Clinical and Histopathological Relevance of *Helicobacter pylori* BabA2 Genotype

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### INTRODUCTION

*H. pylori* is a spiral-shaped Gram-negative organism that uniquely colonizes the human stomach and induces a persistent inflammation. Gastric colonization with *H. pylori* is associated with a wide spectrum of gastric diseases, including gastritis, peptic ulcer, non-ulcer dyspepsia, and gastric cancer.\(^1\) The risk of developing any of these disorders in the presence of *H. pylori* infection depends on several bacterial, host, and environmental factors that relate to the type and severity of gastric disease.\(^2\)

*H. pylori* genome encodes for a large set of outer membrane proteins (OMP) that contribute to the pathogenicity of the bacteria. The variable expression of these proteins among *H. pylori* clinical strains may confer a selective advantage to certain strains in different hosts.\(^3,4\) Therefore, the genotype of *H. pylori* is a potentially useful predictor of the clinical outcomes of gastric mucosal colonization. *H. pylori* blood group antigen-binding adhesin A (BabA) is a surface protein encoded by babA2 gene. It mediates binding to the fucosylated Lewis b (Leb) blood group antigen, and to the terminal fucose residues of A, B, and O blood group antigens, that are expressed on the gastric mucosa.\(^5,7\) BabA-mediated binding to Leb antigen on the gastric epithelial surface triggers the production of the proinflammatory cytokines (CCL5 and IL-8) and the precancer-related factors (CDX2 and MUC2), and induces DNA double strand breaks (DSB) and DNA damage response in host cells.\(^9\)

A number of studies have shown that babA2-positive strains are highly associated with increased risk of developing severe mucosal injury and adverse clinical outcomes. However, the correlation between babA2 genotype status and infection outcomes does not seem to be universal.\(^10\) The babA2-positive status has been linked to severe gastric atrophy and intestinal metaplasia in patients in the United States of America (USA) and Colombia.\(^11\) Conversely, babA2 genotype status was not associated with any of the clinical outcomes of *H. pylori* infection in Thai patients.\(^12\)
The inconsistency among different reports underscores the importance of further investigations for elucidating the clinical and histopathological relevance of *H. pylori* babA2 genotype status. This study aimed to correlate between *H. pylori* babA2 genotype status and the clinical and histopathological findings in Malaysian patients.

**MATERIALS AND METHODS**

**Study description:**
This is a laboratory based cross sectional investigation of the clinical and histopathological relevance of *H. pylori* babA2 genotype status. Endoscopy was performed to visualize the gastric mucosal abnormalities, and to collect 3 biopsy specimens from each patient. CLO test was used in conjunction with endoscopy to diagnose *H. pylori* infection. Conventional and molecular procedures were performed to isolate the organism, confirm its identity and to identify its babA2 genotype status. Histopathological examination was carried out to evaluate the severity of *H. pylori*-associated disease. The study protocol was reviewed and granted ethical approvals by International Islamic University Malaysia Research Ethics Committee (IREC), and the Ministry of Health Malaysia at the Institute for Medical Research (IMR).

**Study participants:**
This study was conducted on 30 consecutive CLO test-positive patients who attended the Endoscopy Unit, Department of Surgery, Hospital Tengku Ampuan Afzan (HTAA), Kuantan, Pahang, Malaysia, from 15th January 2015 to 30th June 2015. Patients who had received nonsteroidal anti-inflammatory drugs, antibiotics, H2-receptor blockers or proton pump inhibitors (PPI) within two weeks prior to endoscopy were excluded from the study. The endoscopic findings of patients were recorded as either gastritis, or peptic ulcer disease. Three gastric mucosal biopsy specimens were obtained from each patient for CLO test, bacterial isolation, and histopathological examination.

**Initial diagnosis of *H. pylori* infection:**
Campylobacter-like organism (CLO) test (Kimberly-Clark, Roswell, GA, USA) was performed in conjunction with the endoscopy procedure according to the manufacturers’ instructions. One gastric biopsy specimen was immediately embedded into the CLO test medium after collection. A positive result was reported if the colour changed from yellow to red within 24 hours (usually within 1 hour) of incubation at room temperature. CLO test-positive gastric biopsy specimens were stored at -70°C in normal saline until DNA extraction was performed.

**Isolation of *H. pylori* clinical strains:**
One gastric biopsy specimen collected during the endoscopy was immediately placed in a Bijou bottle containing 3ml of semi-solid Stuart's Transport Medium (Thermo Scientific Microbiology, Melaka, Malaysia). The bottle was then sealed in a plastic bag and kept chilled during transport to the laboratory in a polystyrene box containing pre-frozen freezer bricks.

The biopsy specimen was transferred from the transport medium to a Petri dish, and it was teased and minced in a drop of saline using a scalpel to release the bacteria from the mucosal surface. The bacterial suspension was then spread over the whole surface of a plate of *H. pylori* selective medium containing Columbia agar, Dent’s antibiotic mixture, and 7% horse blood (Thermo Scientific Microbiology, Melaka, Malaysia).

The inoculated plate was incubated at 37°C under microaerobic conditions generated by CampyGen Compact pouch system (Oxoid, Basingstoke, UK). The plate was inspected after 5-7 days for the presence of the typical tiny translucent *H. pylori* colonies. Suspected colonies were identified and confirmed as *H. pylori* if spiral or curved Gram-negative, urease-positive, catalase-positive, and oxidase-positive organisms were present.

A single colony of the isolated *H. pylori* was picked from the primary culture and sub-cultured using the same medium and incubation conditions mentioned above for purification. Multiple bacterial colonies were harvested from each secondary culture plate, and stored at -70°C in Tryptone Soy Broth with 15% glycerol (Thermo Scientific Microbiology, Melaka, Malaysia) until DNA extraction was performed.

**Detection of glmM and babA2 genes:**
Genomic DNA was extracted from 2 sources, *H. pylori* colonies harvested from the secondary culture plate and CLO test-positive gastric biopsy specimen obtained from the assay medium. Extraction was performed using PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Extracted DNA was stored at -70°C until amplification reaction was performed.

PCR was performed to confirm the presence of *H. pylori*-specific glmM gene and to determine the babA2 genotype status using primer sets described previously12-13 (Table I). Amplification was performed in a total volume of 25 µl. The reaction mixture contained 12.5 µl of 2x Taq Master Mix (Bioron, Ludwigshafen, Germany), 1 µl of 5 µM babA2 or 12.5 µM glmM forward and reverse primers (Bio Basic, Ontario, Canada), 5 µl of template DNA, and 5.5 µl of sterile deionized water. Amplification was carried out using GeneAmp PCR System 9700 automated thermal cycler (PE Applied Biosystems, Singapore) under the thermal cycling conditions described previously12-13 (Table I).
DNA extracted from *H. pylori* reference strain J99 (ATCC 700824) was used as a positive control, and deionized distilled water was used as a non-template negative control. The positive and negative controls were examined by a single, experienced pathologist in a blinded fashion.

**Table I. Primers sets and thermal cycling conditions**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer*</th>
<th>Nucleotide sequence (5’-3’)</th>
<th>Product</th>
<th>Amplification conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>glmM</em></td>
<td><em>glmM</em>-F</td>
<td>AA-</td>
<td>294 bp</td>
<td>93°C, 1 min; 55°C, 1 min; 72°C, 1 min (35 cycles)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCTTTTAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGGGTGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGGGTGGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>glmM</em>-R</td>
<td></td>
<td>AA-</td>
<td>294 bp</td>
<td>93°C, 1 min; 55°C, 1 min; 72°C, 1 min (35 cycles)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCTTACTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTAAACACTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>babA2</em></td>
<td><em>babA2</em>-F</td>
<td>CCAACAC-</td>
<td>271 bp</td>
<td>94°C, 1 min; 45°C, 1 min; 72°C, 1 min (30 cycles)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAAACAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGCGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCTTGTG-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TAAAAGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTCGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*F: Forward primer, R: Reverse primer

The slides were evaluated according to the Updated Sydney Classification System as described previously.

**Table II. Definition and grading guideline for each of the histological variables according to the Updated Sydney Classification System**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Grading guideline*</th>
</tr>
</thead>
</table>
| Density of *H. pylori* colonization: 
Density of *H. pylori* overlying the epithelium | 0: No *H. pylori* colonization; 1: Scattered *H. pylori* covering <1/3 of the surface; 2: Moderate colonization covering 1/3 to 2/3 of the surface; 3: Dense colonization covering >2/3 of the surface |
| Degree of inflammatory activity: 
Infiltration of PMN in the gastric mucosa | 0: No PMN infiltration; 1: Scattered PMN infiltrating <1/3 of the mucosa; 2: Moderate PMN infiltrating 1/3 to 2/3 of the mucosa; 3: Marked PMN infiltrating >2/3 of the mucosa |
| Degree of chronic inflammatory infiltrate: 
Infiltration of Mononuclear cells (lymphocytes, plasma cells) in the gastric mucosa | 0: Normal mononuclear cells level (up to 5/HPF); 1: Mild increase in mononuclear cells (6-20/HPF); 2: Moderate increase in mononuclear cells (20-50/HPF); 3: Diffuse increase in mononuclear cells (>50/HPF) |
| Degree of glandular atrophy: 
Loss of specialized gastric glandular tissue | 0: No atrophy; 1: Few gastric glands are lost; 2: Up to 50% of gastric glands are lost; 3: More than 50% of gastric glands are lost |
| Degree of intestinal metaplasia: 
The amount of gastric glandular tissue replaced by intestinal-type epithelium | 0: No intestinal metaplasia; 1: Few glands are replaced by intestinal cells; 2: Up to 50% of glands are replaced by intestinal cells; 3: More than 50% of glands are replaced by intestinal cells |

* If areas with widely different grades were present on the same specimen, an average grade was considered after a general evaluation of the specimen.

PMN: Polymorphonuclear Cells; HPF: High Power Field (x40 objective)

**Statistical analysis:**
Chi-square and Fisher’s exact tests were used to evaluate the association between *H. pylori* *babA2* genotype status and the clinical outcomes. Mann-Whitney U test was used to determine the relationship between *H. pylori* *babA2* genotype status and the severity of different histopathological variables. A p-value of less than 0.05 was considered statistically significant. Calculations were performed using IBM SPSS Statistics software version 23 (IBM Corporation, NY, USA).

**RESULTS:**

**Study population:**
A total of 30 *H. pylori* positive patients, 16 with gastritis and 14 with peptic ulcer, were recruited in our study. Patients were from different ethnic groups comprising 16 Malay (12 males and 4 females, with age range of 19 to 73 years and mean age of 44 years), 6 Chinese (2 males and 4 females, with age range of 19 to 73 years and mean age of 44 years), and 8 Caucasian (2 males and 6 females, with age range of 20 to 73 years and mean age of 44 years).

The PCR product was electrophoresed on 2% agarose gel stained with ethidium bromide. Electrophoresis was conducted at a constant voltage of 80 V for 50 minutes using PowerPac Basic electrophoresis apparatus (Bio-Rad, CA, USA). The gel was visualized and photographed using Gel Doc System (Bio-Rad, CA, USA). The presence of 294 bp and 271 bp sized bands indicated a positive genotype status of *glmM*, *babA2* genes, respectively.

**Gastric histopathological examination:**
One biopsy specimen from each patient was fixed in 10% formalin, embedded in paraffin, and cut in sequential 5µm sections. The obtained sections were stained with Modified Giemsa stain to detect the presence of *H. pylori*, and Hematoxylin and Eosin (H&E) stain to evaluate the inflammatory cells infiltrate and the mucosal abnormalities. Slides were examined by a single, experienced pathologist who was blinded to the patient’s clinical presentation, endoscopic findings, and CLO test result.

The slides were evaluated according to the Updated Sydney Classification System as described previously.

A semi-quantitative scoring of 5 morphological variables, including *H. pylori* density, neutrophil activity, mononuclear cell infiltration, glandular atrophy, and intestinal metaplasia, was made based on a standard visual analogue scale graded from 0, indicating the normal status, up to 3, indicating the maximal intensity (Table II).
range of 25 to 72 years and mean age of 48 years), 6 Indians (3 males and 3 females, with age range of 34 to 73 years and mean age of 51 years), and 2 other males, 26 and 34 years old. The relatively low number of Chinese patients in this study may reflect the racial distribution of the Malaysian population with Malays predominance especially in and around Kuantan. Also many Chinese patients, being wealthier, attend the private health sector rather than the government health facilities.

Data showed that more Chinese patients had peptic ulcer disease compared to Malay and Indian patients. Males and females were nearly equally distributed among the different clinical outcomes. The mean age

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Clinical status</th>
<th>n=16</th>
<th>n=14</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethnic group</td>
<td>Gastritis</td>
<td>Peptic ulcer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malay</td>
<td>9 (56.3%)</td>
<td>7 (50.0%)</td>
<td>16 (53.3%)</td>
<td></td>
</tr>
<tr>
<td>Chinese</td>
<td>2 (12.5%)</td>
<td>4 (28.6%)</td>
<td>6 (20.0%)</td>
<td></td>
</tr>
<tr>
<td>Indian</td>
<td>3 (18.8%)</td>
<td>3 (21.4%)</td>
<td>6 (20.0%)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>2 (12.5%)</td>
<td>0 (0.0%)</td>
<td>2 (6.7%)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>10 (62.5%)</td>
<td>9 (64.3%)</td>
<td>19 (63.3%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>6 (37.5%)</td>
<td>5 (35.7%)</td>
<td>11 (36.7%)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>19-73</td>
<td>23-73</td>
<td>19-73</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>42</td>
<td>49</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

Overall, the \( \text{babA2} \) genotype \( \text{H. pylori} \) was positive in 86.7% (26/30) of the studied patients. The \( \text{babA2} \) gene was almost equally frequent in \( \text{H. pylori} \) strains of all disease groups. The frequency of \( \text{babA2} \) gene in specimens obtained from gastritis patients and peptic ulcer patients was 81.2% (13/16) and 92.9% (13/14), respectively. There was no statistically significant difference between the above patients' groups (Table IV).

**Detection of** \( \text{H. pylori} \) infection:
The initial diagnosis of \( \text{H. pylori} \) infection was made using CLO test. In which, a positive \( \text{H. pylori} \) status was indicated by a colour change from yellow to red. In culture, \( \text{H. pylori} \) was identified based on its colonial morphology (small translucent colonies), Gram staining (spiral or curved Gram-negative rods), and biochemical activity (positive urease, catalase, and oxidase tests). The identity of \( \text{H. pylori} \) was further confirmed by detecting the presence of species-specific \( glmM \) gene in both, CLO test-positive gastric biopsies and bacterial isolates. A 294 bp segment of \( glmM \) gene was successfully detected in all tested biopsies and isolates (Figure I).

**Clinical relevance of** \( \text{babA2} \) **genotype status:**
\( \text{H. pylori} \ \text{babA2} \) genotype status was determined by conventional PCR amplification and visualization of a 271 bp segment of the \( \text{babA2} \) gene (Figure II).
Histopathological relevance of \textit{babA2} genotype status:
A semi-quantitative scoring of 5 morphological variables was performed according to the Updated Sydney Classification System (Figure III). In general, mild \textit{H. pylori} density, moderate granulocytic and lymphocytic infiltration, and absent gastric atrophy and intestinal metaplasia were the most frequently encountered patterns during grading (Figure IV).

The average score of \textit{H. pylori} colonization density was slightly higher in patients infected with \textit{babA2}-positive strains than in patients infected with \textit{babA2}-negative strains; however, the difference was not statistically significant. The \textit{babA2}-positive status was associated with a higher degree of inflammatory activity.

A moderate to severe grade of PMN infiltration was observed in the majority of patients infected with \textit{babA2}-positive strains, whereas no severe infiltration was found in those infected with \textit{babA2}-negative strains; however, the associations did not reach to a statistically significant level. Conversely, the \textit{babA2} genotype status had no effect on the degree of lymphocytic infiltration, glandular atrophy, or intestinal metaplasia (Table V).

DISCUSSION
Several studies have shown that \textit{babA2}-positive strains are closely associated with increased risk of developing more severe clinical and histopathological outcomes. However, the relationship between \textit{H. pylori} \textit{babA2} genotype status and the severity of gastric mucosal damage does not seem to be universal. In the current study, we investigated the presence of \textit{babA2} gene in 30 \textit{H. pylori} clinical strains, and evaluated the relationship between the \textit{babA2} genotype status and the clinical and histopathological outcomes of the infection.

We found that \textit{babA2} gene was present in 86.7\% of the tested \textit{H. pylori} strains, and there was no significant association between \textit{babA2} genotype status and the severity of gastric disease.
Our findings are in agreement with several previous investigations performed in different geographical regions and failed to correlate between babA2 genotype status and any specific disease outcomes or mucosal changes. However, some other studies have successfully confirmed the association between babA2 genotype status and infection outcomes. For instance, babA2-positive strains have been shown to be more commonly associated with peptic ulcer and gastric cancer in Germany, peptic ulcer in India, severe intestinal metaplasia and gastric atrophy in US and Colombia. Conversely, reports from Japan, Korea, China, Thailand, Brazil, and France have found no clinical or histopathological relevance of babA2 genotype status. This inconsistency between different studies indicates that there are probably important geographical variations.

**CONCLUSION**

In conclusion, our data represent the first report of the clinical and histopathological relevance of *H. pylori* babA2 genotype status in Malaysian patients. The babA2 gene was present in the majority of studied strains, and there was no influence of babA2 genotype status on the severity of gastric disease. Our findings suggest that babA2 gene may not be considered as a sole marker for determining the infection outcomes.

It is more probable that multiple bacterial factors contribute to the differential outcome of *H. pylori* infection. Therefore, it is necessary to consider multiple virulence factors in the prediction of the severity of disease. Additionally, it is important to define what other multiple environmental and host factors that may influence the response to infection and determine the progression of gastritis to peptic ulcer disease or gastric cancer.

The conclusion from this study is limited by the relatively small size of the study population comprising 30 patients. This limitation might have led to inconclusive analysis of some data. This was due to constraints related to unexpected cut off of proposed research funds. Hence a bigger study should be carried out to confirm or negate the association.

**Conflict of Interest:**
The authors declare no potential conflicts of interests.

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