host and exposure to antimicrobials, the bacteria are able to adapt by mutations and DNA rearrangements, rendering novel genotypes that are more resistant to the antimicrobials used to eradicate these bacteria. It is unlikely that many novel antimicrobials will be available in the near future, thus treatment including two antimicrobials (triple therapy) will have to be replaced with therapy that employ three or even four antimicrobials in order to stay effective. This will raise the issue on how to reduce the long duration of the treatment as including more antimicrobials will enhance side effects and thus result in poor patient compliance. Vaccines could be an alternative to antimicrobials, but until now this has not been a very promising approach and it is unlikely that there will be an effective vaccine any time soon. Hence we need a completely alternative strategy and the detection of both bacterial virulence factors and host factors that contribute to the pathology of this infection may allow the design an effective algorithm to predict if a patient is infected with either a 'good' *Helicobacter* or a pathogenic one. But given that some of these host and bacterial virulence factors have been identified, and do show significant associations with disease induction suggests that such an algorithm can be designed. But still much effort needs to go into these algorithms and they may need tweaking for each region and ethic population in order to be effective.

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Summary

Helicobacter pylori (*H. pylori)* is a bacterium that can colonize the epithelial mucosa of the human stomach. With approximately 50% of the world's population infected by *H. pylori*, this makes it the most common cause of chronic gastritis, gastroduodenal ulcer disease, and also gastric adenocarcinoma. It is not clear why some people with *H. pylori* get disease symptoms while most infected individuals never show any symptoms in spite of suffering from serious chronic gastritis. Currently, the main topics for studies on *H. pylori* are antibiotic resistance and the presence of virulence factors and their association with specific clinical outcomes. To date, different virulence factors such as *babA*, *iceA* and *vacA* have been suggested; however, there is no clue how these factors can affect the disease outcomes associated with *H. pylori* infection. Another major topic with *H. pylori* is that over the last years, the efficacy of therapeutic regimens seems to be decreasing due to the development of antibiotic resistance. This is an alarming issue with regard to treatment efficacy.

Virulence Factors

Among the *H. pylori* virulence factors, *cagA* is the most important determinant and it has been shown to be undeniably associated with serious *H. pylori* induced disorders. The *cagA* gene is located at one end of the *cag* pathogenicity island (PAI), which is an approximately 40 kbp region that is incorporated into the *H. pylori* genome by horizontal transfer from an unknown source. In **chapter 2** the frequency of *cagA* gene in *H. pylori* strains isolated from symptomatic patients were investigated. Of 128 strains, 84 (65.6%) were *cagA* positive. Much to our surprise no significant association was observed between specific disease types and *cagA* positive isolates ($P>0.05$). A lack of correlation between the cagA and more severe diseases has been reported before and was attributed to geographical differences in the studied regions. The duodenal ulcer-promoting gene (*dupA*) was recently reported as a novel *H. pylori* virulence factor associated with an increased rate of occurrence of duodenal ulcer (DU) and a decreased risk for gastric cancer (GC). As described in **chapter 3,** *dupA* could serve as independent predictor for the clinical outcome of *H. pylori* infection, as was suggested previously. While our association study provides only circumstantial evidence for an obvious role of *dupA* as a disease causing factor it cannot prove that it is a true virulence factor. This would e.g. require additional infection experiments with isogenic mutants. In an attempt to explain this we tested the growth of DupA positive and negative strains at various pH conditions. We observed that DupA positive strains are more resistant to low pH, making it likely that DupA positive strains reside in the lower parts of the stomach (antrum vs corpus). This difference in the distribution of the gastric colonization would explain with the different patient populations.

The *Helicobacter* outer membrane (Hom) adhesion molecules constitute a paralogous family of proteins that contain a signal sequence at the C terminus and several alternating hydrophobic/hydrophilic motifs, which are typical markers of outer membrane proteins. In 2009, it has been suggested that *homB* can be a good biomarker to predict the presence of gastric adenocarcinoma in *H. pylori* infected individuals. The authors indicated that more studies with higher sample size in different geographical regions are required to confirm their findings. In the Northern parts of Iran there is a high risk of getting gastric cancer as a result of *H. pylori* infection. Our results in **chapter 4** showed that the *homB* gene can be a valuable biomarker for the prediction of gastric cancer in *H. pylori* infected patients ($P<0.001$). Moreover, our study was the first to confirm this association in an Asian country. But again, this is an association study only and to proof that *homB* is a true virulence factor one would need to further investigate the exact biological mechanisms.

Presumably, the type and location of the *H. pylori* colonization in the gastric mucosal epithelium plays a critical role with regards to the clinical symptoms of the *H. pylori* infection. Thus it is no surprise that the presence/absence of certain *H. pylori* adhesions is associated with the occurrence of various digestive disorders. In **chapter 5**, we confirmed the previously reported association between the presence of the adhesion factor encoding $babA_2$ and $iceA_1$ genes and disease outcome. Also in the tested North Iranian population both genes are more prevalent in patients suffering from gastric cancer than in the other patients groups. To date, only a few studies have investigated the possible association of $babA_2$ and clinical outcome. In most Asian countries studies the prevalence of $babA_2$is around 90%. Interestingly we found that in the Iranian population the prevalence of $babA_2$ in *H. pylori* strains is 40%. Due to this more even distribution between $babA_2$ positive and negative strains in our relatively small study population, we were able to draw statistically significant conclusions on the association between the presence of this adhesion factor encoding gene and disease outcome. However, we need more studies in different geographical regions to confirm current findings. Also here further experiments with higher number of samples in different geographic population are needed to substantiate the general validity of our findings.

In **chapter 6** we studied the recently proposed *H. pylori* virulence factors *jhp0652*, *tnpA* and *tnpB*. The results from our study confirm the significant association of the *cagA* gene and gastric cancer, and a weak, correlation was observed between the presence of the *cagA* gene and duodenal ulcer. For the *tnpA* gene an association with GC was observed, but no significant association was observed for *tnpB* and gastroduodenal diseases. The exact biological role these genes play in virulence is currently still unclear. Recently, it was suggested that *jhp0562* is responsible for encoding a glycosyltransferase involved in LPS biosynthesis. Thus it is tempting to speculate that the presence of the *jhp0562*gene affects the LPS composition and thereby the colonization properties of the bacterium. The *H. pylori tnpA* and *tnpB* genes

encode a transpose that can induce deletions in the *cag* region. As this cag region encodes several virulence factors that are known to affect disease outcome, the disruption of this region is likely to affect the virulence of this gastric pathogen.

As mentioned above in **chapter 3** we have used previously published PCR assays to confirm that the presence of the *dupA* gene is higher in duodenal ulcers patients and lower in gastric cancer patients compared to gastritis only patients. An *in silico* analysis of all available *dupA* genes sequences from the public DNA databases revealed significant mismatches of various available *dupA* primers. In **chapter 7**, we designed new primers against more conserved *dupA* sequences and showed that our newly designed primer set (AF-*dupA*) found more *H. pylori* isolates positive for the *dupA* gene than the old primers as designed by others (and used in chapter 3 by ourselves). While we did show that the *dupA* gene is more prevalent than was previously estimated based on the old PCR assays, we do not know if and how this would affect the associations with the various patient populations as we do not know the clinical symptoms for the patients that we isolated our strains from. Hence this is an aspect that definitely would warrant further testing.

Resistance

With *H. pylori* the increase in antibiotic resistance has become an alarming issue that seriously affects the efficacy of *H. pylori* treatment. While monitoring the *H. pylori* resistance does not deal with the consequences of this issue, it does aid the physicians in their rational choice of therapeutic formulations. In **chapters 8** and **9** we showed that the prevalence of antibiotic resistance is increasing. Unfortunately, resistance against moxifloxacine is also already 4.6% in the tested strains. Frequently used antibiotics such as clarithromycin in respiratory diseases and also metronidazole for gastrointestinal parasites can be an explanation for having such high rate of resistance among the *H. pylori* strains. However, as we found in **chapter 8**, moxifloxacine can be an alternative in current therapeutic regimens. Additionally, the next intervention might be considering the use of non-invasive tests such as screening the *H. pylori* DNA isolated from feacal samples for the resistance of the infecting *H. pylori* strains.

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Lastly, I would like to dedicate this work to my Grandfather Abbas Talebi Bezmin Abadi, who left us too soon, I hope that this work makes you proud of me. Love you!

Curriculum Vitae

Amin Talebi Bezmin Abadi was born on September 8, 1983 in Sari, Iran. He grew up in Sari and obtained his diploma at high school '' 29 Aban'' Sari, Iran in 10 May 2000. In 2001, he started his study at biology at Gorgan University, Iran. Afterward, he started his MSc. in Tarbiat Modares University, Tehran, Iran in 2007 under supervision of Dr. Ashraf Mohabbati Mobarez and Dr. Tarang Taghvaei. He graduated in 2010. In November 2010, he started his PhD training at department of Medical Microbiology of the University Medical Center Utrecht under supervision of Prof. dr. J. A. Wagenaar and Dr. J.G. Kusters.

List of publications

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2- **Amin Talebi Bezmin Abadi**, Johannes G Kusters. *Helicobacter pylori* Infection in Iran: Epidemiology, Treatment and Diagnosis. *Helicobacter pylori:* A Worldwide Perspective 2013. 2013, 216-233. (**Chapter 10**). Editor: Dr. Buzás György Miklós.