Mixed Infections of *Helicobacter pylori* Isolated from Patients with Gastrointestinal Diseases in Taiwan

Chih-Ho Lai,1,2,3,4 Ju-Chun Huang,3,4 Chuan Chiang-Ni,1,2 Ju-Pi Li,3,6 Lii-Tzu Wu,3 Hua-Shan Wu,4,5 Yu-Chen Sun,7 Mei-Ling Lin,1,7 Ju-Fang Lee,1,7 and Hwai-Jeng Lin8,9

1Graduate Institute of Biomedical Sciences, Department of Microbiology and Immunology, College of Medicine, Chang Gung University, Taoyuan, Taiwan
2Molecular Infectious Disease Research Center, Department of Pediatrics, Chang Gung Children’s Hospital and Chang Gung Memorial Hospital, Taoyuan, Taiwan
3Graduate Institute of Basic Medical Science, School of Medicine, China Medical University, Taichung, Taiwan
4Department of Medical Research and Department of Laboratory Medicine, China Medical University and Hospital, Taichung, Taiwan
5Department of Nursing, Asia University, Taichung, Taiwan
6Rheumatology Research Center, China Medical University Hospital, Taichung, Taiwan
7Department of Laboratory Medicine, Chang Gung Memorial Hospital, Taoyuan, Taiwan
8Division of Gastroenterology and Hepatology, Department of Internal Medicine, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
9Division of Gastroenterology and Hepatology, Department of Internal Medicine, Shuang-Ho Hospital, New Taipei, Taiwan

Correspondence should be addressed to Chih-Ho Lai; chlai@mail.cgu.edu.tw and Hwai-Jeng Lin; buddhistlearning@gmail.com

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**Background.** Persistent *Helicobacter pylori* infection may induce several upper gastrointestinal diseases. Two major virulence factors of *H. pylori*, vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA), are thought to be associated with the severity of disease progression. The distribution of vacA and cag-pathogenicity island (cag-PAI) alleles varies in *H. pylori* isolated from patients in different geographic regions. *Aim.* To assess the association between mixed infection of *H. pylori* clinical isolates from Taiwanese patients and the severity of gastrointestinal diseases. *Methods.* A total of 70 patients were enrolled in this study. Six distinct and well-separated colonies were isolated from each patient and 420 colonies were analyzed to determine the genotypes of virulence genes. *Results.* The prevalence of mixed infections of all *H. pylori*-infected patients was 28.6% (20/70). The rate of mixed infections in patients with duodenal ulcer (47.6%) was much higher than that with other gastrointestinal diseases (*P* < 0.05). *Conclusions.* *H. pylori* mixed infections show high genetic diversity that may enhance bacterial adaptation to the hostile environment of the stomach and contribute to disease development.

**1. Introduction**

*Helicobacter pylori* is a gram-negative, spiral shaped microaerophilic bacterium that colonizes the human gastric mucosa throughout life [1]. Persistent *H. pylori* infection is associated with several gastrointestinal disorders, including chronic gastritis, peptic ulcer, lymphoid tissue lymphoma, and gastric adenocarcinoma [2]. It has been reported that *H. pylori* may select a particular niche on the mucosa where the bacteria can evade host immune responses by utilizing delicate strategies to manipulate immune cells as well as protect against antibiotic attack, leading to the progression of gastrointestinal diseases [3, 4].

Several virulence factors involving *H. pylori*-induced pathogenesis and the underlying mechanisms have led to different clinical sequelae [5–7]. Vacuolating cytotoxin (VacA), one of the major virulence factors secreted from *H. pylori*, has been detected in bacterial culture supernatants [8]. Upon
H. pylori colonization on cells, bacterial surface-contacted VacA is secreted directly from bacteria, followed by the intoxication of cells by vacuolation [9]. Previous studies reported that vacA was diversified among clinical H. pylori isolates, particularly in the region encoding the signal sequence (type s1 or s2) and the mid-region (type ml or m2) [10]. Additionally, the distribution of vacA alleles varies among different geographic regions [11–14].

Another virulence factor of H. pylori is the cag-pathogenicity island- (cag-PAI-) encoded type four secretion system, which mediates the translocation of cytotoxin-associated gene A (CagA) into host cells [15, 16]. Once translocated into cells, CagA is phosphorylated at one or more tyrosine phosphorylation motifs to induce cell pathogenesis [17]. Diversity within cag-PAI is found among people from Eastern and Western parts of the world [18]. Nearly all East Asian isolates carry cag-PAI, and one-half to two-thirds of the isolates from Western countries carry cag-PAI [19, 20]. Of note, cagA, cagE, and cagT were found to be present in 100% of the domestic strains isolated from patients in Taiwan [21]. These findings indicate that H. pylori isolates possess unusually high genetic heterogeneity and are diverse in different geographic regions.

H. pylori mixed infections have been found to involve more than one allele of either the s-region or m-region of vacA [22–24]. The rates of mixed infections may differ in cag-PAI of H. pylori isolated from the corpus and antrum [22] or there may be discrepancies in the antimicrobial susceptibility tests [25]. The rates of mixed infections vary from 0% to 85% in different populations worldwide [14, 22, 26–28]. However, the prevalence of H. pylori mixed infections isolated from patients in Taiwan remains unknown. In this study, we characterized six isolates from each patient using genotyping analysis. The association between mixed infections in H. pylori clinical isolates from Taiwanese patients and disease severity was assessed.

2. Materials and Methods

2.1. Patient Selection. From January 2011 to December 2014, a total of 70 patients with H. pylori infection were selected and diagnosed with upper gastrointestinal problems. Patients were excluded if they presented with any of the following: unwillingness to give written informed consent; bleeding tendency; and usage of H2-receptor antagonists or proton pump inhibitors within two weeks of enrollment [29]. H. pylori status was assessed by 13C urea breath test and bacterial culture was performed on biopsies before therapy [30]. Among the enrolled patients, there were 9 patients with chronic gastritis, 21 with duodenal ulcer, 22 with gastric ulcer, and 18 with gastric carcinoma. The severity of gastrointestinal disorders was evaluated using endoscopic examination and confirmed by histology as previously described [29]. All the patients had completed a self-administered questionnaire prior to being enrolled in the study. This study was approved by the Clinical Research Committee of Taipei Medical University, Taipei, Taiwan.

2.2. H. pylori Isolates and Bacterial Culture. Two biopsied specimens of each patient were taken: one specimen from antrum (lesser curvature side) and another from low body (greater curvature side). H. pylori isolates were cultured from the biopsies specimen and identified by biochemical reactions [24]. H. pylori were diagnosed with positive reaction in catalase, urease, and oxidase tests. The bacterial isolates were routinely cultured on Brucella agar plates (Becton Dickinson, Franklin Lakes, NJ) with appropriate antimicrobial agents as described previously [31].

2.3. Preparation of Genomic DNA and Polymerase Chain Reaction. After obtaining positive cultures from the biopsies, 6 isolated colonies from a single culture plate were examined for the genotypes using polymerase chain reaction (PCR) approach as described previously [29, 30, 32]. Briefly, the genomic DNA was extracted from the colonies by the sterile micropestle in guanidinium isothiocyanate, and the prepared DNA was dissolved in 10 mM Tris–HCl (pH 8.3). Two microliters of the eluted DNA was subjected to each PCR reaction. Twelve paired primers (Table 1) were then used to amplify specific DNA fragments. The PCR was performed under the following condition: 30 cycles at 94°C for 1 min, 50.9–63°C for 2 min, 72°C for 1 min, and final extension at 72°C for 5 min. Mixed infection was defined as distinct expression of cagA, cagE, cagT, cagM, and vacA s- or m-regions among the 6 isolates isolated from one host.

2.4. Statistical Analysis. The relationship of between-group comparisons was performed using the Chi-square test with Fisher’s exact test. A P value of less than 0.05 was considered significant.

3. Results

From January 2011 to December 2014, 70 patients diagnosed with upper gastrointestinal diseases and H. pylori-positive status (9 with chronic gastritis, 21 with duodenal ulcer, 22 with gastric ulcer, and 18 with gastric carcinoma) were enrolled in this study. From each patient, the biopsies were taken from the antrum and body of stomach. We obtained 6 colonies from pooled isolates in one culture plate, for a total of 420 isolates, to study the H. pylori genes. cag-PAI status and the s-region or m-region of vacA in H. pylori were assessed by PCR using unique primers. Mixed infection was defined as distinct expression of cagA, cagE, cagT, cagM, and vacA s- or m-regions among the 6 isolates in one host. The PCR distribution of positive/negative results for individual genes was shown in Table 2. All the isolates from single infection (n = 300) and mixed infection (n = 120) were positive for cagA. There were no major differences of the gene distributions between the two groups.

We then analyzed the association between mixed infections in H. pylori clinical isolates from patients and disease severity. As shown in Table 3, a total of 20 patients with H. pylori mixed infections, two patients (22.2%) with chronic gastritis, 10 patients (47.6%) with duodenal ulcer, 4 patients (18.2%) with gastric ulcer, and 4 patients (22.2%) with gastric carcinoma, had mixed infections. Additionally, patients with duodenal ulcer showed a higher prevalence of H. pylori mixed infection compared to that in other gastrointestinal diseases.
Table 1: PCR primers used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Nucleotide sequence (5'-3')</th>
<th>Length of PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>cagA</td>
<td>cagA-F</td>
<td>GATAACAGGCAAGCTTTTGAGG</td>
<td>349</td>
</tr>
<tr>
<td></td>
<td>cagA-R</td>
<td>CTGCCAAAAGATTTGTTCGAGA</td>
<td></td>
</tr>
<tr>
<td>cagE</td>
<td>cagE-F</td>
<td>GTTACATCAAAAAAAAAAGGGAAGG</td>
<td>735</td>
</tr>
<tr>
<td></td>
<td>cagE-R</td>
<td>CAATAATTTTGGAAGAGTTTCAAAGGC</td>
<td></td>
</tr>
<tr>
<td>cagT</td>
<td>cagT-F</td>
<td>TCTAAAAAGATTACGCTCATAGGCCG</td>
<td>490</td>
</tr>
<tr>
<td></td>
<td>cagT-R</td>
<td>CTTTGGCTTGCATGTTCAAGTTGCCC</td>
<td></td>
</tr>
<tr>
<td>cagM</td>
<td>cagM-F</td>
<td>ACAAATACAAAAAAGAAAAAGAGGC</td>
<td>587</td>
</tr>
<tr>
<td></td>
<td>cagM-R</td>
<td>ATTTTTCAACAAGTTAGAAAAGCC</td>
<td></td>
</tr>
<tr>
<td>s1a</td>
<td>VA1-F</td>
<td>AGCGACATACACAAACACACAG</td>
<td>259</td>
</tr>
<tr>
<td></td>
<td>VA1-R</td>
<td>CTGCCATGGAATGCGCCAACTTTATC</td>
<td>286</td>
</tr>
<tr>
<td>s1b</td>
<td>VA3-F</td>
<td>AGCGACATACACAAACACACAG</td>
<td>259</td>
</tr>
<tr>
<td></td>
<td>VA3-R</td>
<td>CTGCCATGGAATGCGCCAACTTTATC</td>
<td>286</td>
</tr>
<tr>
<td>s1c</td>
<td>VA4-F</td>
<td>AGCGACATACACAAACACACAG</td>
<td>259</td>
</tr>
<tr>
<td></td>
<td>VA4-R</td>
<td>CTGCCATGGAATGCGCCAACTTTATC</td>
<td>286</td>
</tr>
</tbody>
</table>

Table 2: PCR analysis for cag-PAI status and the s-region or m-region of vacA in single and mixed infections of H. pylori.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Single infection, ( n = 300 ) (%)</th>
<th>Mixed infection, ( n = 120 ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cagA</td>
<td>300 (100.0)</td>
<td>120 (100.0)</td>
</tr>
<tr>
<td>cagE</td>
<td>295 (98.3)</td>
<td>110 (91.7)</td>
</tr>
<tr>
<td>cagT</td>
<td>296 (98.7)</td>
<td>116 (96.7)</td>
</tr>
<tr>
<td>cagM</td>
<td>300 (100.0)</td>
<td>114 (95.0)</td>
</tr>
<tr>
<td>vacA</td>
<td>300 (100.0)</td>
<td>117 (97.5)</td>
</tr>
<tr>
<td>vacA</td>
<td>273 (91.0)</td>
<td>100 (83.3)</td>
</tr>
<tr>
<td>vacA</td>
<td>110 (36.7)</td>
<td>53 (44.2)</td>
</tr>
<tr>
<td>vacA</td>
<td>214 (71.3)</td>
<td>99 (82.5)</td>
</tr>
</tbody>
</table>

\( P < 0.05 \). In all studied subjects, mixed infections of H. pylori strains were found in 20 (28.6%) patients.

4. Discussion

H. pylori mixed infections were defined as having more than one allele of either the s-region or m-region of vacA or both iceA1 and iceA2 genotypes [22–24]. The rates of mixed infections were varied from 0% to 85% [14, 22, 26–28]. However, most studies used pooled cultures or biopsies for PCR, which may have yielded misleading results. Additionally, the sampling methods, including bacterial cultures from the sites of biopsies and analysis of the antimicrobial-resistant fractions or unselected strains, might have been attributed to the discrepant results [33]. In this study, we used 6 distinct colonies from each culture plate for genotyping of the cagA, cagE, cagT, cagM, and vacA s- or m-regions. We evaluated a large number of colonies (\( n = 420 \)) isolated from 70 patients. Moreover, 11 pairs of primers, including those for the genes cag-PAI and vacA s- or m-regions, were used. Therefore, we accurately and sensitively analyzed H. pylori mixed infections.

Mixed genotypes were found in 24% of patients who were Chinese residents of Hong Kong [33]. Another report indicated that the prevalence of mixed infections was 23.3% of all H. pylori-infected samples isolated from patients who lived in southern Taiwan [28]. In this study, we found that the prevalence of H. pylori mixed infections was 28.6% (20/70) in patients who were residents of northern Taiwan. This discrepancy in the prevalence of mixed infections in Chinese populations may be explained by the different analysis strategies and the fact that enrolled subjects were from different geographic regions [34, 35].

A high percentage of subjects (77%) carrying a mix of metronidazole-susceptible/resistant strains have been reported [33]. Mixed infections with metronidazole-resistant strains may not be eradicated by metronidazole-based therapy [36–38]. Additionally, patients with mixed infections in
the corpus showed a significantly higher rate of intestinal metaplasia in the antrum [28]. Our study showed that patients with duodenal ulcer have higher rates of mixed infections than of chronic gastritis. These findings support those of previous studies indicating that mixed infections facilitate interstrain gene transfer and genetic diversity, enhancing survival in the harsh environment of the stomach where disease progression occurs [39, 40].

In conclusion, our study reported that the prevalence of \textit{H. pylori} mixed infections was high in residents living in northern Taiwan and that the rates differed from those in other Chinese populations from other geographic regions. The mixed infections in \textit{H. pylori} with high genetic diversity may promote bacterial adaptation to the stomach and contribute to disease development.

### Competing Interests
All authors have no competing interests to declare.

### Authors’ Contributions
Chih-Ho Lai and Hwai-Jeng Lin were responsible for conception and design. Ju-Chun Huang, Chuan Chiang-Ni, and Ju-Pi Li were responsible for collection of samples. Lii-Tzu Wu, Hua-Shan Wu, and Yu-Chen Sun were responsible for experimental study. Yu-Chen Sun and Mei-Ling Lin were responsible for data analysis and interpretation. Chih-Ho Lai and Hwai-Jeng Lin were responsible for manuscript writing. All authors were responsible for final approval of manuscript.

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