**Mixed Infections of** *Helicobacter pylori* **Isolated from**

**Patients with Gastrointestinal Diseases in Taiwan, a three year project**

**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 underlyingmechanisms 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 frombacteria, 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 m1 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*-athogenicity

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 phosphorylationmotifs to induce cell pathogenesis [17]. Diversity within *cag*-PAI is found among people from Eastern andWestern 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 *vac*A [22–24].The rates ofmixed infectionsmay 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 frompatients 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 gastroenterological 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 biopsie 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 10mMTris-HCl (pH8.3). 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∘Cfor 1min, 50.9–63∘C for

2min, 72∘C for 1min, and final extension at 72∘C for 5min.

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.

Table 1: PCR primers used in this study.

Gene Primer Nucleotide sequence (5󸀠-3󸀠) Length of PCR product

*cag A* cagA-F GATAACAGGCAAGCTTTTGAGG 349

cagA-R CTGCAAAAGATTGTTTGGCAGA

*cag E* cagE-F GTTACATCAAAAATAAAAGGAAGCG 735

cagE-R CAATAATTTTGAAGAGTTTCAAAGC

*cag T* cagT-F TCTAAAAAGATTACGCTCATAGGCG 490

cagT-R CTTTGGCTTGCATGTTCAAGTTGCC

*cag M* cagM-F ACAAATACAAAAAAGAAAAAGAGGC 587

cagM-R ATTTTTCAACAAGTTAGAAAAAGCC

*s1 and s2* VA1-F ATGGAAATACAACAAACACACC 259

VA1-R CTGCTTGAATGCGCCAAACTTTATC 286

*s1a* SS1-F GTCAGCATCACACCGCAAC 190

*s1b* SS3-F AGCGCCATACCGCAAGAG 187

*s1c* S1C-F CTTGCTTTAGTTGGGTTA 213

*m1* VA3-F GGTCAAAATGCGGTCATGG 290

VA3-R CCATTGGTACCTGTAGAAAC

*m1T* m1T-F GGTCAAAATGCGGTCATGG 290

m1T-R CTCTTAGTGCCTAAAGAAACA

*m2* VA4-F GGAGCCCCAGGAAACATTG 352

VA4-R CATAACTAGCGCCTTGCAC

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