Original Article

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Microbiology Section

Low Rate of *babA2* Genotype among Iranian *Helicobacter pylori* Clinical Isolates

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ABSTRACT

Introduction: The Blood Group Antigen-Binding Adhesion (*babA*), Outer Inflammatory Protein (*oipA*) and Sialic Acid-Binding Adhesin (*sabA*) as outer membrane proteins involved in *Helicobacter pylori* adherence to gastric mucosa have been suggested to have a role in the pathogenesis.

Aim: To investigate the frequency of *H. pylori* isolates *babA2*, *oipA* and *sabA* genes in Iranian dyspeptic patients.

Materials and Methods: DNAs were extracted from *H. pylori* –positive cultures taken from 100 different dyspeptic patients. Genotyping was performed by Polymerase Chain Reaction (PCR), using the specific primers for *babA2*, *oipA* and *sabA* genes. Chi square test was used to investigate association between variables, $p<0.05$ was considered statistically significant.

Results: All (100%) isolates possessed *oipA* and *sabA* genotypes, whereas *babA2* was detected in 22% of isolates. There was no significant relationship between presence of genes with clinical outcome. The combined genotype *oipA* +/ *sabA* +/ *babA2*- was correlated with gastritis. The rate of *babA2* genotype in our isolates was lower than other Iranian reports.

Conclusion: Frequency of *babA2* genotype among *H. pylori* isolates from Southwest of Iran is considerably less than other regions of Iran. Due to heterogeneity of *H. pylori* strains in different geographic regions, further work will be needed to understand the role of these virulence genes in *H. pylori* pathogenesis and their possible association with disease outcome.

Keywords: Gastroduodenal diseases, *oipA*, *sabA*, Virulence genes

INTRODUCTION

Helicobacter pylori as a human common gastroduodenal pathogen colonizes the gastric mucosa more than half of the world's population and induces disorders ranging from gastritis and Peptic Ulcer Disease (PUD) to gastric malignancies [1,2]. Many genes are involved in virulence of *H. pylori*, which includes cell wall synthesis gene glmM (*ureC*) one of the key genes for molecular confirmation of all *H. pylori* strains and is different from ureA (for urease production) gene [3,4].

Adherence of *H. pylori* to the gastric epithelium cells is believed to be a major process for stomach inflammation. About 32 *H. pylori* Outer Membrane Proteins (OMPs) as adhesins are involved in this process [5]. These adhesins as virulence factors have been recognized in the Hop (*Helicobacter* outer membrane protein) group [6,7]. Three of the most common Hop adhesins are blood group antigen-binding adhesin (*BabA* or *HopS*), outer inflammatory protein A (*OipA* or *HopH*) and sialic acid-binding adhesin (*SabA* or *HopP*). ABO blood group and Lewis B (leb) antigens on erythrocytes and gastric mucosa are receptors for babA [8,9]. The *babA* gene contains *babA1* and *babA2* alleles; but, due to an insertion at the 3' end of the gene [10], only the *babA2* product is functional for binding [9,11]. It has been shown that 70% of Western *H. pylori* isolates associated with severe diseases were harbored *babA2* [11]. *BabA2*- mediated strong adhesion to gastric epithelial cells might enhance delivery of CagA (Cytotoxin-Associated Gene A) and VacA (Vacuolating Cytotoxin A) cytotoxins into the host cells; therefore, may be associated with peptic ulcer and gastric cancer [12].

Apart from the role of bacterial colonization, *OipA* is a powerful inducer of Interleukin-8 (IL-8) secretion by gastric epithelia, which results in elevated levels of gastric inflammation. This process is due to phosphorylation of multiple signaling pathways that interact to cag PAI (the cag pathogenicity island)-related pathways [13]. To date, the specific receptor for *OipA* has been remained unknown [6,9,14].

H. pylori infection induces expression of sialylated glycans, namely sialyl-Lewis x/a antigens (sLeX and sLea) in the gastric mucosa. The expression of these antigens, in particular sLea, has been reported to be associated with gastric malignancies in both developed and developing countries. Hence, further investigations on *SabA* in developing countries has been suggested [15,16]. Both *oipA* and *SabA* genes, together with babA in some strains [16], are regulated by phase variation (switch “off”=non functional and switch “on”= functional), through a slipped-strand mispairing mechanism based on the number of calcitonin or Cysteine-Threonine (CT) dinucleotide repeats in the 5' coding region [7,17-19].

The association of *H. pylori* different genotypes with clinical manifestations varies within societies. However, investigation on putative virulence factors is crucial to provide useful clinical data. Therefore, as there is no data available about geographic prevalence of *H. pylori oipA*, *SabA* and *babA2* genotypes from our region, we decided to assess the frequency of these genes among *H. pylori* clinical isolates from Iran.

MATERIALS AND METHODS

Study Population

In this cross-sectional study 100 *H. pylori* isolates were obtained from antral biopsies of 318 patients referred for endoscopy in Shiraz Faghihi Hospital, Southwest of Iran, from January to May 2014 [20]. The *H. pylori* positive subjects were classified on the basis of endoscopic and histopathological findings into gastritis (Ga, n= 63), Gastric Ulcer (GU, n=15), Duodenal Ulcer (DU, n= 13) and Non-Ulcer Dyspepsia (NUD, n=9) groups. According to written informed consent, no patient had received anti-*H. pylori* therapy and endoscopy within the last month. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (EC-9379-7059).

H. pylori Identification and PCR-based Genotyping

Antral biopsy samples were processed and cultured as previously described [20]. Briefly, transported specimens within Brucella Broth (Quelab, Montreal, QC, Canada) to the Microbiology laboratory, were plated onto Columbia agar (Merck kGaA, Darmstadt, Germany) supplemented with 7% sheep blood and specific antibiotics (vancomycin 10 mg, polymyxin B 2500 IU, trimethoprim 5 mg and amphotericin B 2.5 mg/L). After 5-10 days, incubation at 37°C under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2), identification of *H. pylori* was done based on typical morphology in Gram staining and conventional biochemical tests including catalase, oxidase and urease production tests. Indeed, DNA extraction from all 100 *H. pylori* pure cultures and storage conditions were carried out as previously described [20].

PCR was used for detection of *H. pylori*-specific glmM (for molecular confirmation) and presence of *oipA, SabA* and *babA2* genes. Primer sets used were provided from the published literature [1,4,21,22]. Each PCR reaction was done in a final volume of 25 µl containing 1X PCR buffer, 400 nM of each primer, 1.5 mM $MgCl_2$, 200 µM of dNTPs mix, 1U Taq DNA polymerase and 200 ng (2 µl) of genomic DNA. PCR amplifications were performed in a thermocycler (Eppendorf AG 22331; Eppendorf, Hamburg, Germany) under the specific conditions [Table/Fig-1]. The amplicons were resolved on 1.5% agarose gel with ethidium bromide and visualized under U.V light. In all runs, one negative (DNase-free water) and positive (*H. pylori* ATCC® 26695™) control was included.

Region amplified	Primer sequence (5′ to 3′)	Size of amplicon	PCR cycles
glmM-F	AAGCTTTTAGGGGTGTTAGGGGTTT	294 bp	Initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 45 sec, annealing at 50 °C for 45 sec and extension at 72 °C for 35 sec. The final extension was at 72 °C for 10 min.
glmM-R	AAGCTTACTTTCTAACACTAACGC		
oipA-F	GTTTTTGATGCATGGGATTT	401 bp	5 min predenaturation at 95 °C, followed by 35 cycles of 35 sec at 95 °C, 35 sec at 53 °C and 35 sec at 72 °C
oipA-R	GTGCATCTCTTATGGCTTT		
sabA-F	CCGCTAGTGTCCAGGGTAAC	364 bp	35 sec at 95 °C, 35 sec at 50 °C and 35 sec at 72 °C
sabA-R	CACCGCGATTTGCGTTGGTA		
babA2-F	AATCCAAAAAGGAGAAAAATATGAAA	832 bp	35 sec at 95 °C, 35 sec at 55 °C and 45 sec at 72 °C. The final extension was at 72 °C for 5 min.
babA2-R	TGTTAGTGATTTCGGTGTAGGACA		

[Table/Fig-1]: Primers and thermal conditions in genotyping of *H. pylori* clinical isolates.
F: Forward primer, R: Reverse primer

DNA Sequence Analysis

The *oipA*, *SabA* and *babA2* amplicons (one sample from each gene) were sequenced to verify that they represented the studied virulence genes. The sequencing was performed by Bioneer Company (Munpyeongseoro, Daedeok-gu, Daejeon, South Korea). The resulting sequences were edited, aligned and analyzed using the CLC Sequence Viewer (version 6.4; CLC Bio Co., Aarhus, Denmark).

STATISTICAL ANALYSIS

The Chi-square (χ^2) test was used to analyze significant differences between the studied virulence genes with the clinical outcome. The data were analyzed using SPSS (version 21.0; IBM Co., Armonk, NY, USA) software. The results of demographic and clinical manifestations presented as descriptive statistics in terms of relative frequency. Differences were considered significant when p-value was less than 0.05 for data analysis.

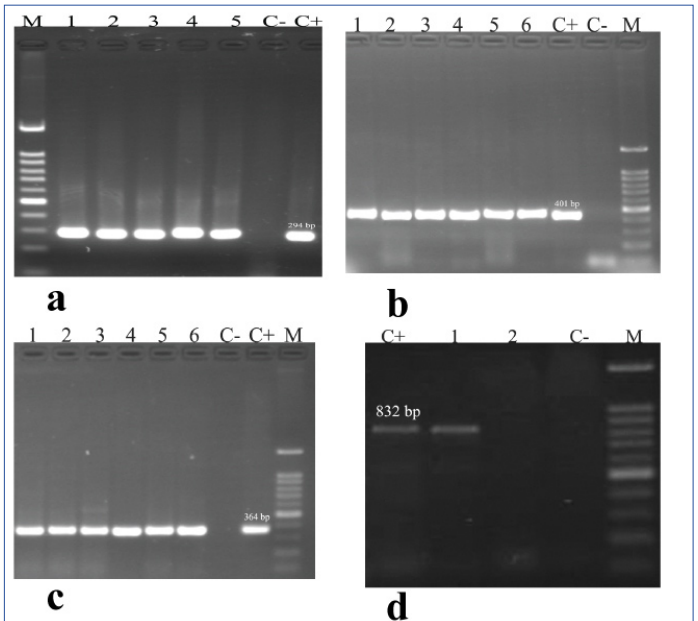
RESULTS

H. pylori Status and Clinical Outcomes

The isolation rate of *H. pylori* was 31.4%. The *H. pylori*-specific glmM (ureC) gene (294 bp) was confirmed in all isolates. The study population comprised of 50 men and 50 women with an average age of 42.9, SD=15.32 and 40.3, SD=13.40, respectively (range 18 to 75 years). A statistically significant difference between patients gender with four studied disease groups ($p<0.014$) and isolates distribution with disease status ($p<0.001$) was determined [20].

Distribution and Combined Presence of Virulence-Associated *oipA*, *SabA* and *babA2* Genotypes in Different Disease Groups

The primers used for *oipA* and *SabA* genes amplified the corresponding genes in all isolates (100%). Nevertheless, the 832-bp amplicon indicating the presence of *babA2* genotype [Table/Fig-2] was only detected in 22% (22 of 100) of isolates and the remaining isolates (78%) failed to obtain the *babA2* gene amplification [Table/Fig-3]. There was no significant difference between each of the studied genes with clinical outcome ($p>0.05$). Moreover, no statistically significant difference was determined between *oipA*, *SabA* and *babA2* genes and patient's age or gender.



[Table/Fig-2]: Agarose gel electrophoresis of glmM, ***oipA***, ***sabA*** and *babA2* genes in *H. pylori* isolates. A: M=DNA ladder (100 bp), C+ is positive control (*H. pylori* ATCC 26695, glmM= 294 bp), C- is negative control (DNase-free water), lanes 1-5 (positive samples). B: M=DNA ladder (100 bp), lanes 1-6 (positive samples of ***oipA***=401 bp). C: M=DNA ladder (100 bp), lanes 1-6 (positive samples of ***sabA*** =364 bp). D: M=DNA ladder (100 bp), lane 1 (positive sample of babA2 =832 bp) and lane 2 (negative sample).

Based on analysis of the *oipA, SabA* and *babA2* genes (positive and negative), two different genotypic combinations were determined [Table/Fig-3]. The most prevalent genotype was *oipA +/SabA +/ babA2-* (78%). The *oipA +/SabA +/ babA2-* genotype was significantly associated only with Ga cases ($p<0.001$).

Virulence gene	Ga No. (%)	GU No. (%)	DU No. (%)	NUD No. (%)	Total No. (%)
oipA	63 (100)	15 (100)	13 (100)	9 (100)	100 (100)
sabA	63 (100)	15 (100)	13 (100)	9 (100)	100 (100)
babA2	13 (20.6)	4 (26.7)	3 (23.1)	2 (22.2)	22 (22)
Genotype					
oipA+/*sabA*+/*babA2*-	51 (65.3)	10 (12.8)	11 (14)	6 (7.6)	78 (78)
oipA+/*sabA*+/*babA2*+	12 (54.5)	5 (22.7)	2 (9)	3 (13.6)	22 (22)

[Table/Fig-3]: Distribution of *H. pylori* virulence genes and combined genotypes within disease groups.
*Ga: Gastritis, GU: Gastric ulcer, DU: Duodenal ulcer, NUD: Non-ulcer dyspepsia

DISCUSSION

Three factors including host's genetics, environmental elements such as diet and *H. pylori* virulence genes are related to the severity of clinical manifestations [11, 23]. Among virulence genes, adhesins are very crucial for bacterial colonization and pathogenesis [6,15]. In recent years, there has been a great interest about the role of *H. pylori* OMPs, such as *oipA*, *SabA* and *babA2* in developing of digestive diseases. Therefore, the presence and relationship of these virulence genes with clinical outcome needs to be further investigated. In the present study, *H. pylori* were isolated from 31.4% of patients. This result is similar to Abdollahi H et al., report from Iran [24], which implies the improvement of hygiene and the socio-economic conditions. Moreover, all studied isolates possessed both *oipA* and *SabA* genes, while the *babA2* genotype has been only detected in 22% of isolates. However, no significant difference between each of studied genes with patients' gender or age was determined.

Mansour KB et al., detected the *oipA* genotype in 90.8% of *H. pylori* clinical isolates from Tunisia [21]. In another study from Bulgaria, all 69 clinical isolates were detected positive for *oipA* and there was a strong association between *oipA* and peptic ulcer status [25]. In the survey of Yamaoka Y et al., [5], *oipA* positive isolates from the US and Colombian patients were significantly recognized in duodenal ulcers (88%) and gastric cancer (89%) subjects. Several studies in Iran showed different ranges for prevalence of *oipA* from 40.8% to 95.9% [26-30]. Nevertheless, in none of these studies, except two of them [29,30] an association has been found between the genes and clinical outcome. Our result is similar to the obtained data by Lehours P et al., [12], who found that all *H. pylori* isolates were *oipA* positive. However, they did not find any correlation between *oipA* gene with different gastroduodenal diseases. Our finding is also in accordance with reports from some other studies [17, 28, 31], which reflects the importance of geographic differences in *H. pylori* virulence factors distribution [25]. Nevertheless, some works have shown an association between *OipA* with peptic ulcers and gastric malignancies [5,6,16].

The role of *SabA* in gastroduodenal disorders is progressively apparent. Due to absence of sLeX in the healthy gastric mucosa, it was proposed that the *SabA* is related to the *H. pylori* chronic persistence [9]. Therefore, it is suggested that *SabA* is related to severe clinical outcomes, such as gastric cancer [16]. In two studies from Japan, a high frequency of *SabA* gene among *H. pylori* clinical isolates (with frequencies of 91. 3% and 81%, respectively) has been reported [1, 32]. In the latter one, there was no significant correlation between the *SabA* genotype with gastric diseases. Conversely, in other report from Japan [33], the rate of *SabA* was found 47.7% and it was also suggested that the presence of *SabA* gene might not correlated with Ga. In some previous studies, no association between *SabA* and clinical manifestations was reported [12,17,34,35]. Yamaoka Y and co-workers [5] assessed 200 *H. pylori* isolates from the US and Colombian patients, in which the *SabA* positive status was associated with gastric cancer and Ga. In two different studies from Iran, the prevalence of *SabA* have reported 83.3%-100% and 83.6%, respectively [36,37], with no association with the severity of clinical manifestations. In our study, the *SabA* genotype showed a high frequency (100%), which is somewhat similar to Shao L et al., investigation [1]. In the study of Yanai A et al., [32], inspite of the positivity of the most of *H. pylori* isolates for functional *SabA*; but, this gene was not as a marker for gastroduodenal disorders, which is consistent with our findings. This discrepancy between our results and other reports, underlines that *SabA* genetically varies among different strains and geographical areas [1] and also may not be present in all *H. pylori* isolates [16]. It is necessary to note that patient selection to establish an association between *H. pylori* virulence genes and clinical outcome is very important, because the studied groups should be sufficiently large and heterogeneous [21].

It has been mentioned that *H. pylori babA2* positive isolates are associated with increased risk of acute gastritis, duodenal ulcers and gastric cancer and that the *babA2* detection in clinical isolates by PCR does not necessarily correlates with its adhesive properties and vice versa [12,38]. In two studies conducted among Colombian patients, it was found that *babA2* gene was more related to duodenal ulcers and gastric cancer [5,22], which is not in agreement with our observation. In contrast, in a research from Turkey [31], no significant difference was found between babA and other virulence genes with Ga and ulcer. Con SA et al., [39] reported a high prevalence of *babA2*, 73.7% and 96.8% in Costa Rican and Japanese isolates, respectively. In Brazil, 46.15% strains were *babA2* positive and a strong association has been observed between the presence of *babA2* and duodenal ulcers as well as gastric carcinoma [40]. Yu J et al., [41] have shown that around 80% of studied patients were infected with *babA2* positive strains. The *babA2* prevalence among South-American *H. pylori* isolates was reported between 46-82.3% [38]. In Iran, these fluctuations vary from 49.5% to 96.7% [29,30,35,42,43], with no observed correlation between *babA2* and peptic ulcer disease or gastric cancer. Our result (*babA2*=22%) is in agreement with Paniagua GL and co-workers survey [38]; however, it is considerably lesser than other studies from Iran. Moreover, no significant association was seen between the presence of *babA2* genotype and clinical manifestations. This result is supported by some other works [12,34]. Notably, frequency of *babA2* in our isolates was low similar to data from Colombia and Cuba, countries with low incidence of gastric cancer [22,44] and in contrary with Japan, Costa Rica, Brazil and China where a high rate of gastric cancer have been found [39-41]. On the other hand, the most prevalent combined genotype in the present study was *oipA*+/*SabA*+/*babA2*- (78%), which was significantly associated with Ga.

The results of the present study highlight the importance of *oipA* and *SabA* genes in *H. pylori* infection associated complications. Indeed, it seems that the most important point is low frequency of *babA2* genotypes in our isolates which is in contrast to other regions of Iran.

LIMITATION

There are some limitations in our study such as sample size, which was relatively small. Lack of gastric cancer cases among our studied samples was another limitation for our study; thereby we could not evaluate the studied genes among *H. pylori* isolates from those patients. We also did not evaluate the *oipA* functional status among our isolates.

CONCLUSION

Our study showed a considerably low frequency of *babA2* genotype among *H. pylori* isolates examined from Southwest of Iran. This result is different from other Iranian reports; however, in agreement with countries with a low rate of gastric malignancy. Therefore, with respect to heterogeneity of *H. pylori* strains and diverse results from different geographic regions about above mentioned genotypes, further investigations are required to assess the role of these genes in *H. pylori* pathogenesis and their association with clinical outcomes.

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