Reviewer Recommendation and Comments for Manuscript Number GUTP-D-20-00232

Prevalence of Helicobacter pylori genotypes and their association with upper gastrointestinal diseases: A cross sectional study in southern Iran

Original Submission
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Reviewer Comments to Author

1. Although there is a correlation between cagA and hiatal hernia, and also the normal esophageal finding is correlated with glmM genotype, the author failed to explain the possible mechanisms of this relationship. Whereas association is not the same as causation, perhaps, this is just a coincidence factor. However, if this is not a coincidence, the relationship in between them should be explained. For example, the presence of vacAs1bm1 and vacAs1bm2 had a significant association with gastric erythema; according to this passage, the relationship in between them can still be understood. This result may be influenced by the use of endoscopic method, instead of histology. Hence, result could be misleading.
2. It is necessary to carry out a multivariate analysis to rule out any accidental relationships. Multivariate analysis could be done by determining and entering significant variables.
3. Discussion section need to described relevant reasons within the study, rather than explaining results or comparing with other studies. In addition, please state the study limitation.

Gut Pathogens

Prevalence of Helicobacter pylori genotypes and their association with upper gastrointestinal diseases: A cross sectional study in southern Iran

--Manuscript Draft--

Manuscript Number:	GUTP-D-20-00232
Full Title:	Prevalence of Helicobacter pylori genotypes and their association with upper gastrointestinal diseases: A cross sectional study in southern Iran
Article Type:	Research
Abstract:	Background Investigating the prevalence of vacuolating cytotoxin (vacA), cytotoxin associated gene A (cagA), glm M genotypes, and subtypes of vacA of Helicobacter pylori (H. pylori) isolate in Jahrom, Southern Iran. DNA extracted from H. pylori samples retrieved from gastric biopsy isolated from 113 dyspeptic patients with positive rapid urease test (RUT). Genotyping was done by polymerase chain reaction (PCR) technique, using primers for vacA (s1a, s1b, s1c, s1, s2, m2, and m1), cagA, and glmM. Endoscopy was done for all the patients to screen upper gastrointestinal (GI) disorders. Results GlmM was detected in 100% of the cases. VacA subtypes s1am2, s2m2, s1a, s1b, and s1c were detected in 27.9%, 25.6%, 50%, 3.5% and 2.4% of the isolates, respectively, while cagA was detected in 60.5% of the isolates. VacA alleles m1, s1, and s2 were detected in 54%, 50%, and 44% of isolates respectively. Also, 60.5% of the isolates were cagA-vacA-positive. A significant correlation was observed between vacAs1bm1 and gastroesophageal reflux disease (GERD) and glmM and normal esophagus. The presence of vacAs1bm1 and vacAs1bm2 has a significant association with gastric erythema. The presence of cagA showed a significant association with normal esophagus and hiatal hernia. Conclusions In our research, the number of glmM and cagA positive isolates is higher among other genotypes and cagA is correlated with hiatal hernia, and normal esophageal finding is correlated with glmM genotype. There was no association between age or sex of the patients and bacterial genotype.

Prevalence of Helicobacter pylori genotypes and their association with upper gastrointestinal diseases: A cross sectional study in southern Iran

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Abstract

Background: Investigating the prevalence of vacuolating cytotoxin (vacA), cytotoxin associated gene A (cagA), glm M genotypes, and subtypes of vacA of Helicobacter pylori (H. pylori) isolate in Jahrom, Southern Iran. DNA extracted from H. pylori samples retrieved from gastric biopsy isolated from 113 dyspeptic patients with positive rapid urease test (RUT). Genotyping was done by polymerase chain reaction (PCR) technique, using primers for vacA (s1a, s1b, s1c, s1, s2, m2, and m1), cagA, and glmM. Endoscopy was done for all the patients to screen upper gastrointestinal (GI) disorders.

Results: GlmM was detected in 100% of the cases. VacA subtypes s1am2, s2m2, s1a, s1b, and s1c were detected in 27.9%, 25.6%, 50%, 3.5% and 2.4% of the isolates, respectively,

while cagA was detected in 60.5% of the isolates. VacA alleles m1, s1, and s2 were detected in 54%, 50%, and 44% of isolates respectively. Also, 60.5% of the isolates were cagA-vacA-positive. A significant correlation was observed between vacAs1bm1 and gastroesophageal reflux disease (GERD) and glmM and normal esophagus. The presence of vacAs1bm1 and vacAs1bm2 has a significant association with gastric erythema. The presence of cagA showed a significant association with normal esophagus and hiatal hernia.

Conclusions: In our research, the number of glmM and cagA positive isolates is higher among other genotypes and cagA is correlated with hiatal hernia, and normal esophageal finding is correlated with glmM genotype. There was no association between age or sex of the patients and bacterial genotype.

Keywords: Prevalence; Helicobacter pylori; genotype; Gastrointestinal diseases; Iran

Background

Helicobacter pylori (H. pylori) is a gram-negative bacillus and one of the most common bacterial infections which affect nearly half of the world's population, which has naturally colonized humans for at least 100,000 years, and probably throughout human evolution [1]. H. pylori colonize the stomach in approximately 50% of the world's human population. In the United States and other developed countries, the prevalence of H. pylori is approximately 30% of the population [2]. Moreover, DNA-level analyses have indicated that H. pylori are one of the most genetically diverse bacterial species [3].

The sequencing of the H. pylori genome in 1997 has led to considerable progress in the understanding of the biology of this organism. H. pylori are considered an important etiological agent in the development of gastritis, peptic ulcers, and gastric carcinoma [4, 5]. Colonization with this organism is the main risk factor for peptic ulceration as well as for gastric

adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [6]. H. pylori infection is virtually always associated with chronic active gastritis, but less than 15% of patients develop peptic ulcer disease (PUD), gastric adenocarcinoma, or gastric lymphoma [7]. Studies suggested that more than 90% of duodenal ulcers (DU) and 85% of gastric ulcers (GU) are associated with H. pylori [7]. There is a strong connection between H. pylori and gastric lymphoma. H. pylori antigen stimulates T-cells which promotes B-cell proliferation, leading to low-grade gastric MALT lymphoma. Eradication of H. pylori can result in regression or cure of this lymphoma [8].

The clinical outcome following infection with this pathogen has been related to environmental conditions, host immunological factors, and microorganism virulence [9]. H. pylori genotypes and their geographic distribution are linked to the severity of PUD [10, 11]. The VacA protein induces vacuolation and apoptotic processes in epithelial cells, as well as immunosuppressive actions in immunological cells [12].

The vacA, cagA, and glmM are the most commonly studied virulence factors of H. pylori, and in this study, we investigate the prevalence of the genotypes and their association with GI pathologies.

The clinical outcome of this bacterial infection seems to be influenced by the distribution of the above-mentioned pathogenic factors in H. pylori strains [13]. Due to the lack of H. pylori genotyping in southern Iran, in this study we investigate H. pylori genotyping in Jahrom, southern Iran. Furthermore, we aim to establish the main virulence strain and its association with clinical outcomes.

Results

A total of 113 patients with an average age of 39.15 (SD:16.18, range 13-89) were studied which consisted of 44 (38.9%) male and 69 (61.1%) female patients. Furthermore, 15.9% of

the patients were smokers. Based on PCR evaluation for H. pylori, 86 samples out of 113 (76.1%) patients were positive. Table 1 demonstrates the study population characteristics.

Table 1 Characteristic of patients with persistent gastrointestinal problems in our study

Variable		**Frequency** n=113	**Percentage**
Gender	Male	44	38.9
	Female	69	61.1
Positive PCR for H.Pylori		86	76.1
Endoscopy finding	NUD	52	46
	Erosive gastropathy	47	41.6
	GU	3	2.7
	DU	11	9.7
Erythema in NUD	Antrum	45	39.8
	Antrum with extension to body or fundus	6	5.3
Edema in NUD	Antrum	4	5.3
	Antrum with extension to body or fundus	2	1.8
Nodularity	Antrum	5	4.4
	Antrum with extension to body or fundus	0	0
Esophageal finding	Esophagitis	9	8
	GERD	5	4.4
	Hiatal hernia	57	50.4
	Normal	42	37.2

PCR: polymerase chain reaction; H, pylori: Helicobacter Pylori; NUD: non-ulcer dyspepsia; GU: Gastric ulcer; DU: Duodenal ulcer; GERD: Gastroesophageal reflux disease;

Vac A gene was present in all H. pylori samples and was accompanied by the Cag A gene in 60.5% of samples. Table 2 demonstrates the result of different genotypes that we found in our samples.

Table 2 Distribution of H. pylori genotypes in southern Iran n=86

Genotypes	Positive		Negative	
	Frequency	Percentage	Frequency	Percentage
Vac Am1	39	45.3	47	54.7
Vac Am2	47	54.7	39	45.3
Vac As1a	43	50	43	50
Vac As1b	3	3.5	83	96.5
Vac As1c	2	2.3	84	97.7
Vac As2	38	44.2	48	55.8
Cag a	52	60.5	34	39.5
Glm m	86	100	0	0

The vac A gene and its subtypes distribution are shown in table 3. The most common genotypes were vac As1am2 with a prevalence of 28% then vac As2bm2 and vac As1am1.

Table 3 Distribution of the vac A gene and its subtypes in southern Iran.

Genotypes	Positive		Negative	
	Frequency	Percentage	Frequency	Percentage
vacAs1am1	19	22.1%	67	77.9
vacAs1am2	24	27.9	62	72.1

vacAs1bm1	2	2.3	84	97.7
vacAs1bm2	1	1.2	85	98.8
vacAs1cm1	1	1.2	85	98.8
vacAs1cm2	1	1.2	85	98.8
vacAs2m1	16	18.6	70	81.4
vacAs2m2	22	25.6	64	74.4

The patients were divided into 4 groups based on their endoscopic findings: a) Non-ulcer dyspepsia (NUD): no erosion, but erythema, edema, or nodularity was observed. The location of these findings in the stomach was also studied. b) Erosive gastropathy c) Gastric Ulcer (GU) d) Duodenal ulcer (DU).

Out of 52 patients with NUD, 51 of them had gastric erythema, 6 of them had gastric edema and 5 patients had gastric nodularity. The erythema was located in the antrum for 39.8% of them. Out of 6 patients with edema, 5.3% had antrum involvement.

Esophageal findings of patients are shown in table 1. Esophagitis in 8% of the cases, Gastroesophageal reflux in 4.4% of the cases, and hiatal hernia in 50.4% of the cases.

Table 4 shows the relationship between the different H. pylori genotypes and age, gender, smoking, gastric endoscopy, and esophageal findings.

[INSERT TABLE 4]

There was no significant relationship found between the age, gender, smoking of the patients, and the genotype of H. pylori they were colonized with. However, the Vac As1cm1 genotype had the highest mean age (70 ± 0) and the vacAs1am1 gene had the lowest mean age (33.6 ± 10.8). Also, there is no relationship between gastric endoscopy finding and the genotype of H. pylori.

Based on table 4, there is a significant correlation among Vac As1bm1 genotype and GERD (p=0.002); Cag A genotype and hiatal hernia (p=0.02); Cag A genotype and normal esophagus; Glm M genotype and normal esophagus (p=0.02). We found that genotype vac As1bm2 is linked with gastric edema (P>0.001); however, there is only 1 person in this group.

Discussion

H. pylori genotypes, environmental and epidemiological factors can play a role in its pathogenicity. The genetic diversity and epigenetic modifications of H. pylori give rise to the different levels of pathogenicity. Several studies have shown that the incidence and/or severity of gastroduodenal pathologies related to H. pylori may vary between geographic areas, such as studies have shown genotype of H. pylori in Europe differs from South-East Asia but is the same for the North of Iran and Uzbekistan [14, 15]. The predominant H. pylori strain circulating among geographic locations differs with regard to the genomic structure [16]. The present study reports common H. pylori genotypes in Jahrom, Iran, and their clinical relevance.

The vacA gene was present in all H. pylori strains and it is a useful marker in predicting disease outcome [17]. The vacA strain's structure determines its in vitro cytotoxic activity, with m1 vacA type being more active than m2 type, s1a more active than s1b, and s2 vacA not producing detectable activity [18]. We found an apparent correlation between vacAs1bm1 positive cases and GERD (P=0.002). Genotype vacA s1/m2 is a dominant H.pylori genotype in Iran, but no relationships were found between these genotypes and clinical outcomes. In this study, we found the vac A gene of H. pylori has 8 different combinations with its subtypes, and their prevalence are; vacAs1am1 (22.1%), vacAs1am2 (27.9%), vacAs1bm1 (2.3%), vacAs1bm2 (1.2%), vacAs1cm1 (1.2%), vacAs1cm2 (1.2%), vacAs2m1 (18.6%) and vacAs2m2 (25.6%). The most common genotype is vac A s1am2 with a prevalence of approximately 28%; however, S1m1 was the most common genotype in some research studies conducted on

Afghan, Iranian, Turkish, and Thai patients [19-22], however in our research prevalence of s1m1(22.1%) is the third common genotype. A study from Shiraz, southern Iran reported that vacA-positive strains were more frequently found in PUD patients than in NUD patients [23]. Another study in Tehran reported that the vacA s1 genotype was detected in 79% and 68% of patients with PUD and NUD, respectively [24]. However, in our study, we didn't observe any correlation between any vacA subtypes and PUD or NUD.

The cagA toxin can induce severe inflammation of gastric mucosa and is related to peptic ulcer and gastric cancer [18]. Based on literature regarding H. pylori genotype in Iran and around the world, the prevalence of cag A genotype varies in different areas. The prevalence of cag A genotype is high based on studies conducted on Iranian and Iraqi patients [24]. However, in research conducted on Malaysian and Jordanian patients, the prevalence of cag A is relatively low [25, 26]. The cagA was present in 60-70% of isolates from the Western population [27]. In our research cagA was present in 60.5% of isolates. We observed a significant relationship between cag A and hiatal hernia (P=0.02). Several European and North American studies have shown that infection with cagA-positive H. pylori strains also increases the risk for atrophic gastritis and gastric cancer [27]. However, several studies in Asian populations did not confirm these relationships, indicating that there are important geographic differences, which is consistent with our research finding [28].

The association of the cagA-positive, vacA s1 genotypes with peptic ulcer disease (PUD) and gastric cancer was reported in Western countries, which was not consistent with our research and a research in East Asian countries [16]. Patients infected with less virulent genotypes are more likely to have mild gastritis throughout their entire life, whereas patients infected with more virulent genotypes have a higher probability of developing peptic ulcer disease, atrophic gastritis, and eventually gastric carcinoma [28].

Our results showed that the most common allele in our isolates is m2 (55.9%), and s1 (55.9%), then s2 (44.2%). Although in other research on Iranian and Afghan patients [21] the most common allele is s1.

Conclusions

In this study, we analysed the H. pylori gene and its association with clinical outcomes. Our data suggested that cagA is associated with normal esophageal findings, which support the hypothesis of virulent strains may provide some protection for the esophagus. Furthermore, no significant relationship was observed between the H. pylori gene and patients' characteristics (such as age, sex, smoking status). This study provides the first report of H. pylori gene diversity in Jahrom, Iran, and serves as an epidemiological tool for future studies on this bacterium to better understand the clinical outcome of this pathogen.

Materials and methods

Patients

We used a descriptive cross-sectional method in this study. Patients who visited Jahrom's Honary clinic (a city in Fars province, south of Iran) with persistent gastrointestinal problems, above 18 years of age, and did not respond to 6 months treatment with proton pump inhibitor (PPI), and positive rapid urease test (RUT) were studied. Esophagogastroduodenoscopy (EGD) and 2 biopsy samples were retrieved from their stomach antrum.

This study was approved by the ethics committee of Jahrom University of Medical Sciences. All the participants were informed about the study and signed consent forms and reassured their confidentiality.

The patients included must have dyspepsia and positive RUT at the time of EGD. Patients were excluded from this study if they had a history of antibiotic therapy in the last month or if they used a PPI or H2 blocker in the last week.

DNA Extraction and PCR

Two samples were retrieved by EGD from every patient gastric antrum and with microbiology techniques one sample was used for RUT and another was kept on a transport medium which contains; Agar 1.3 g/L and yeast extract 3%. If the urease test came positive, PCR was conducted for the second sample. We used fermentas Company kits for DNA extraction.

The primers that were used for genotyping and PCR conditions are shown in table 5. The following cycle conditions were used: for vacA: 35 cycles of 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C; for cagA: 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C; for glmM: 1 min at 93^{o}C, 1 min at 55^{o}C, and 1 min at 72^{o}C. GlmM PCR product has 294 bp, cagA has 298 bp and vacA subtypes (s1/s2, s1a, s1b, s1c, m1/m2) has 259, 286, 190, 187, 213, 567 and 642 bp respectively.

Table 5 Primer sequence and polymerase chain reaction (PCR) conditions

Genes	Primer sequence (5′ → 3′)	PCR product (bp)	PCR conditions	References
glmM	AAGCTTTTAGGGGTGTTAGGGGTTT AAGCTTACTTTCTAACACTAACGC	294	93 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min (35 cycles)	[29]
vacA				
s1/s2	ATGGAAATACAACAAACACAC	259/286		[18, 30]

	CTGCTTGAATGCGCCAAAC		94 °C, 1 min; 52 °C, 1 min; 72 °C, 1 min (35 cycles)	
s1a	GTCAGCATCACACCGCAAC CTGCTTGAATGCGCCAAAC	190	94 °C, 1 min; 52 °C, 1 min; 72 °C, 1 min (35 cycles)	[18]
s1b	AGCGCCATACCGCAAGAG CTGCTTGAATGCGCCAAAC	187	94 °C, 1 min; 52 °C, 1 min; 72 °C, 1 min (35 cycles)	[18]
s1c	CTCTCGCTTTAGTGGGGYT CTGCTTGAATGCGCCAAAC	213	94 °C, 1 min; 52 °C, 1 min; 72 °C, 1 min (35 cycles)	[31]
m1/m2	CAATCTGTCCAATCAAGCGAG GCGTCAAAATAATTCCAAGG	567/642	94 °C, 1 min; 52 °C, 1 min; 72 °C, 1 min (35 cycles)	[18]
cagA	ATAATGCTAAATTAGACAACTTGAGCGA TTAGAATAATCAACAAACATCACGCCAT	298	94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min (45 cycles)	[32]

Data analysis

Based on Prevalence of 69%, ci=95%, absolute error=80% and population(N) of 1,000,000, a sample size of n=113 was calculated [33]. Data collected from this study were analyzed using chi-square, fisher's exact test and spearman methods, and SPSS software ver 22.0.

Abbreviations

vacA: Vacuolating cytotoxin A; cagA: Cytotoxin associated gene A; H. pylori: Helicobacter pylori; RUT: Rapid urease test; PCR: Polymerase chain reaction; GI: Gastrointestinal; GERD: Gastroesophageal reflux disease; MALT: Mucosa-associated lymphoid tissue; PUD: Peptic ulcer disease; DU: Duodenal ulcers; GU: Gastric ulcers; NUD: Non-ulcer dyspepsia; PPI: Proton pump inhibitor; EGD: Esophagogastroduodenoscopy.

Declarations

Ethical approval of the study

Written inform consent was obtained from the patients in our study. The purpose of this research was completely explained to the patient and was assured that their information will be kept confidential by the researcher. This research was approved by the ethical committee of Jahrom University of Medical Sciences issued with code No.: Jums.REC.1392.028.

Consent for publication

Consent was obtained from the patients regarding the publication of this study**.**

Availability of data and materials

SPSS data of the participant can be requested from the authors. Please write to the corresponding author if you are interested in such data.

Competing interests

The authors declare that they have no competing interests.

Funding

No financial support was received for this study.

Authors' contributions

Study concept and design: Rahim Raufi, Nikta Taghipour. Acquisition of data: Rahim Raufi, Seyedeh Maryam Pishva. Analysis and interpretation of data: Reza Shahriarirad, Nikta Taghipour. Drafting of the manuscript: Seyedeh Maryam Pishva, Reza Shahriarirad. Critical revision of the manuscript for important intellectual content: Rahim Raufi, Nikta Taghipour . Statistical analysis: Reza Shahriarirad, Nikta Taghipour. Study supervision: Rahim Raufi. All authors have approved the final version of the manuscript.

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Table 4 Relationship between different H.pylori genotypes and variables in our study

Variable		Genotype *n=86*																			
		S1am1 *n=19*	**P**	**S1am2** *n=24*	**P**	**S1bm1** *n=2*	**P**	**S1bm2** *n=1*	**P**	**S1cm1** *n=1*	**P**	**S1cm2** *n=1*	**P**	**S2m1** *n=16*	**P**	**S2m2** *n=22*	**P**	**Cag a** *n=52*	**P**	**Glm m** *n=86*	**P**
Mean Age (SD)		33.6 (10.8)	-	39.2 (14.3)	-	40.5 (27.6)	-	29 (0)	-	70 (0)	-	60 (0)	-	38.1 (17.2)	-	41.8 (19.3)	-	37.8 (15)	-	38.9 (16.1)	-
Gender	*Male*	7 (36.8)	1	9 (37.5)	1	2 (100)	0.149	1 (100)	0.389	1 (100)	0.389	1 (100)	0.389	4 (25)	0.275	9 (40.9)	1	20 (35.5)	1	34 (39.5)	1
	Female	12 (63.2)		15 (62.5)		0 (0)		0 (0)		0 (0)		0 (0)		12 (75)		13 (59.1)		32 (61.5)		52 (60.5)	
Smoker	*Yes*	3 (15.8)	1	3(12.5)	0.76	1 (50)	0.294	0 (0)	1	0 (0)	1	1 (100)	0.159	3 (18.8)	0.718	6 (4.5)	0.19	10 (29.2)	0.4	12 (14)	0.4
	No	16 (84.2)		21 (87.5)		1 (50)		1 (100)		1 (100)		0 (0)		13 (81.2)		21 (95.5)		42 (80.8)		74 (86)	
Endoscopy	*NUD*	7 (36.8)	0.454	13 (54.2)	0.489	1 (50)	1	1 (100)	0.46	1 (100)	0.46	0 (0)	1	8 (50)	0.791	11 (50)	0.812	24 (46.2)	1	42 (48.8)	0.4
	Erosive gastropathy	10 (52.6)	0.316	6 (25)	0.101	1 (50)	1	0 (0)	1	0 (0)	1	0 (0)	1	7 (43.8)	1	9 (40.9)	1	19 (36.56)	0.3	33 (38.4)	0.3
	GU	0 (0)	1	2 (8.3)	0.114	0 (0)	1	0 (0)	1	0 (0)	1	0 (0)	1	0 (0)	1	1 (4.5)	0.481	1 (1.9)	1	3 (3.5)	1
	DU	2 (10.5)	1	3 (12.5)	0.698	0 (0)	1	0 (0)	1	0 (0)	1	1 (100)	0.097	1 (6.2)	1	1 (4.5)	0.689	8 (15.4)	0.1	8 (9.3)	0.7
Esophageal Findings	*Esophagitis*	0 (0)	0.353	3 (12.5)	0.398	0 (0)	1	0 (0)	1	0 (0)	1	0 (0)	1	2 (12.5)	0.613	1 (4.5)	1	3 (5.8)	0.5	5 (5.8)	0.4
	GERD	1 (5.3)	1	1 (4.2)	1	2 (100)	0.002	0 (0)	1	0 (0)	1	0 (0)	1	0 (0)	1	0 (0)	0.581	3 (5.8)	0.6	4 (4.7)	1
	Hiatal Hernia	9 (47.4)	0.806	10 (41.7)	0.366	1 (50)	1	0 (0)	0.496	0 (0)	0.496	1 (100)	1	7 (43.8)	0.6	11 (50)	1	20 (38.4)	0.02	39 (45.3)	0.07
	Normal	9 (47.4)	0.439	10 (41.7)	0.813	0 (0)	0.524	1 (100)	0.381	1 (100)	0.381	0 (0)	1	7 (43.8)	0.782	10 (45.5)	0.468	26 (50)	0.02	38 (44.2)	0.02

PCR: polymerase chain reaction; H,pylori: Helicobacter Pylori; NUD: Non-ulcer dyspepsia; GU: Gastric ulcer; DU: Duodenal ulcer; GERD: Gastroesophageal reflux disease;

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