Published in final edited form as:
Clin Gastroenterol Hepatol. 2007 January ; 5(1): 49–58. doi:10.1016/j.cgh.2006.09.015.

Helicobacter pylori BabA Expression, Gastric Mucosal Injury, and Clinical Outcome

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Abstract

Background & Aims—The blood group antigen binding adhesin (BabA) has been proposed to play a role in disease pathogenesis. This hypothesis is based on the functional BabA status as determined by polymerase chain reaction (PCR) analysis to distinguish functional *babA2* genes from nonfunctional *babA1* genes.

Methods—We compared the ability of published PCR-based methods to assess BabA status with BabA immunoblotting and Lewis b (Le^b) binding activity assays. We also used immunoblotting to examine the relationship between clinical presentation and levels of BabA expression.

Results—Immunoblotting and Le^b binding assays for 80 strains revealed 3 levels of BabA expression: BabA high producers (BabA-H) with Le^b binding activity, BabA low producers (BabA-L) without Le^b binding activity, and BabA-negative. BabA-negative strains lacked the *babA* gene. PCR methods to determine BabA status yielded poor results. *babA1* sequences were never detected. BabA expression was examined in 250 strains from Western countries and 270 strains from East Asia. The results failed to confirm any relationship between triple-positive status (*cagA*-positive/*vacA* s1/BabA-H) and clinical outcome. BabA-negative strains typically were *cagA*-negative/*vacA* s2 and were associated with gastritis. BabA-L strains showed a higher level of mucosal injury and were associated more frequently with duodenal ulcer and gastric cancer than the other groups.

Conclusions—Information gained from currently used PCR-based methods must be interpreted with caution. Le^b binding activity does not accurately reflect the severity of mucosal damage or the clinical outcome. Quantitation of BabA expression revealed that Le^b-nonbinding BabA-L strains are associated with higher levels of mucosal injury and clinical outcome.

Bacterial adherence is believed to play an important role in the colonization of gastric epithelium by *Helicobacter pylori*. Fucosylated ABO blood group antigens and the related sialyl-Lewis x/a antigens are thought to serve as one group of functional receptors for *H pylori* adherence.[1,2] The ABO antigens are recognized by the blood group antigen binding

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adhesin (BabA)[3,4] and sialyl-Lewis x/a antigens are recognized by the *H pylori* sialic acid binding adhesin.[2]

A number of studies have suggested a relation between *babA2*-positive *H pylori* and increased cellular mucosal inflammations and an increased risk of developing clinical outcomes (Table 1). However, the role of BabA in the pathogenesis of *H pylori*–related disease remains unclear. The *babA* genes initially were cloned from the strain CCUG17875, which contains a silent *babA1* gene and an expressed *babA2* gene.[3] The sequence of these 2 genes differed only by the presence of a 10 – base pair (bp) deletion in the signal peptide sequence of *babA1* that eliminates its translational initiation codon.[3] Most studies evaluating BabA status have used polymerase chain reaction (PCR) techniques based on detection of the 10-bp deletion to distinguish between the *babA2* and *babA1* genes (Table 1).[5–22] However, the ability of such PCR-based strategies to detect the presence of a functional *babA* gene is questionable. For example, *H pylori* strains unable to bind to Lewis b antigen (Le^b) have been reported to contain signal peptide sequences that are identical to those of strains with a functional *babA2* gene (eg, strain 26695).[23] It also has been suggested that *babA* expression may be regulated transcriptionally[24] and that there are chimeric *babA*–*babB* genes (chimera *babA/B* or *babB/A*).[24,25] Finally, not all of these PCR-based studies have shown an association between *babA2* and intense cellular mucosal inflammations and/or increased risk of peptic ulcer diseases and gastric cancer (Table 1).[10–13,19,20,22] Therefore, in the current study, we have compared results from PCR-based strategies that currently are used to predict BabA functional status with those from strategies that measure actual *babA* expression (ie, immunoblotting) and Le^b binding.

Materials and Methods

Helicobacter pylori

We examined *H pylori* isolates cultured from patients in East Asia and North and South America with clinical presentations including simple *H pylori* gastritis, duodenal ulcer (DU), and noncardiac gastric adenocarcinoma. Ulcers were defined endoscopically, and patients with either ulcer scars or DU and gastric ulcers were excluded. Gastritis was defined as histologic gastritis with no peptic ulcers or gastric cancer. No patients received treatment for their *H pylori* infection, and those patients who used nonsteroidal anti-inflammatory drugs were excluded. Because of the small number of patients available, gastric cancer patients from the United States were not included.

We obtained gastric biopsy specimens to isolate *H pylori*. Isolation was performed using standard culture methods according to protocols approved by local ethics committees. To minimize the risk of phase variations, all *H pylori* samples were obtained from a single bacterial colony that was passaged in vitro less than 4 times.[26] The control strain was strain J99,[27] which contains a *babA* gene and possesses Le^b binding ability.[3] We also used strain 26695,[23] which contains a *babA* gene but does not bind to Le^b,[3] and strain TN2GF4,[28] which contains a *babA* gene and possesses Le^b binding ability. In addition, we constructed isogenic *babA* mutants from strains J99 and TN2GF4 using methods previously described.[29]

Histology

Gastric mucosal biopsy specimens were stained with H&E for morphologic observations and either Genta stain[30] or El-Zimaity triple stain[31] (for US, Colombian, and Korean specimens) or modified Giemsa stain (for Japanese specimens) for detection of *H pylori*. We examined up to 5 biopsy specimens from the antrum and up to 6 specimens from the corpus. Each specimen was scored for *H pylori* density, neutrophil infiltration, and atrophy. All of the variables were scored using a visual analogue scale graded from 0 (absent/normal) to 5

(maximal intensity), as described previously.[32] Scores for each site were averaged in both the antrum and the corpus specimens.

Detection of BabA Protein and Lewis b Antigen Binding

Whole protein extracts from *H pylori* isolates were obtained by suspending the bacteria in Laemmli sample buffer and boiling this suspension at 100°C for 10 minutes. Immunoblotting was performed using standard methods. Anti-BabA antiserum (AK277)[33] was used as the primary antibody at a 1:5000 dilution. Horseradish-peroxidase– conjugated protein A (1:3000) (Bio-Rad Laboratory, Hercules, CA) was used as the secondary antibody. Detection was performed using enhanced chemiluminescence reagents (Amersham Life Science, Arlington Heights, IL) and radiographic film exposure. The specificity of this antiserum for BabA during immunoblotting was confirmed previously.[33] For semiquantitation, 4, 2, and 0.5 μg of J99 strain protein extracts were transferred to each membrane, and the density of the band detected in the 4-μg sample from each strain was expressed numerically using the Image J 1.36 software from the National Institutes of Health (available at: http://rsbweb.nih.gov/ij/). We generated a standard curve using extracts from the J99 strain, and the density of bands detected in the samples from each strain was expressed as the percentage density relative to the J99 strain 4-μg sample. BabA status also was evaluated as the ability to bind to ^{125}I-labeled fucosylated blood group Le^b antigens using previously described methods.[3]

babA *Genotypes*

Genomic *H pylori* DNA was extracted using a commercially available DNA extraction kit (Qiagen Inc., Valencia, CA). *babA* status (*babA2* positive or negative) was determined by PCR using primer pairs and amplification conditions previously described (Figure 1).[5–22] Most of these primers were designed to detect the 10-bp signal sequence deletion of *babA1*, which is absent in *babA2*. Most previous studies used primer pair A (*babA2*S and *babA2*AS) (Table 1) in which 2 nucleotides of the forward primer were changed from the known sequences of the *babA2* gene from strain CCUG17875 (ie, AC to GT) (Figure 1). Other published primer pairs (denoted as primer pairs B–D) are modified from this original primer pair (Figure 1). A few studies have used a forward primer (*bab*7F) that is within the promoter region of the *babA* gene, a region that is identical to the sequence of *babA2* but different from that of *babA1* in the strain CCUG17875.[20–22] In these studies, they also used a unique reverse primer (*bab*7R) that is within the 5′ region of the *babA* gene, a region that is identical in both *babA1* and *babA2* in the strain CCUG17875 (primer pair E). In some experiments, PCR products were sequenced directly at Macrogen, Ltd. in Seoul, Korea.

Because *babA* status has been reported to be related closely to *cagA* status and *vacA* genotypes (s region) (Table 1), we also examined *cagA* status (*cagA* positive or negative) and *vacA* s genotypes (s1 or s2) using methods previously described.[34]

Presence of the babA *Gene*

By using primers designed by Pride et al,[35,36] we tested for the presence of the *babA* gene (both *babA1* and *babA2*). The presence of the *babA* gene was confirmed by Southern blotting using 1 μg of genomic DNA digested with either *Hind*III or *Ssp*I and a 542-bp *babA*-specific probe that was amplified from strain J99 using the following primers: 5′-GCTTACCCGCGCTCAAAG-3′ and 5′-CTCCGTGAAAGGGTTGAAAG-3′. The probe was labeled with horseradish-peroxidase and chemiluminescence was detected using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) and radiographic film exposure. Samples were scored as *babA*-gene positive if the PCR and/or Southern blot data yielded a positive result.

babA *Messenger RNA Expression*

Total *H pylori* RNA was extracted using a commercially available RNA extraction kit (Qiagen Inc.) and treated with DNase I (Roche Molecular Biochemical, Nutley, NJ). Primers specific for the 16S ribosomal RNA and *babA* genes were designed by using the Prime3 software (Whitehead Institute for Biomedical Research, Cambridge, MA) (available at: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). The sequences of the primers were as follows: 16S ribosomal RNA, 5′-CGTAAGGGCCATGATGACTT-3′ and 5′-CAGCTCGTGTCGTGAGATGT-3′, and *babA*, 5′-CACGATCAGTTCAAAAG-3′ and 5′-TTRATGAGCGTGCTCGCTTGCG-3′. Reverse transcription, complementary DNA amplification, and real-time PCR were performed using previously described methods,[25] with the exception that we used relative quantitative analysis with 16S ribosomal RNA levels as a control for the standardization of *babA* gene transcriptional activity. In our preliminary experiments, 16S ribosomal RNA levels were similar among the strains we used (data not shown).

Data Analysis

χ^2 tests were used for the univariate analysis of the relationship between clinical outcomes and bacterial factors. Mann–Whitney rank sum tests and Kruskal–Wallis tests with the Scheffe test were used for the univariate analysis of the relationship between histology and bacterial factors (*cagA* status/*vacA* s genotypes and BabA types, respectively).

A multiple logistic regression analysis was performed to determine which bacterial factor(s) was the most predictive of clinical outcome. Bacterial factors, age, sex, and country were used as explanatory variables. Multiple linear regression analyses were used for analyzing the histologic data. In these analyses, the bacterial factors, sex, age, country, and clinical outcomes were the explanatory variables, and the mutually adjusted associations with the criterion variables were calculated. A *P* value of less than .05 was accepted as statistically significant. Calculations were performed using the statistical software HALBAU (Gendai-sugaku-sha, Kyoto, Japan).

Results

Lewis b Antigen Binding Assay

We first evaluated the accuracy of assessing BabA status by immunoblotting and PCR analysis as compared with assessing Le^b binding activity. Eighty *H pylori* strains from our Colombian and Japanese *H pylori* stocks (40 strains from each country) were examined. Le^b binding activity was detectable in 68 (85%) of these strains (83% of the Colombian strains and 88% of the Japanese strains).

Comparison Between BabA Immunoblotting and Lewis b Antigen Binding Assay

Based on the results of immunoblotting analyses, the *H pylori* strains were divided into 2 major groups: BabA positive or BabA negative. The BabA-negative strains included 4 Colombian strains with no detectable BabA-specific band even after a long (60-min) exposure (Figure 2). Semiquantitative analyses of the BabA-positive strains allows this group of strains to be classified further into 2 distinct groups: those with high levels of BabA expression (68 strains that express BabA to levels more than 20% of that detected in the J99 strain) or those with low levels of BabA expression (8 strains that express BabA to levels less than 10% of that detected in the J99 strain). All of the strains that showed Le^b binding activity were high BabA producers. Both the low- and no-producer strains did not show Le^b binding activity. Based on this finding, we classified the strains into 3 distinct groups based on their expression levels of BabA: (1) BabA-high producers (BabA-H), which produce BabA protein to high enough levels to mediate Le^b binding, (2) BabA-low producers

(BabA-L), which produce a small amount of BabA but not enough to mediate Le^b binding, and (3) BabA-negative strains, which do not produce any BabA protein (Figure 2*A*). Five (10%) Japanese strains and 3 (6%) Colombian strains were classified as BabA-L strains.

Relation Between the babA *Gene and* babA *Messenger RNA Expression*

We tested for the presence of the *babA* gene using various PCR primer pairs located within the middle regions of the gene.[35,36] By using these primers, none of the 4 (5%) clinical isolates classified as BabA-negative contained a *babA* gene. We also examined the *babA* gene by PCR using the primer pairs A–E, which can distinguish between *babA2* and *babA1*. As expected, the primer pairs A–D did not yield any PCR products in the BabA-negative strains. Southern blot analyses confirmed that BabA-negative strains lacked the *babA* gene (data not shown). Interestingly, primer pair E resulted in specific amplification in 2 of the BabA-negative strains, and these products were confirmed by sequence analysis to be the *babB* gene (data not shown). Analysis of the sequence of primer pair E (*bab*7F and *bab*7R) revealed that they are homologous to the *babB* gene, with 29% of the 72 GenBank-deposited *babA* sequences being identical to that of the *bab*7R primer. Because the primer pair E can detect both *babA* and *babB*, we did not use this primer pair in subsequent experiments. By definition, *babA1* strains do not produce detectable BabA protein and therefore are categorized as BabA-negative strains, although they do possess a *babA* gene. None of the BabA-negative strains used in the present study contain a *babA* gene, suggesting that naturally occurring *babA1* sequences must be very rare. This hypothesis is supported by the fact that none of the GenBank-deposited *babA* sequences contain the reported 10-bp deletion. Therefore, assigning BabA status based on PCR techniques to detect the 10-bp deletion is not a reliable method.

We also performed real-time reverse-transcription PCR to analyze *babA* expression in all BabA-L strains, including the 26695 strain and 10 randomly selected BabA-H strains and the J99 and TN2GF4 strains (Figure 3). *babA* messenger RNA (mRNA) levels correlated with BabA protein levels as detected by immunoblot, confirming the prediction that BabA expression is regulated at the transcription level.

Sequencing of the Promoter and Signal Region of the babA *Gene*

Although all strains containing a *babA* gene are capable of expressing *babA* mRNA and producing BabA protein, some strains (BabA-L strains) do not express sufficient *babA* mRNA/BabA protein levels to mediate Le^b binding in vitro. We tested for possible differences between BabA-H and BabA-L strains using direct PCR-based sequencing of the promoter region and signal peptide regions of the *babA* gene using the following primers: *bab*7F and *babA2*AS or *babA2*R607.[5,18,20] We sequenced all 8 of the BabA-L strains and 12 randomly selected BabA-H strains.

BabA status has been reported to be regulated by the number of adenine [poly(A)] residues within the −10 to −35 region of the *babA* gene promoter.[24,25] This region is stable when the number of adenines was 14, but would become nonfunctional when the number was reduced to 10.[24] The number of adenine residues was 8 –14 in the strains we analyzed. However, we were unable to confirm any relationship between the number of adenine residues within the −10 to −35 region and BabA status (Figure 4). Although previous reports have suggested that chimeric *babA/B* or *babB/A* points were located close to the start codon,[24,25] we did not detect any chimeric formation within the region 600 bp from the start codon (data not shown).

Accuracy of Polymerase Chain Reaction Using Previously Published Primers for babA *Gene*

The earlier-described results suggest that previously used PCR methods that found the 10-bp difference between the *babA1* and *babA2* genes are unreliable for determining BabA status. By using Le^b binding ability as the gold standard for assigning BabA status, we performed PCR with the previously reported primer pairs and confirmed that these PCR methods had low sensitivity and specificity (Table 2). By definition, BabA-H and BabA-L strains contain *babA2* gene sequences within the signal peptide region. However, the primer pairs had very low sensitivity and could not even detect the *babA2* gene in the BabA-H and BabA-L strains (eg, only 45% [34 of 76] of the strains yielded a positive result using primer pair A). These data suggested there are sequence variations within these primer regions. Therefore, we examined the 137 GenBank-deposited sequences, including the sequence of the primer *babA2*AS (from primer pair A, B, and D) and 98 GenBank-deposited sequences, including the sequence of the primer *babA2*R607 (from primer pair C). Only 14 (10%) and 36 (37%) of the GenBank-deposited sequences were identical to the sequences of the *babA2*AS and *babA2*R607 primers, respectively. Importantly, 92 (67%) of the 137 deposited sequences differed from the sequence of primer *babA2*AS at 1 nucleotide from the 3′ annealing site, a site known to be critical for successful PCR amplification. These results provide further explanation as to why commonly used PCR methods have low sensitivity for assigning BabA status.

Relationship Between BabA Status and Clinical Presentation

We examined the relationship between the BabA-H, BabA-L, and BabA-negative strain classification, which is based on the presence or absence of detectable BabA protein, and in vitro Le^b binding activity, clinical presentation, gastric injury, and the presence of the virulence factors *cagA* and *vacA*. We analyzed BabA protein levels in 520 *H pylori* isolates (including 250 strains from Western countries [150 strains from Colombia, 100 from the United States] and 270 from East Asia [150 from Korea and 120 from Japan]) using immunoblotting.

As noted earlier, the BabA-H strains are defined as those strains that express 20% more BabA protein than the J99 strain, and the BabA-L strains are defined as those strains that express BabA protein to a level that is less than 10% of the level expressed in the J99 strain. BabA-negative strains are defined as those strains with no detectable BabA expression. The 10 strains (1.8%) that expressed BabA protein to levels between 10% and 20% of that in the J99 strain were regarded as strains with a borderline BabA status and were excluded from further analyses.

All strains from East Asia expressed BabA protein. Twenty-four (9.8%) of the Western strains were BabA-negative (Table 3). For these strains, the BabA-negative status was correlated inversely with *cagA* or *vacA* s status (ie, only 1 [4.2%] and none [0%] of these BabA-negative strains were *cagA*- or *vacA* s1-positive, respectively). Most (91%) Western strains were classified as either *cagA*-positive/*vacA* s1-positive/BabA-H (triple positive, 76%), *cagA*-positive/*vacA* s1-positive/BabA-L (6.1%), or *cagA*-negative/*vacA* s2-positive/BabA-negative strains (9.4%). We were unable to confirm the reported relationship between the triple positive strains and clinical outcome (Table 3).[5]

Both BabA-L and BabA-negative strains lacked Le^b binding ability in vitro and therefore were either functional or actual BabA-negative strains. However, these strains showed marked differences in their associations with gastric cancer and DU. Patients with BabA-L strains were twice as likely to develop these conditions as gastritis (Table 3). In contrast, BabA-negative strains typically (79%) were associated with gastritis only and their presence

was related inversely to the development of DU or gastric cancer (Table 3). Multiple logistic regression analyses confirmed that BabA-negative strains were related inversely to the development of DU or gastric cancer in Western countries (Table 4).

Relationship Between BabA Status and Gastric Mucosal Histology

Histologic analyses were performed in gastritis and DU cases. Twenty samples from Korea were not included in this analysis because there were 2 or fewer biopsy specimens available. Independent univariate analysis indicated that BabA status was related to gastric histology both in Western and East Asian countries (Table 5). Gastric mucosal damage and *H pylori* density was most severe in patients infected with BabA-L strains, less severe in patients infected with BabA-negative strains, and even less severe in patients infected with BabA-H strains. In East Asian countries, no BabA-negative strain samples were found, yet gastric mucosa damage and *H pylori* density were greater in patients with BabA-L strains than in those with the BabA-H strain. Backward stepwise multiple linear regression analysis confirmed these findings (Table 6).

Discussion

We show that PCR-based methods designed to detect a 10-bp deletion in the signal region of the *babA* gene do not reliably reflect BabA expression as determined by immunoblotting or Le^b binding activity. These results call into question the conclusions of previous studies using those techniques to relate BabA functional status and histology or clinical outcome.

BabA-negative status is associated with mild gastric injury and lower *H pylori* density. BabA-negative strains also are associated infrequently with DU or gastric cancer. However, because BabA-negative status is linked closely to *cagA*-negative/*vacA* s2 status, potential interactions between these different putative virulence factors cannot be ruled out. Although we did not measure OipA status in this study, we previously showed that *cagA*-positive status also is related closely to functional OipA status,[37] suggesting that less-virulent strains are BabA negative and CagA negative and contain *vacA* s2 and a nonfunctional OipA.

We identified a small class of strains (BabA-L) that were BabA-positive but produced low levels of the BabA protein and lacked Le^b binding activity. These strains are functionally BabA negative and typically are CagA positive. They are more likely to be associated with DU, gastric cancer, and increased mucosal inflammation and atrophy than BabA-positive strains that show in vitro Le^b binding activity (BabA-H strains) and BabA-negative strains. Interestingly, neither BabA-negative nor low-producing BabA strains showed Le^b binding activity, yet they differ markedly in their correlation with gastric injury. The finding that BabA-L strains lack Le^b binding activity suggests that either gastric mucosal injury is not dependent on Le^b binding activity or that in vitro binding activity does not accurately reflect in vivo conditions.

It remains unclear how BabA expression is regulated or if expressing low levels of BabA has a direct role in the pathogenesis of DU or gastric cancer. Although the quantitative analysis of BabA protein levels by immunoblotting has led to the identification of BabA-L strains that infrequently are associated with simple gastritis, the clinical importance of this finding is unclear. It is possible that BabA expression is influenced by the intragastric environment and that the phenotype of the BabA-L strains is a reflection rather than a cause of disease. It also is possible that BabA expression down-regulates the proinflammatory effects of other putative virulence factors, such as the *cag* pathogenicity island and OipA. Overall, these results suggest that in vitro Le^b binding activity does not reflect virulence accurately.

Recent evidence suggests that BabA status may be regulated by transcription[24] and/or by the formation of chimeric *babA–babB* genes.[24,25,36] We examined the prediction of Bäckström et al[24] that the *babA1* gene is transcriptionally silenced by 4 additional adenines between the −10 and −35 sites of the promoter. Such repetitive poly(A) sequences between the −10 and −35 sites also should make this region prone to slippage mutations, thereby possibly altering the transcription levels of downstream genes as well. Our sequence data and the fact that there were 11 adenines in control strain J99, which contains a functional BabA, do not support the hypothesis that differences in the promoter region influence the expression level of BabA. However, because we did not perform primer extension analyses, we are unable to make a definitive statement regarding whether the −10 to −35 spacing plays an important role in BabA expression.

It is possible that strong Le^b binding activity is associated with an enhanced immune response resulting in a severely inflamed mucosa. If so, the ability to change BabA status from a high producer to a low producer (ie, Le^b binding to Le^b nonbinding) would be advantageous for the organism. Solnick et al[25] reported the chimeric *babA/B* formation, which resulted in loss of BabA protein expression during experimental infection of rhesus monkeys with *H pylori* J166 strains that contained both the *babA* and *babB* genes. Those data suggested that, in rhesus monkeys, BabA-producing/Le^b binding activity might be disadvantageous and result in a survival advantage for an organism in which binding was switched off. Although we did not find chimeric *babA/B* or *babB/A* near the start codon in strains from human beings, it is possible that the mechanism(s) that might regulate BabA status in monkeys is similar to that in human beings. Our finding that a BabA-positive/Le^b nonbinding phenotype is associated with severe inflammation, DU, and gastric cancer is consistent with the hypothesis that BabA-L reflects an adaptation of *H pylori* that enhances survival in inflamed stomachs.

Finally, it should be noted that in a previous study we evaluated the relationship between clinical outcome (gastritis vs DU) and BabA status using immunoblotting with the anti-BabA antiserum AK253, which is directed against amino acid 55-725.[38] In the present study, we used the anti-BabA antiserum AK277 that is directed against recombinant BabA containing amino acid 123-432. Our preliminary data show that AK277 more accurately reflects Le^b binding activity than does AK253 (data not shown). Possibly, the use of the longer peptide fragments resulted in production of antibodies that are capable of cross-reacting with other *H pylori* outer-membrane proteins.

Acknowledgments

This report is based on work supported in part by grants from the National Institutes of Health (R01 DK62813 to Y.Y.), the Office of Research and Development, Medical Research Service Department of Veterans Affairs (to D.Y.G.), the Deutsche Forschungsgemeinschaft (project OD 21/1-1) (to S.O.), and a Public Health Service grant (DK56338) that funds the Texas Gulf Coast Digestive Diseases Center. O.O.O. was supported by a UNESCO BAC short-term fellowship and a Macarthur Foundation/University of Ibadan Staff Training Grant.

The authors thank Dr Oscar Gutierrez (Universidad Nacional de Colombia, Bogota, Colombia), the late Dr Jong G. Kim (Guru Hospital, Korea University College of Medicine, Seoul, Korea), Dr Tadashi Kodama and Dr Shoji Mitsufuji (Kyoto Prefectural University of Medicine, Kyoto, Japan), and Dr Masahiro Asaka and Dr Mototsugu Kato (Hokkaido University of Medicine, Sapporo, Japan) for providing clinical samples, and Dr Hala El-Zimaity (Baylor College of Medicine, Houston, TX) for histologic evaluations.

Abbreviations used in this paper

BabA	blood group antigen binding adhesin
BabA-H	BabA high producers

BabA-L	BabA low producers
bp	base pair
DU	duodenal ulcer
Le^b	Lewis b antigen
PCR	polymerase chain reaction

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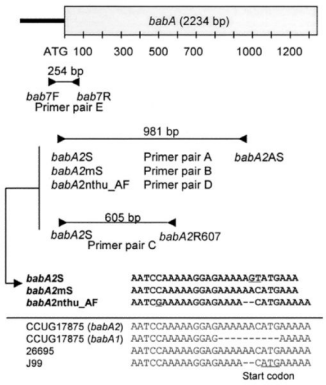

Figure 1.

Location of primer pairs used in this study. Most previous studies used primer pair A (*babA2*S and *babA2*AS); 2 nucleotides in the forward primer sequence were changed (AC to GT) from the GenBank sequence of the *babA2* gene from the strain CCUG17875. The forward primer *babA2*mS has the same sequence arrangement as the *babA2* gene from the strain CCUG17875. This primer was used in combination with the original reverse primer (primer pair B). *babA2*R607 is a newly designed reverse primer used in combination with the original forward primer (primer pair C). nthu_*babA2*F is a modified forward primer design based on GenBank sequence data from Taiwanese strains. It has 2 nucleotides deleted near the 3′ end of the original forward primer sequences. This primer was used in combination with the original reverse primer (primer pair D). The forward primer *bab*7F is located in the promoter region of the *babA* gene and was used in combination with a unique reverse primer from the 5′ region of the *babA* gene (*bab*7R) (primer pair E).

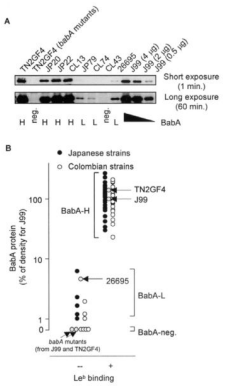

Figure 2.

Immunoblot analyses of BabA protein levels. (*A*) Semiquantitative analyses for BabA protein levels. For semiquantitation, 4, 2, and 0.5 μg of protein extracted from strain J99 was transferred to each membrane along with 4 μg of protein extracted from each experimental strain. The density of the detected band was expressed numerically using Image J 1.36 software (National Institutes of Health). A BabA band was not observed in strain JP79, strain CL74, or strain 26695 (upper panel) after short exposure, whereas the BabA band clearly appeared after longer exposure (lower panel). This result suggests that these strains produced the BabA protein (2.6%, 1.3%, and 6.1% relative to the amount of BabA produced in strain J99 [4 μg]). In contrast, the BabA band did not appear, even after long exposure, in the isogenic *babA* mutant strains TN2GF4 and strain CL43. Strain names beginning with JP signify strains from Japanese patients; CL signifies strains from Colombian patients. (*B*) Relationship between the density of BabA band (BabA protein levels) and Le^b binding activity. Three groups can be distinguished clearly based on BabA expression levels.

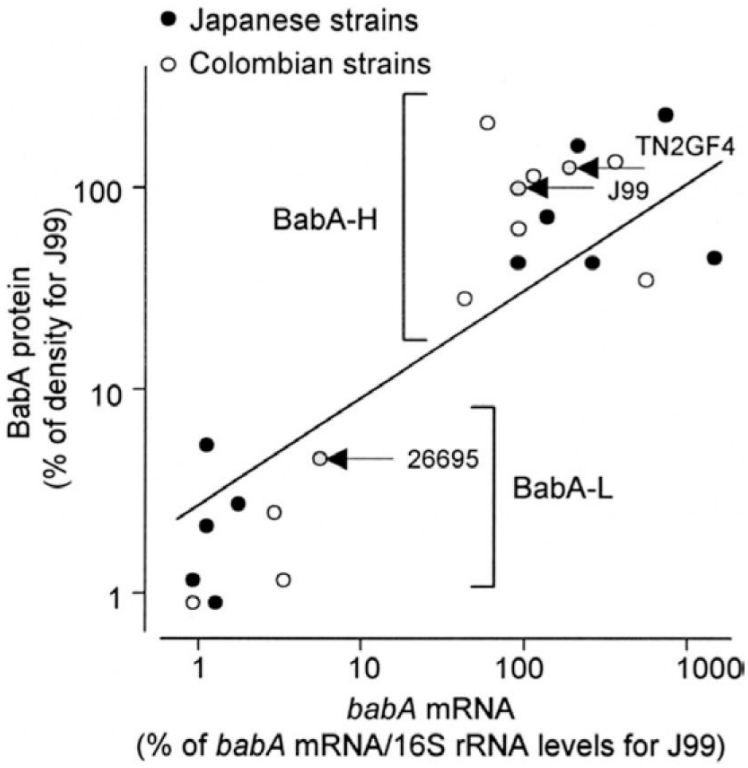

Figure 3.
Relationship between the density of BabA band (BabA protein levels) and *babA* mRNA levels. *babA* mRNA levels were measured using real-time reverse-transcription PCR, and BabA protein levels were measured using semiquantitative immunoblotting.

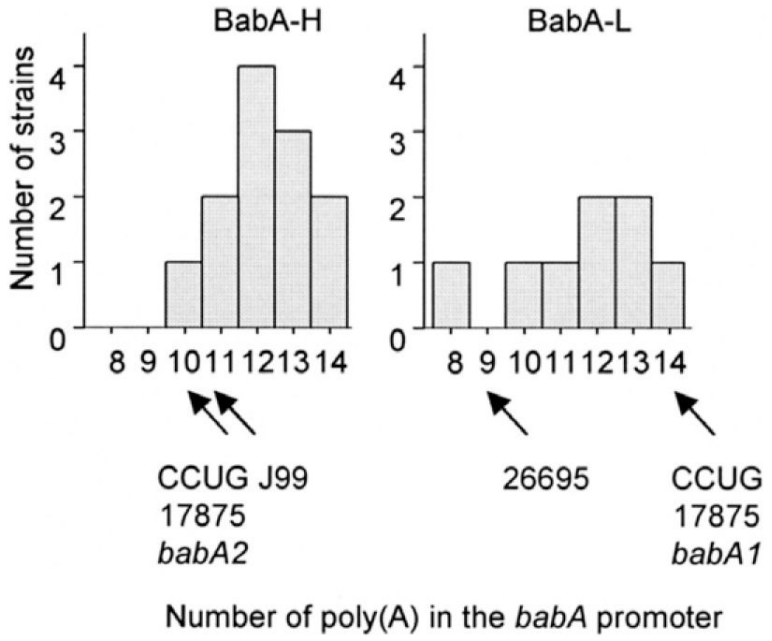

Figure 4.
Number of poly(A)s in the *babA* promoter relative to BabA protein levels. There was no correlation between the number of poly(A)s and BabA expression (eg, between BabA-H and BabA-L strains).

Table 1

PCR-Based Genotyping for the *babA2* Gene

Study	Year	Population	Primer pair	Prevalence of *babA2* gene: Total	Gastritis	PUD	Gastric cancer	MALT	Reflux esophagitis	Related to diseases	Related to *cagA/vacA*
Original strategy using primer pair A											
Western											
Gerhard et al[5]*a*	1999	Germany	A	114 (72%)	18 (51%)	23 (100%)	21 (78%)	20 (69%)		Yes	Yes
Prinz et al[6]*b,c*	2001	Germany	A	145 (39%)	57 (39%)						Yes
Oleastro et al[7]	2003	Portugal	A	140 (32%)	24 (23%)	21 (58%)				Yes	
Podzorski et al[8]	2003	United States	A	61 (36%)	22 (36%)						No
Oliveira et al[9]	2003	Brazil	A	208 (46%)	24 (32%)	43 (54%)	29 (56%)			Yes	Yes
Lehours et al[10]	2004	France	A	82 (49%)	21 (54%)			19 (44%)		No	Yes
Gatti et al[11]	2005	Brazil	A	89 (47%)	37 (53%)	3 (20%)	1 (100%)	1 (33%)		No	Yes
Olfat et al[12]	2005	Germany	A	92 (45%)	19 (28%)	22 (88%)				Yes	Yes
		Sweden	A	74 (45%)	21 (48%)	12 (40%)				No	Yes
		Portugal	A	91 (34%)	12 (20%)	19 (63%)				Yes	Yes
		Finland	A	57 (60%)	12 (46%)	22 (71%)				$P = .06$	Yes
		Western total		1153 (45%)	267 (37%)	165 (61%)	51 (64%)	40 (53%)			
East Asian											
Mizushima et al[13]	2001	Japan	A	179 (85%)	34 (81%)	73 (85%)	36 (90%)	9 (82%)		No	No
Yu et al[14]	2002	China	A	104 (80%)	83 (80%)						No
Han et al[15]	2004	China	A	141 (64%)	28 (65%)	50 (65%)	12 (57%)			Yes (GU vs DU)	No
		Asian total		325 (77%)	145 (77%)	123 (75%)	48 (79%)	9 (82%)			
Modified PCR primer pair from primer pair A											
Western											
Rad et al1[6]*b*	2002	Germany	B	141 (38%)	54 (38%)						Yes
Rad et al[17]*d*	2004	Germany	B	207 (35%)	73 (35%)						Yes
Zambon et al[18]	2003	Italy	C	167 (36%)	26 (28%)	20 (49%)				Yes	Yes
East Asian											

Study	Year	Population	Primer pair	Prevalence of *babA2* gene							
				Total	Gastritis	PUD	Gastric cancer	MALT	Reflux esophagitis	Related to diseases	Related to *cagA/vacA*
Lai et al[19]	2002	Taiwan	D	101 (100%)	41 (100%)	46 (100%)	14 (100%)			No	No
PCR primer pair using different strategy											
Western											
Mattar et al[20]	2005	Brazil	E	104 (69%)		104 (69%)					No
East Asian											
Sheu et al[21]	2003	Taiwan	E	188 (100%)	98 (100%)	90 (100%)				No	No
Lai et al[22]	2005	Taiwan	E	143 (100%)	90 (100%)				53 (100%)	No	No

PUD, peptic ulcer disease; MALT, mucosa-associated lymphoid tissue.

[a] Reverse-transcription PCR also was performed in this study, and the results were identical to those of PCR.

[b] A total of 88% had German nationality and 12% were from other European countries. The same patients were examined in both studies.

[c] Samples were examined from the antrum and the corpus, and the corpus data are presented (in the antrum, 55 were *babA2* positive).

[d] A total of 89% had German nationality and 11% were from other southern European countries.

Table 2

Comparison Among PCR, Immunoblot, and Le^b Binding for Evaluating BabA Status

	BabA-H	BabA-L	BabA negative		
***babA* gene**	+	+	+		
BabA protein	+	**weak**	**−**	**Lebbinding as gold standard**	
Le^b binding	+	**−**	**−**	**Sensitivity, %**	**Specificity, %**
Colombia and Japan	n = 68	n = 8	n = 4		
Primer pair A	29	5	0	43	58
Primer pair B	38	8	0	56	33
Primer pair C	55	8	0	81	33
Primer pair D	46	8	0	68	33
Primer pair A–D	61	8	0	90	33
Colombia	n = 33	n = 3	n = 4		
Primer pair A	12	0	0	36	100
Primer pair B	15	3	0	45	57
Primer pair C	26	3	0	79	57
Primer pair D	26	3	0	79	57
Primer pair A–D	30	3	0	91	57
Japan	n = 35	n = 5	n = 0		
Primer pair A	17	5	—	49	0
Primer pair B	23	5	—	66	0
Primer pair C	29	5	—	83	0
Primer pair D	20	5	—	57	0
Primer pair A–D	31	5	—	89	0

Table 3

Univariate Analysis of Relationship Between *H pylori* Typing and Clinical Outcomes

	Western countries (n = 244)			East Asia (n = 266)		
	Gastritis n = 98	**DU n = 97**	**Cancer n = 49**	**Gastritis n = 86**	**DU n = 90**	**Cancer n = 90**
BabA-H	75 (77%)	85 (88%)	41 (84%)	81 (94%)	80 (89%)	78 (93%)
BabA-L	4 (4.1%)	9 (9.3%)	6 (12%)	5 (5.8%)	10 (11%)	12 (13%)
BabA negative	19 (19%)	3 (3.1%)[a]	2 (4.1%)[b]	0	0	0
cagA positive	77 (79%)	85 (88%)	43 (88%)	82 (95%)	90 (100%)	88 (98%)
vacA s1	76 (78%)	88 (91%)[b]	43 (88%)	86 (100%)	90 (100%)	90 (100%)
cagA positive, s1, BabA-H	72 (73%)	76 (78%)	37 (76%)	77 (90%)	80 (89%)	76 (84%)
cagA positive, s1, BabA-L	3 (3.1%)	8 (8.2%)	4 (8.2%)	5 (5.8%)	10 (11%)	12 (13%)
cagA negative, s2, BabA negative	18 (18%)	3 (3.1%)[c]	2 (4.1%)[b]	0	0	0
Other types	5 (5.1%)	10 (10%)	6 (12%)	4 (4.7%)	0	2 (2.2%)

NOTE. Six samples from Western countries and 4 from East Asia had borderline results for BabA and were excluded. The *P* value was determined by the χ^2 test.

[a] $P < .001$ compared with gastritis.

[b] $P < .05$ compared with gastritis.

[c] $P < .01$ compared with gastritis.

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Table 4

Multiple Logistic Regression Analysis: *H pylori* Factors Associated With Clinical Presentation

	Factors	*P* value	Adjusted odds ratio	95% confidence interval
DU vs gastritis				
Western countries	BabA	.001	BabA-L vs -H, 2.6	0.8–8.9
			BabA-H vs negative, 19.8	2.8–137
			BabA-L vs negative, 54.8	6.4–468
East Asia	None	—	—	—
Gastric cancer vs gastritis				
Western countries	BabA	.021	BabA-L vs -H, 1.9	0.4–8.3
			BabA-H vs negative, 18.2	1.7–198
			BabA-L vs negative, 33.9	2.8–411
East Asia	None	—	—	—

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Table 5

Univariate Analysis of Relationship Between *H pylori* Genotyping and Histology

		Antrum			Corpus		
	n	***H pylori* density**	**Neutrophil infiltration**	**Atrophy**	***H pylori* density**	**Neutrophil infiltration**	**Atrophy**
Western countries							
BabA							
BabA-H	160	2.5 (2.7)	2.6 (2.5)	1.3 (0.8)	2.5 (2.7)	1.8 (1.9)	0.3 (0)
BabA-L	13	3.4 (3.5)	3.3 (3.0)	2.1 (2.0)	3.2 (3.3)	2.4 (2.0)	0.8 (0.5)
BabA negative	22	2.1 (2.3)	1.6 (1.4)	0.8 (0)	1.8 (2.0)	1.0 (0.8)	0.1 (0)
P value (L vs H)		<.001	.083	.002	<.001	.006	.004
P value (H vs negative)		NS	.053	.013	.029	.001	.004
P value (L vs negative)		<.001	.003	<.001	<.001	<.001	<.001
cagA							
Positive	162	2.6 (2.8)	2.7 (2.5)	1.4 (1.0)	2.6 (2.8)	1.9 (2.0)	0.4 (0)
Negative	33	2.3 (2.5)	2.0 (2.3)	0.8 (0)	2.0 (2.0)	1.2 (0.9)	0.1 (0)
P value		NS	.043	.007	<.001	.006	.099
vacA							
s1	164	2.6 (2.8)	2.7 (2.5)	1.3 (1.0)	2.6 (2.7)	1.9 (2.0)	0.3 (0)
s2	31	2.3 (2.5)	2.0 (2.4)	0.9 (0.5)	2.0 (2.0)	1.2 (1.0)	0.2 (0)
P value		NS	.034	.062	.004	.003	NS
East Asia							
BabA							
BabA-H	141	2.6 (2.5)	2.2 (2.0)	1.1 (1.0)	2.2 (2.0)	1.5 (1.5)	0.6 (0)
BabA-L	15	3.3 (3.0)	3.1 (3.0)	2.0 (2.0)	2.8 (3.0)	2.1 (2.0)	1.1 (1.0)
P value		.011	.008	.015	.029	NS	.091
cagA							
Positive	152	2.6 (3.0)	2.3 (2.0)	1.1 (1.0)	2.3 (2.0)	1.6 (1.5)	0.6 (1.0)
Negative	4	2.0 (1.5)	1.8 (1.5)	1.8 (2.0)	2.3 (1.5)	1.4 (1.8)	1.8 (0.5)
P value		NS	NS	NS	NS	NS	NS

NOTE. Analyses were performed by Mann–Whitney rank sum test (*cagA* status/*vacA* s genotypes) and Kruskal-Wallis test with Scheffe test (BabA types). Nine samples (5 from Western countries and 4 from East Asia) had borderline BabA status and were excluded. Twenty samples from Korea also were not included because the number of biopsy specimens was ≤2. For NS, $P > .10$. Data for *vacA* s region in Japan were not presented because all strains studied were *vacA* s1 genotype. For histologic scores (minimum 0, maximum 5), the means (median) are presented.

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Table 6

Final Model Using Multiple Linear Regression Analysis: *H pylori* Factors Associated With Histology

Pathology	Sites	Factors	Partial regression coefficient ± SE	*P* value	Multiple correlation coefficient
Western countries					
H pylori density	Antrum	BabA (L vs H)	0.77 ± 0.29	.022	0.35
		BabA (H vs negative)	0.12 ± 0.25		
		BabA (L vs negative)	0.89 ± 0.36		
	Corpus	*cagA* (positive vs negative)	0.62 ± 0.25	.013	0.43
		BabA (L vs H)	0.71 ± 0.20	.019	
		BabA (H vs negative)	−0.01 ± 0.31		
		BabA (L vs negative)	0.70 ± 0.37		
Neutrophil infiltration	Antrum	BabA (L vs H)	0.54 ± 0.35	.012	0.41
		BabA (H vs negative)	0.76 ± 0.30		
		BabA (L vs negative)	1.30 ± 0.45		
	Corpus	BabA (L vs H)	0.62 ± 0.31	<.001	0.27
		BabA (H vs negative)	0.84 ± 0.26		
		BabA (L vs negative)	1.46 ± 0.39		
Atrophy	Antrum	BabA (L vs H)	0.86 ± 0.33	.022	0.40
		BabA (H vs negative)	0.31 ± 0.41		
		BabA (L vs negative)	1.17 ± 0.49		
		cagA (positive vs negative)	0.69 ± 0.33	.040	
	Corpus	BabA (L vs H)	0.44 ± 0.18	.007	0.42
		BabA (H vs negative)	0.34 ± 0.25		
		BabA (L vs negative)	0.78 ± 0.27		
		cagA (positive vs negative)	0.52 ± 0.22	.106	
East Asia					
H pylori density	Antrum	BabA (L vs H)	0.68 ± 0.29	.023	0.32
	Corpus	BabA (L vs H)	0.53 ± 0.29	.067	0.38
Neutrophil infiltration	Antrum	BabA (L vs H)	0.85 ± 0.26	.001	0.58
	Corpus	BabA (L vs H)	0.61 ± 0.27	.029	0.40

Pathology	Sites	Factors	Partial regression coefficient ± SE	*P* value	Multiple correlation coefficient
Atrophy	Antrum	BabA (L vs H)	0.88 ± 0.32	.006	0.48
	Corpus	BabA (L vs H)	0.48 ± 0.22	.027	0.58

NOTE. In the analyses, the partial regression coefficient 0.77 of BabA (BabA-L vs BabA-H) for *H pylori* density can be interpreted as showing that the *H pylori* density score with BabA-L strains would be expected to be 0.77 points greater than with BabA-H strains.