

World Journal of *Gastroenterology*

World J Gastroenterol 2023 January 7; 29(1): 1-222



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INDEXING/ABSTRACTING

The WJG is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports, Index Medicus, MEDLINE, PubMed, PubMed Central, Scopus, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database. The 2022 edition of Journal Citation Reports® cites the 2021 impact factor (IF) for WJG as 5.374; IF without journal self cites: 5.187; 5-year IF: 5.715; Journal Citation Indicator: 0.84; Ranking: 31 among 93 journals in gastroenterology and hepatology; and Quartile category: Q2. The WJG's CiteScore for 2021 is 8.1 and Scopus CiteScore rank 2021: Gastroenterology is 18/149.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: Ying-Yi Yuan; Production Department Director: Xiang Li; Editorial Office Director: Jia-Ru Fan.

NAME OF JOURNAL

World Journal of Gastroenterology

ISSN

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

LAUNCH DATE

October 1, 1995

FREQUENCY

Weekly

EDITORS-IN-CHIEF

Andrzej S Tarnawski

EDITORIAL BOARD MEMBERS

<http://www.wjgnet.com/1007-9327/editorialboard.htm>

PUBLICATION DATE

January 7, 2023

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INSTRUCTIONS TO AUTHORS

<https://www.wjgnet.com/bpg/gerinfo/204>

GUIDELINES FOR ETHICS DOCUMENTS

<https://www.wjgnet.com/bpg/GerInfo/287>

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<https://www.wjgnet.com/bpg/GerInfo/288>

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<https://www.wjgnet.com/bpg/gerinfo/208>

ARTICLE PROCESSING CHARGE

<https://www.wjgnet.com/bpg/gerinfo/242>

STEPS FOR SUBMITTING MANUSCRIPTS

<https://www.wjgnet.com/bpg/GerInfo/239>

ONLINE SUBMISSION

<https://www.f6publishing.com>



Observational Study

Impact of *Helicobacter pylori* virulence markers on clinical outcomes in adult populations

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Specialty type: Infectious diseases

Provenance and peer review:

Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Netto ERA, Brazil;
Selvin ST, United States; Zhang J, China; Zojaji M, Iran

Received: August 16, 2022

Peer-review started: August 16, 2022

First decision: October 20, 2022

Revised: November 2, 2022

Accepted: November 21, 2022

Article in press: November 21, 2022

Published online: January 7, 2023



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Abstract

BACKGROUND

In recent years, associations between specific virulence markers of *Helicobacter pylori* (*H. pylori*) and gastrointestinal disorders have been suggested.

AIM

To investigate the presence of virulence factors including vacuolating cytotoxin A genotypes (*s1m1*, *s1m2*, *s2m1*, and *s2m2*), cytotoxin-associated gene A (*CagA*), and urease activity in *H. pylori* strains isolated from Arab and Jewish populations in northern Israel and to assess associations between these factors and patients' demographics and clinical outcomes.

METHODS

Patients ($n = 108$) who underwent gastroscopy at the Baruch Padeh Medical Center, Poriya due to symptomatic gastroduodenal pathologies as part of *H. pylori* diagnosis were enrolled in the study. Gastric biopsy specimens were collected from the antrum of the stomach. Clinical condition was assessed by clinical pathology tests. Bacteria were isolated on modified BD *Helicobacter* Agar (BD Diagnostics, Sparks, MD, United States). Bacterial DNA was extracted, and PCR was performed to detect *CagA* and vacuolating cytotoxin A genes. Urease activity was assessed using a rapid urease test.

RESULTS

A significant correlation was found between disease severity and patient ethnicity ($P = 0.002$). A significant correlation was found between *CagA* presence and the *s1m1* genotype ($P = 0.02$), which is considered the most virulent genotype. Further, a higher level of urease activity was associated with isolates originating from the Jewish population. Moreover, higher urease activity levels were

measured among *CagA*-/*s1m1* and *CagA*-/*s2m2* isolates.

CONCLUSION

Our study highlights the importance of incorporating molecular methods for detection of virulence markers of *H. pylori* in order to tailor optimal treatments for each patient. Further investigation should be performed regarding associations between *H. pylori* virulence factors and ethnicity.

Key Words: *Helicobacter pylori*; Virulence factors; Vacuolating cytotoxin A; Cytotoxin-associated gene A; Urease activity

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Core Tip: In recent years, associations have been found between virulence markers of *Helicobacter pylori* and gastrointestinal disorders. In parallel, several physicians in northern Israel noted a higher treatment failure rate among Arab patients compared to Jewish patients. This work found a significant correlation between disease severity and patient ethnicity ($P = 0.002$). Further, a higher level of urease activity was associated with isolates originating from the Jewish population. Moreover, higher urease activity levels were measured among *CagA*-/*s1m1* and *CagA*-/*s2m2* isolates. These findings are expected to advance personalization of treatment to specific strains based on their virulence factors.

Citation: Roshrosh H, Rohana H, Azrad M, Leshem T, Masaphy S, Peretz A. Impact of *Helicobacter pylori* virulence markers on clinical outcomes in adult populations. *World J Gastroenterol* 2023; 29(1): 190-199

URL: <https://www.wjgnet.com/1007-9327/full/v29/i1/190.htm>

DOI: <https://dx.doi.org/10.3748/wjg.v29.i1.190>

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a microaerophilic, Gram-negative bacterium that colonizes the gastric mucosa and infects the stomach epithelium, causing peptic ulcer disease[1]. Unrelenting *H. pylori* infections cause chronic inflammation, which can result in gastritis, intestinal metaplasia, and even gastric cancer[1].

The genetic variability of *H. pylori* and its host, combined with environmental factors, have been suggested to affect the clinical outcome[1-3]. *H. pylori* pathogenesis is mediated *via* distinct virulence factors, including the secreted vacuolating cytotoxin A (*VacA*), cytotoxin-associated gene A (*CagA*) protein, and urease[1,2]. *VacA* is one of the main toxins secreted by the bacterium; once bound to host cells and internalized, it causes “vacuolation,” characterized by the accumulation of large vesicles that disrupt protein trafficking pathways. Two polymorphic regions in the *VacA* gene sequence are the signal sequence region (s-region) and the mid region (m-region), with allelic variations classified as *s1* or *s2* and *m1* or *m2*, respectively[3]. It was suggested that isolates with a *s1/m1* genotype cause more severe chronic inflammation compared to the other *VacA* genotypes[4].

H. pylori strains can be divided into two main subpopulations according to their ability to produce *CagA*, a 120-145 kDa protein associated with gastric carcinoma. The protein interferes with cellular signal transduction, provoking cellular dysfunction that can eventually lead to cell transformation and cancer[5].

Along with *VacA* and *CagA*, urease activity in *H. pylori* is an indicator of bacterial virulence. Urease is a metalloenzyme that requires nickel for its activity and consists of two components, *UreA* and *UreB*. The enzyme breaks down urea into ammonia and carbon dioxide, promoting bacterial survival in the acidic environment of the stomach. In addition, urease is involved in *H. pylori* colonization in the gastric tissue, prevention of phagocytosis, and induction of proinflammatory cytokines[6,7].

In addition to the virulence factors that may affect the efficacy of *H. pylori* eradication regimens, antibiotic resistance poses a major challenge to treatment success. Several physicians in northern Israel noted that the Arab population has a higher treatment failure rate compared to the Jewish population, a finding which triggered a study that examined antibiotic resistance of *H. pylori* among these two groups in Israel[8]. Indeed, isolates from the Arab population were more resistant to both clarithromycin and levofloxacin and exhibited simultaneous resistance to more antibiotics as compared to isolates from Jewish patients[8]. The current study further examined the characteristics of *H. pylori* in adult Arab and Jewish populations in northern Israel, with an emphasis on the three virulence markers of *H. pylori* and their correlation with clinical outcomes.

MATERIALS AND METHODS

Study population

The study group included 108 isolates of patients who underwent gastroscopy at the Baruch Padeh Medical Center, Poriya, due to symptomatic gastroduodenal pathologies as part of the *H. pylori* workup between November 2018 and December 2019. We included the first 108 biopsies from which *H. pylori* was successfully isolated at the microbiology laboratory of the medical center. The study was approved by the Helsinki Committee of the Baruch Padeh Medical Center, Poriya (Approval no. POR 0007-20). The Institutional Review Board committee waived the need for participant approval. Gastric biopsy specimens were collected from the antrum of the stomach. Clinical pathology tests were performed to assess the patients' clinical conditions. Demographic and clinical data were retrospectively collected from the patients' medical records.

Histology staining and pathology

Gastric biopsy specimens were stained using hematoxylin and eosin staining method in order to identify *H. pylori* and to evaluate the degree of inflammation. The bacterium has an actively dividing spiral shape that changes to coccoid morphology under stressful environments.

All histologic slides were reviewed by a single blinded gastrointestinal pathologist at the pathology laboratory of the Baruch Padeh Medical Center, Poriya and graded unremarkable (none), mild, moderate, or severe, based on the presence of acute (polymorphonuclear cells) or chronic (monocytes, lymphocytes, plasma cells) inflammation, lymphoid aggregates, and metaplasia.

Bacterial isolation and identification

H. pylori identification was carried out in accordance with the routine identification tests of the clinical microbiology laboratory including a Gram stain, oxidase, catalase, and urease tests.

Biopsy specimens were manually minced with a sterile scalpel, seeded on modified BD Helicobacter Agar plates (BD Diagnostics, Sparks, MD), and incubated for 7 d at 35 °C in a microaerobic atmosphere (5% O₂ and 10% CO₂) produced by a gas-generating system adapted for *Campylobacter* (CampyGen™; Gamidor Diagnostics, Petah Tikva, Israel).

Final identification of the bacteria was performed by matrix-assisted laser desorption ionization-time of flight mass spectrometry[9], using the Bruker Biotyper system (Bruker Daltonics, Bremen, Germany) with MALDI BIOTYPER 3.3 (Bruker Daltonics) software.

Molecular characterization of genes associated with bacterial virulence

DNA extraction: Tissue collected from gastroscopic biopsy was finely chopped with a sterile scalpel and then lysed by tissue lysis buffer supplemented with proteinase K enzyme (Bioneer, Daejeon, Korea). Total DNA was extracted using the AccuPrep Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea), according to the manufacturer's instructions.

Multiplex PCR assay: DNA was amplified with a multiplex PCR, designed to detect the *VacA* and *CagA* genes in a single run, using specific primers (Table 1). For this purpose, 10 µL Taq ReadyMix2 (Hy labs, Rehovot, Israel) was added to 0.2 µL forward primer, 0.2 µL reverse primer, 4 µL template DNA, and 5.6 µL nuclease-free water. Reaction conditions were 35 cycles of: Denaturation of the pre-amplified templates at 95 °C for 1 min, followed by an annealing step at 72 °C for 1 min, an extension step at 25 °C for 1 min and one additional extension step for 7 min. PCR products were visualized by 1% agarose gel electrophoresis.

Urease activity

Urease activity was quantified using the rapid urease test in a 96-well plate. For this purpose, bacteria were placed in a sterile Eppendorf tube containing sterile physiological solution until 0.5 McFarland turbidity was reached. Rapid Urease Test Brute solution (100 µL; Novamed, Jerusalem, Israel), containing 2% urea, pH 6.8, was then added to each well, along with 100 µL of the bacterial stock. Then, absorbance at 570 nm (optical density, O.D₅₇₀) was measured after 1 min, 5 min and 10 min using the Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA, United States)[10]. A change in the solution color from orange to pink was considered a positive result.

Statistical analysis

Continuous variables (age, urease activity) were presented as means and ranges with standard deviations and categorical variables (sex, disease severity, ethnicity, living area, genotypes) were presented as absolute numbers and percentages. *T* test or one-way analysis of variance was used to compare continuous variables of two or more groups and Fisher's exact test to assess the relationship between categorical variables. *P* value < 0.05 indicated statistical significance. Statistical analysis was performed using R Statistical Software (version 4.1; R Foundation for Statistical Computing, Vienna, Austria).

Table 1 List of genes and primer sequences used in the study

Primer	Gene	Primer sequence	Product size (bp)
CAGAF	<i>CagA</i>	5'-GATAACAGCAAGCTTTGAGG-3'	349
CAGAR		5'-CTGCAAAAGATTGTTGGCAGA-3'	
VA1-F	<i>VacA</i> signal region	5'-ATGGAATACAACAAACACAC-3'	259/286 (s1/s2)
VA1-R		5'-CTGCTTGAATGCGCCAAAC-3'	
VAG-F	<i>VacA</i> middle region	5'-CAATCTGTCCAATCAAGCGAG-3'	567/642 (m1/m2)
VAG-R		5'-GCGTCTAAATAATTCCTCAAGG-3'	

VacA: Vacuolating cytotoxin A; *CagA*: Cytotoxin-associated gene A.

RESULTS

Demographic characteristics

In total, 108 patients [average of 42.3 (18.0-88.0) years] were enrolled in this study, 24% of whom were males and 76% of whom were females (Table 2). Within the study group, 56 were Arabs and 52 were Jews; 61.1% lived in a city and 33.9% lived in a village. A larger percentage of the Arab *vs* Jewish cohort lived in villages (62.5% *vs* 13.5%, respectively).

VacA and *CagA* genotypes

To characterize the common *CagA* and *VacA* genotypes in the patient population, 108 isolates were randomly selected, of which 56 originated from the Arab cohort and 52 from the Jewish cohort (Table 3). *CagA* was identified in 24 (22.2%) samples and was distributed equally between the two ethnic groups. However, it had a higher frequency among isolates from patients living in villages (67.7%) compared to isolates from those living in cities (33.3%) ($P = 0.149$). The most prevalent *VacA* genotype was *s2m1*. No statistically significant associations were noted between the different genotypes and demographic characteristics.

A significant association was found between the presence of *CagA* and specific *VacA* genotypes ($P = 0.002$) (Figure 1); 33.3% of the *CagA*-positive strains had the *s1m1* genotype, which is considered the most virulent genotype[7]. Additionally, 61.9% of the *CagA*-negative strains had the *s2m1* genotype.

Urease activity

Urease activity was found to be faster in isolates from the Jewish population compared to isolates from the Arab population. This was observed at all tested time points (1 min, 5 min, and 10 min; $P < 0.05$ for all). The results of the first-minute measurements are presented in Figure 2. No significant associations were found between urease activity and patient's sex, place of residence and the presence of the *CagA* gene.

In addition, urease activity in *CagA* (-) *s2m2* strains (mean O.D 0.33) and in *CagA* (-) *s1m1* isolates (mean O.D 0.32) were significantly higher than urease activity in *CagA* (-) *s1m2* (mean O.D 0.28), ($P = 0.013$ and $P = 0.016$, respectively) (Figure 3).

Associations between virulence factors, patient ethnicity and disease severity

A significant association was found between disease severity and ethnicity ($P = 0.002$) (Table 4); in patients with mild disease, 58% were Jews while 42% were Arabs. In patients with moderate disease, 75.7% were Arabs compared to 24.3% Jews. Finally, the severe group included 66.7% Arabs compared to 33.3% Jews. No significant links were found between disease severity and urease activity, *CagA* gene occurrence, *VacA* alleles, and genotype combinations.

DISCUSSION

The main purpose of this study was to evaluate and compare the virulence markers of *H. pylori* among two adult populations in northern Israel and their correlation with clinical outcomes. It should be noted that, due to its special growth requirements and slow growth, cultivation of *H. pylori* is difficult. As a result, the diagnosis of *H. pylori* infection is usually performed, as opposed to diagnosis of other bacterial infections, on the basis of indirect tests that do not require bacterial isolation[11]. This is the reason why there is limited data on the distribution of virulence factors and their associations with clinical outcomes among *H. pylori* isolates in Israel.

Table 2 Demographic characteristics of the study participants, *n* (%)

Characteristic	Arabs, <i>n</i> = 56	Jews, <i>n</i> = 52	Total, <i>n</i> = 108
Sex			
Male	14 (25)	39 (23)	26 (24)
Female	84 (75)	64 (77)	82 (76)
Age, yr			
Average (range)	40.93 (18-81)	43.65 (18-88)	42.30 (18-88)
Area of residence			
City	21 (37.5)	45 (86.5)	66 (61.1)
Village	35 (62.5)	7 (13.5)	42 (33.9)

Table 3 The prevalence of vacuolating cytotoxin A alleles and cytotoxin-associated gene A vs patient demographics

Characteristic	CagA-positive, <i>n</i> = 24	CagA-negative, <i>n</i> = 84	<i>P</i>	VacA genotype				<i>P</i>
				<i>s1m1</i>	<i>s1m2</i>	<i>s2m1</i>	<i>s2m2</i>	
Ethnicity			0.834					0.729
Arabs	12 (50.0)	40 (47.6)		11 (21.2)	11 (21.2)	27 (51.9)	3 (5.8)	
Jewish	12 (50.0)	44 (52.4)		8 (14.3)	15 (26.8)	31 (55.4)	2 (3.6)	
Sex			0.623					0.214
Male	7 (29.2)	29 (34.5)		6 (16.7)	5 (13.9)	22 (61.1)	3 (8.3)	
Female	17 (70.8)	55 (65.5)		13 (18.1)	21 (29.2)	36 (50.0)	2 (2.8)	
Residence			0.149					0.055
Village	16 (67.7)	42 (50.0)		8 (13.8)	17 (29.3)	28 (48.3)	5 (8.6)	
City	8 (33.3)	42 (50.0)		11 (22.0)	9 (18.0)	30 (60.0)	0 (0)	

VacA: Vacuolating cytotoxin A; CagA: Cytotoxin-associated gene A.

CagA was found in 22.2% of the detected strains. This rate is a bit lower than expected according to previous studies; the prevalence of the cytotoxin-associated gene pathogenicity island, which includes CagA, varies between different geographic areas, ranging from 95% in Western and South Africa and East and Central Asia to 28% in Latin America. In Europe, approximately 58% of the *H. pylori* strains carry the CagA gene, while in the Middle East, CagA was detected in approximately 50% of the strains [12,13]. As only a sample of our isolates were tested for the presence of CagA, it is possible that a higher prevalence of this gene exists among *H. pylori* strains in Israel.

We did not find significant differences in the distribution of CagA between the two populations. Interestingly, Muhsen *et al* [14], who also investigated *H. pylori* isolates in Arab and Jewish populations, demonstrated higher CagA IgG antibodies in the Arab population. Another study, performed in Israeli children and adolescents, found higher *H. pylori* seroprevalence among Arab participants as compared to Jewish participants [15].

Regarding the virulence factor VacA, *s2m1* genotype was the most common, present in 53.3% of the isolates, while only 17.6% carried the *s1m1* genotype. In a study conducted in South Africa, *s1m1* was the most common genotype (56.4%), while the *s2m1* genotype was present in only 10.3% [16]. In a similar study conducted in Iran, the most common genotype was *s2m2*, found in 50% of the isolates [17]. It should be noted that the frequency of the alleles in different populations is influenced by several factors, including evolution, natural selection, mutations, and genetic drift [18].

CagA was identified most frequently together with the VacA genotype *s1m1* ($P = 0.02$), which is considered the most virulent [4]. This result reinforces evidence from a previous study, which found that most VacA *s1* strains carry CagA as well [19,20]. Previous studies suggested that the presence of CagA together with certain genotypes of VacA can indicate disease severity. For example, its appearance with the genotype *s1m2* or *s1m1* was correlated with the appearance of peptic ulcers, while its appearance with *s2m2* was correlated with gastritis [17]. In light of the above, it is important to profile the VacA and CagA variants in patients in order to assess disease progression. This may aid in optimizing medical treatment.

Table 4 Associations between virulence factors, patient ethnicity, and disease severity, *n* (%)

	Disease severity					<i>P</i> value
	Unremarkable, <i>n</i> = 18	Mild, <i>n</i> = 50	Moderate, <i>n</i> = 37	Severe, <i>n</i> = 3	Total, <i>n</i> = 108	
Ethnicity						0.002
Arab	5 (27.8)	21 (42.0)	28 (75.7)	2 (66.7)	56 (51.9)	
Jewish	13 (72.2)	29 (58.0)	9 (24.3)	1 (33.3)	52 (48.1)	
Urease activity						
t1	0.32 (0.05)	0.29 (0.06)	0.29 (0.07)	0.30 (0.06)	0.30 (0.06)	0.273
t5	0.32 (0.05)	0.30 (0.05)	0.29 (0.07)	0.34 (0.02)	0.30 (0.06)	0.174
t10	0.32 (0.05)	0.30 (0.06)	0.30 (0.06)	0.35 (0.04)	0.30 (0.06)	0.171
t15	0.33 (0.04)	0.31 (0.05)	0.31 (0.06)	0.36 (0.03)	0.31 (0.05)	0.245
<i>CagA</i> gene						0.534
<i>CagA</i> -	13 (72.2)	41 (82.0)	27 (73.0)	3 (100)	84 (77.8)	
<i>CagA</i> +	5 (27.8)	9 (18.0)	10 (27.0)	0 (0)	24 (22.2)	
<i>VacA</i> s allele						0.651
<i>VacA</i> s1	5 (27.8)	22 (44.0)	16 (43.2)	1 (33.3)	44 (40.7)	
<i>VacA</i> s2	13 (72.2)	28 (56.0)	21 (56.8)	2 (66.7)	64 (59.3)	
<i>VacA</i> m Allele						0.652
<i>VacA</i> m1	3 (16.7)	14 (28.0)	7 (18.9)	1 (33.3)	25 (23.1)	
<i>VacA</i> m2	15 (83.3)	36 (72.0)	30 (81.1)	2 (66.7)	83 (76.9)	
Genotype						0.926
<i>CagA</i> +/ <i>s1m1</i>	0 (0)	3 (6.0)	4 (10.8)	0 (0)	7 (6.5)	
<i>CagA</i> +/ <i>s1m2</i>	1 (5.6)	2 (4.0)	3 (8.1)	0 (0)	6 (5.6)	
<i>CagA</i> +/ <i>s2m2</i>	2 (11.1)	2 (4.0)	2 (5.4)	0 (0)	6 (5.6)	
<i>CagA</i> +/ <i>s2m1</i>	1 (5.6)	1 (2.0)	1 (2.7)	0 (0)	3 (2.8)	
<i>CagA</i> -/ <i>s1m1</i>	2 (11.1)	7 (14.0)	2 (5.4)	1 (33.3)	12 (11.1)	
<i>CagA</i> -/ <i>s1m2</i>	2 (11.1)	11 (22.0)	7 (18.9)	0 (0)	20 (18.5)	
<i>CagA</i> -/ <i>s2m2</i>	10 (55.6)	22 (44.0)	18 (48.6)	2 (66.7)	52 (48.1)	
<i>CagA</i> -/ <i>s2m1</i>	0 (0)	2 (4.0)	0 (0)	0 (0)	2 (1.9)	

VacA: Vacuolating cytotoxin A; *CagA*: Cytotoxin-associated gene A.

Apart from *VacA* and *CagA*, urease activity is another indicator of *H. pylori* virulence. We found a higher urease activity among isolates from the Jewish population as compared to those from Arabs ($P < 0.005$). No previous study has investigated this issue. As urease activity is influenced by specific food ingredients such as isothiocyanates[10] and essential oils[21], variations in nutrition habits may explain the difference in urease activity between isolates from Arab and Jewish patients. Further studies are needed to confirm our result and investigate its meaning.

Significant differences in urease activity were noted among isolates with specific *CagA* and *VacA* genotype combinations. Both *CagA* (-) s1m1 and *CagA* (-) s2m2 showed the highest urease activity. This result shows that the absence of *CagA* may result in increased urease activity, especially when combined with the most virulent alleles of the *VacA* gene, s1m1, as previously suggested[6].

Given that antibiotic resistance was found to be higher in the Arab population[8], we cautiously suggest that increased urease activity does not coincide with increased antibiotic resistance. In our preliminary analysis, no significant correlation was found between disease severity and urease activity. These results were quite surprising as previous studies did report on such associations[22]. It is possible that this contradiction is due to our relatively small sample size. However, we did find a significant correlation between ethnicity and disease severity ($P = 0.002$). This finding may be ascribable to differences in health-related lifestyle between different ethnic groups as well as differences in socioeconomic conditions along with cultural and social customs between groups related to their

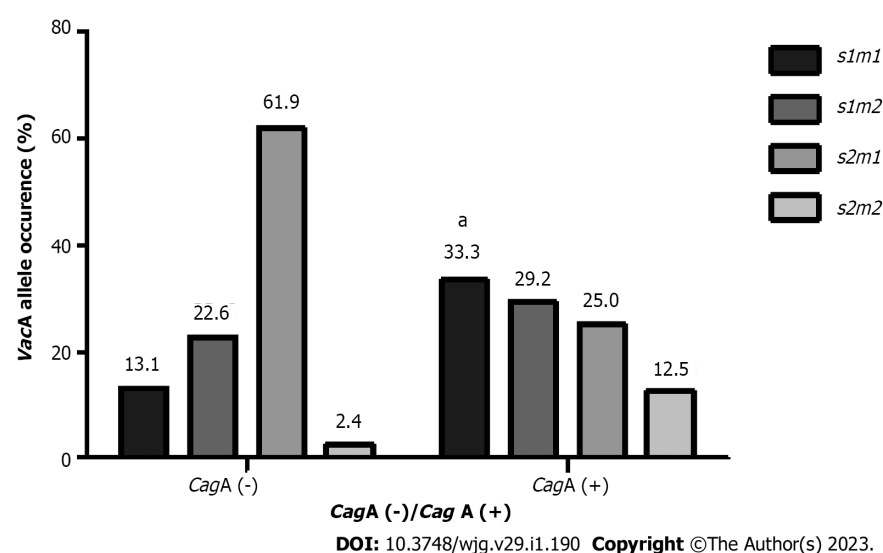


Figure 1 Distribution of the different vacuolating cytotoxin A genotypes (percentage) among 108 isolates, according to cytotoxin-associated gene A presence. VacA: Vacuolating cytotoxin A; CagA: Cytotoxin-associated gene A. ^a $P < 0.05$.

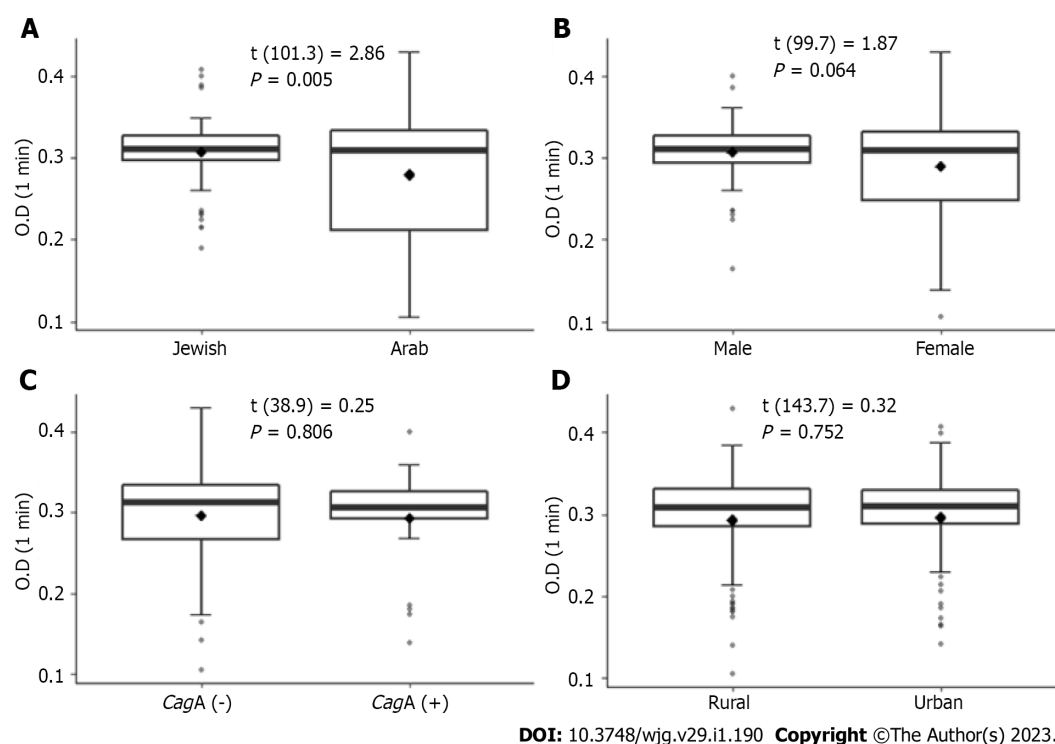


Figure 2 Urease activity in relation to ethnicity, sex, cytotoxin-associated gene occurrence, and place of residence. A: Ethnicity; B: Sex; C: Cytotoxin-associated A gene occurrence; D: Place of residence. Urease activity (indicated by O.D) was measured 1 min after incubation of bacteria with urea solution, as described in the Materials and Methods section. VacA: Vacuolating cytotoxin A; CagA: Cytotoxin-associated gene A.

residential environments, which can undoubtedly affect morbidity. Furthermore, it can also be explained by the high antibiotic resistance found among the Arab population[8].

CONCLUSION

Our study highlighted the importance of incorporating molecular methods for detection of virulence markers of *H. pylori* in order to tailor optimal treatments for each patient. Further investigation should be performed regarding associations between *H. pylori* virulence factors and ethnicity.

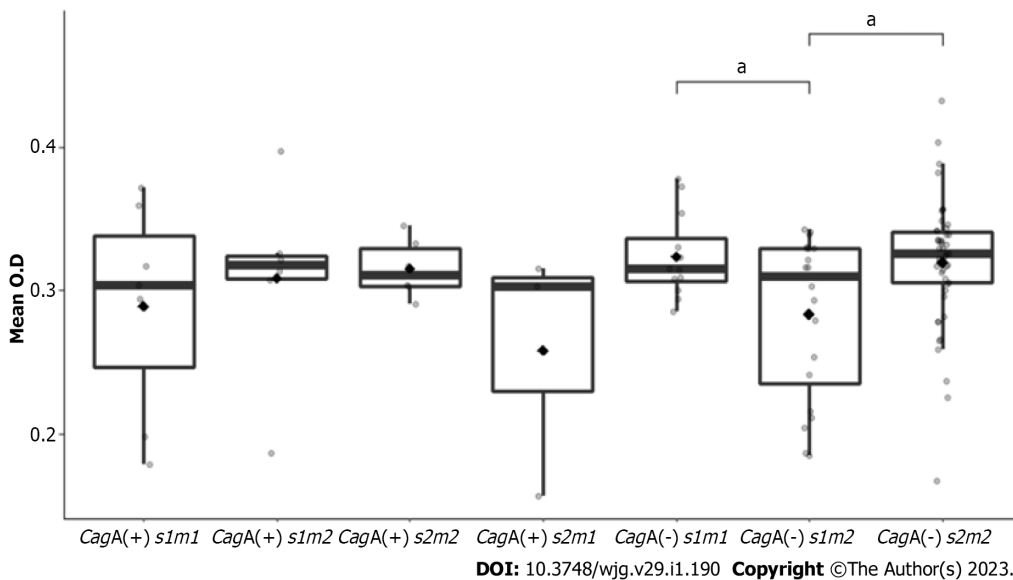


Figure 3 Urease activity as a function of isolate genotype combination. Urease activity was measured 1 min, 5 min, and 10 min after incubation of bacteria with urea solution, as described in the Materials and Methods section. The minimal, maximal, and median of mean urease activity values are shown (the median is indicated by the line within the bar) per each group of isolates with a different genotype of cytotoxin-associated gene A and vacuolating cytotoxin A genes. O.D: Optical density; VacA: Vacuolating cytotoxin A; CagA: Cytotoxin-associated gene A. ^a $P < 0.05$.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori (*H. pylori*) is a microaerophilic, Gram-negative bacterium that colonizes the gastric mucosa and infects the stomach epithelium, causing peptic ulcer disease. The genetic variability of *H. pylori* and its host, combined with environmental factors, have been suggested to affect the clinical outcome. *H. pylori* pathogenesis is mediated *via* distinct virulence factors, including the secreted vacuolating cytotoxin A, cytotoxin-associated gene A, and urease.

Research motivation

In recent years, associations between specific virulence markers of *H. pylori* and gastrointestinal disorders have been suggested.

Research objectives

To investigate the distribution of three virulence factors among isolates from both Arab and Jewish populations and to assess their impact on clinical presentations.

Research methods

We enrolled 108 patients tested for the presence of vacuolating cytotoxin A and cytotoxin-associated gene A genes and evaluated the urease activity levels. We assessed the clinical state of the patients by hematoxylin and eosin staining of the gastric biopsies from which the bacteria were recovered.

Research results

We found associations between disease severity and ethnicity and between some of the virulence factors to ethnicity.

Research conclusions

Our study highlighted the importance of incorporating molecular methods for detection of virulence markers of *H. pylori* in order to tailor optimal treatments for each patient.

Research perspectives

Further investigation should be performed regarding associations between *H. pylori* virulence factors and ethnicity.

ACKNOWLEDGEMENTS

We thank Mr. Wadie Abu Dahoud for the statistical analysis.

FOOTNOTES

Author contributions: Roshrosh H, Rohana H, and Peretz A contributed to conceptualization; Roshrosh H, Rohana H, Azrad M, and Leshem T contributed to data curation; Roshrosh H, Rohana H, Azrad M, Leshem T, and Peretz A contributed to formal analysis; Roshrosh H, Rohana H, and Leshem T contributed to investigation and methodology; Leshem T, Azrad M, and Peretz A contributed to project administration and supervision; Roshrosh H, Rohana H, and Leshem T contributed to validation; Roshrosh H, Rohana H, and Leshem T contributed to visualization; Roshrosh H, Rohana H, Azrad M, and Peretz A contributed to writing the original draft; Roshrosh H, Rohana H, Azrad M, Leshem T, and Peretz A contributed to reviewing and editing; All authors read and agreed to the published version of the manuscript.

Institutional review board statement: The study was reviewed and approved by the Helsinki Committee of the Baruch Padeh Medical Center, Poriya (Approval No. POR 0007-20).

Informed consent statement: The Institutional Review Board committee waived the need for participant approval.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Data sharing statement: The data presented in this study are available on request from the corresponding author.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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S-Editor: Chen YL

L-Editor: Filipodia

P-Editor: Chen YL

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