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# High frequency variations of *Helicobacter pylori* isolates in individual hosts in a Chinese population

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SUMMARY

*Background:* Colonization of individual hosts by multiple *Helicobacter pylori* genotypes may be one reason why this infection is persistent and difficult to eradicate.
*Methods:* In order to study the diversity of *H. pylori* in individuals, a modified randomly amplified polymorphic DNA (RAPD) method was applied using primary culture isolates instead of passaged cultures.
*Results:* The results showed that variations in *H. pylori* were prevalent among individuals in the Chinese population, and the incidence of multiple colonization was 99.1% (115/116), significantly higher than in other reports. Moreover, the number of RAPD genotypes was found to be significantly associated with the process of disease development ($p < 0.05$). Indeed, a trend for a higher number of RAPD genotypes within a single host (up to five genotypes) was observed as the disease developed or became more serious. After subculturing for three generations in our experiment, some genotypes present in the primary cultures were lost. The different genotypes in one patient may have originated from a single ancestral strain, as determined by analysis of six *H. pylori* housekeeping gene alleles, most of which were shown to be identical.
*Conclusions:* These results suggest that investigating isolates of the primary culture will better reflect the *H. pylori* diversity in individuals. Also, they indicate that continuous variation of one strain in the gastric microenvironment may be the main cause of *H. pylori* diversity in individuals in the Chinese population.


## 1. Introduction

The human gastric pathogen *Helicobacter pylori* is a Gram-negative proteobacterium, ubiquitous in large parts of the human population.[1] Its long-term colonization of the stomach causes different clinical outcomes, ranging from asymptomatic, to chronic gastritis, to peptic ulcers and cancer.[2–5] As a prerequisite for survival, the *H. pylori* population has followed an efficient adaptive evolutionary strategy. *H. pylori* is characterized by great genetic diversity and a panmictic population structure, as evidenced by an apparently unlimited number of unique strains that differ in genome size, gene order, genetic content, and allelic profile.[6–8]

*H. pylori* diversity in individual hosts has also been confirmed, which may be associated with the recurrence of infection and antibiotic resistance.[9] If so, then elucidating colonization pathways is vital for selecting novel targets to prevent the development of diseases caused by this organism. Several molecular biological methods have been used to analyze *H. pylori* diversity in individual hosts,[10–15] while the results of the incidence of multiple infections are inconsistent, ranging from approximately 10% to 67.5%.

In a previous study in our laboratory, several *H. pylori* isolates failed to survive in culture during the passage process (unpublished results). This led us to suspect the loss of some phenotypes during passage of cultures. In the present study, primary isolates were directly used to reflect *H. pylori* diversity in individual hosts, as opposed to culturing for generations.

The aim of this study was to determine the prevalence and incidence of *H. pylori* diversity in individual Chinese hosts. We analyzed genomic variations using randomly amplified polymorphic DNA (RAPD), which has been widely used to identify different bacterial genotypes among and within individual patients.[9,16–20] The possibility that genetic information is lost during the passaging of isolates was also examined. Furthermore, we sought to determine whether the phenotypic diversity reflected true multi-strain infection or whether it resulted from genotypic changes within one strain. Multilocus sequence analysis was used to investigate the relationships among different genotypes in each individual. The diversity of *H. pylori* in individuals might be an indicator of microevolution during chronic infection. The data from this study should provide an insight into the mechanism of *H. pylori* drug resistance and aid in the treatment of infections.

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**Table 1**
Background information of the patients

| Region of isolation | Gender | Disease | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|
| | | Dyspepsia | Gastritis | Gastric ulcer | Duodenal ulcer | Combined gastric and duodenal ulcer | Atrophy gastritis | Cancer | |
| Chongqing | Male | 1 | 8 | 11 | 7 | 0 | 1 | 3 | 31 |
| | Female | 1 | 6 | 11 | 7 | 1 | 0 | 1 | 27 |
| Hangzhou | Male | 3 | 11 | 7 | 8 | 1 | 2 | 1 | 33 |
| | Female | 0 | 10 | 4 | 5 | 3 | 2 | 1 | 25 |
| Total | | 5 | 35 | 33 | 27 | 5 | 5 | 6 | 116 |

## 2. Materials and methods

### 2.1. Patients

Clinical isolates of *H. pylori* were obtained in 2009–2010 from two areas of China, Chongqing and Hangzhou. No patient had received non-steroidal anti-inflammatory drugs or antacids, and none had recently (during the previous 6 months) been prescribed antibiotics. The patients comprised 64 males and 52 females, and their ages ranged from 25 to 80 years. Patient information is detailed in Table 1. The Chongqing patients were labeled C1–C58 and Hangzhou patients were labeled H1–H58. This study was performed in accordance with the Declaration of Helsinki. Informed consent was obtained from each patient prior to study entry.

### 2.2. Primary culture of H. pylori and DNA extraction from isolates

Biopsies were cultured at 37 °C for 5 days on brain–heart infusion (BHI) agar plates containing 5% rabbit blood under microaerobic conditions (5% $O_2$, 10% $CO_2$, 85% $N_2$) using gas packs (Thermo Fisher Scientific, Inc., Marietta, OH, USA). Twenty-four isolates per patient were randomly selected from primary cultures. *H. pylori* were identified on the basis of typical colony morphology, Gram straining, and a positive urease test. Chromosomal DNA was analyzed by colony PCR.[21] *H. pylori* were also identified using *Helicobacter* genus-specific 16S rDNA primers (446 bp; forward primer 5′-CTGGAGAGACTAAGCCCTCC-3′, reverse primer, 5′-AGGATCAAGGTTTAAGGATT-3′).[17] The PCR conditions were as follows: 30 cycles at 94 °C for 2 min, 38 °C for 2 min, 72 °C for 2 min, followed by a final incubation at 72 °C for 10 min. PCR products were electrophoresed in 1.5% agarose gels for comparison of banding patterns.

### 2.3. RAPD fingerprinting

All extracted DNA was quantified, and suspensions of equal DNA concentrations were prepared for RAPD analysis. To analyze the RAPD fingerprints, random primers 1254, 1281, and A11[12,22] were used for amplification. RAPD was carried out in a final volume of 25 μl containing 4 μl template DNA, 1 μl primer, 12.5 μl 2×Taq Plus PCR MasterMix polymerase (Tiangen Biotech, Shanghai, China), and 7.5 μl $ddH_2O$. A Gene Amp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA) was used for amplification. For primers 1254 and 1281, the PCR conditions were four cycles at 94 °C for 5 min, 36 °C for 5 min, 72 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min, and a final incubation at 72 °C for 10 min. For primer A11, the PCR conditions were 35 cycles at 94 °C for 2 min, 38 °C for 2 min, 72 °C for 1 min, followed by a final incubation at 72 °C for 10 min. PCR products were electrophoresed in 1.5% agarose gels for comparison of banding patterns. Marker I (Tiangen Biotech, Shanghai, China) was used as a size marker in all gels.

### 2.4. H. pylori passages and RAPD fingerprinting of subcultures

Samples from 20 patient cultures were subcultured for three generations as usual. The 24 colonies were analyzed after the third generation of subculture in each case. As a positive control, a single colony from strain 26695 was chosen. All isolates were frozen pending RAPD fingerprinting analysis. The RAPD fingerprinting results were then compared with those of primary cultures.

### 2.5. Multilocus sequence analysis of variation in housekeeping genes

The housekeeping genes *atpA*, *efp*, *ppa*, *trpC*, *ureI*, and *yphC* were selected for analysis in 30 samples (Table 2). PCR products were amplified with oligonucleotide primers designed as previously described (http://pubmlst.org/helicobacter/),[23] and were purified for sequencing using the QIAquick Gel Extraction Kit (Qiagen, Germany) in accordance with the manufacturer's instructions. The DNA sequencing was completed by TaKaRa Bio, Inc. (Otsu, Shiga, Japan). Sequences were assembled from the resultant chromatograms using DNAssist software (Gene Codes Corp., Ann Arbor, MI, USA).

### 2.6. Statistical analysis

Bands from the fingerprint analysis were denoted as variables. Fisher's exact test and the Chi-square test were used to assess the relationship between the number of genotypes and clinical disease. *p*-Values of less than 0.05 were considered statistically significant.

**Table 2**
Gene functions and PCR information for the housekeeping genes

| Gene | Gene function | Forward primer | Reverse primer | Fragment length (bp) |
|---|---|---|---|---|
| *atpA* | ATP synthase subunit alpha | 5′-GACTAGCGTTAAACGCACG-3′ | 5′-TTGAAACCGACAAGCCCAC-3′ | 627 |
| *efp* | Elongation factor P | 5′-CAATTTGGATGAGCGAGCTC-3′ | 5′-TTCACCTTTTCAAGATACTC-3′ | 410 |
| *trpC* | Bifunctional indole-3-glycerol phosphate synthase | 5′-TAGAATGCAAAAAAGCATCGCCCTC-3′ | 5′-TAAGCCCGCACACTTTATTTTCGCC-3′ | 456 |
| *ureI* | Urease accessory protein | 5′-AGGTTATTCGTAAGGTGCG-3′ | 5′-GTTTAAATCCCTTAGATTGCC-3′ | 585 |
| *ppa* | Inorganic pyrophosphatase | 5′-GGAGATTGCAATGAATTTAGA-3′ | 5′-GTGGGGTTAARATCGTTAAATTG-3′ | 398 |
| *yphC* | GTP-binding protein | 5′-CACGCCTATTTTTTTGACTAAAAAC-3′ | 5′-CATTYACCCTCCCAATGATGC-3′ | 510 |

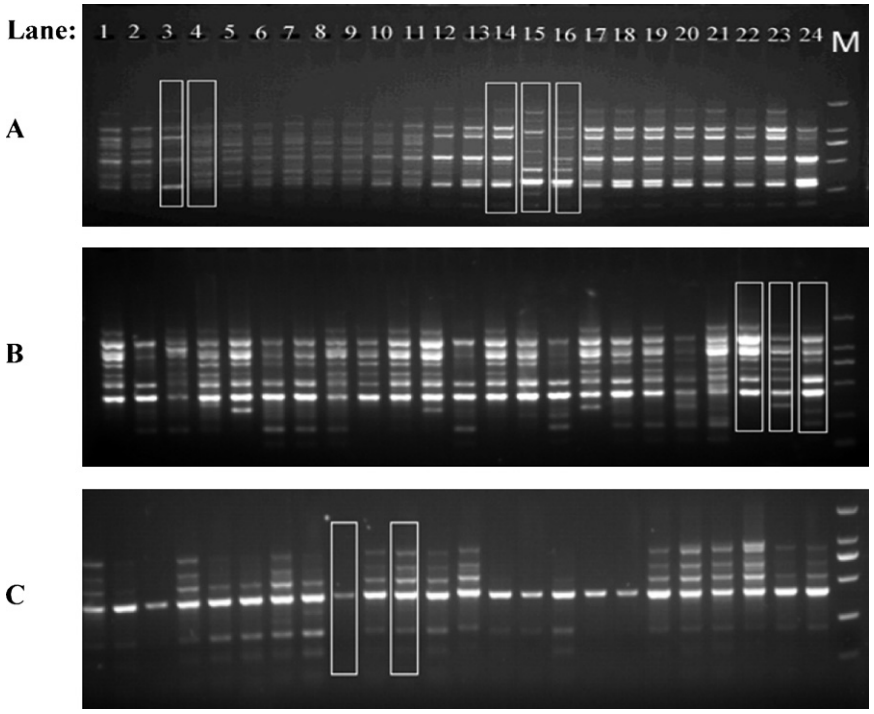


**Figure 1.** RAPD fingerprint patterns of *Helicobacter pylori* with different genotypes. Each lane represents an isolate except for the last lane (marker). (A) Patient C2 harbored five kinds of genotype (lanes 3, 4, 14, 15, and 16) with primer 1254. (B) Patient H9 had three kinds (lanes 22, 23, and 24) using primer 1281. (C) Patient C27 harbored two kinds (lanes 9 and 11) with primer A11.

## 3. Results

### 3.1. Many of the RAPD fingerprinting profiles of primary H. pylori isolates showed variety

RAPD analysis of *H. pylori* isolates from 116 patients showed considerable evidence of intra-patient genetic divergence. Most isolates from individual patients showed slight variations in banding patterns (Figure 1). Only one patient with gastritis had the same genotype fingerprinting using the three random primers in the RAPD analysis. The genotypes of 24 colonies from strain 26695 (control) did not differ from one another.

### 3.2. Relationship between RAPD profiles and clinical diseases

RAPD screening of 116 patients was initially conducted using primer 1254 (Table 3). Fingerprinting showed that the most common RAPD profile in individuals comprised three distinct genotypes. Genotype 1 only existed in patients with ulcers and gastritis. As 19 patients showed only a single genotype using this primer, primers 1281 and A11 were used for further analysis (Table 4). Overall, statistical analysis revealed a correlation between the clinical diseases and the numbers of RAPD genotypes ($p < 0.05$) (Table 5). As the diseases progressed, there was a tendency for the number of individual RAPD genotypes to increase, to a maximum of five. One genotype only existed in patients with gastritis. Two and three genotypes were mainly detected in patients with dyspepsia, gastritis, and gastric ulcers, which occurred in 100%, 91%, and 77% of patients, respectively. Four genotypes were identified mainly in patients with gastric ulcers, atrophic gastritis, and cancer, which occurred in 20%, 20%, and 17%, respectively. Five distinct genotypes were rarely detected and only in patients with severe cases of gastric ulcers, atrophic gastritis, and cancer, which occurred in 3%, 20%, and 33% of patients, respectively (Figure 2).

### 3.3. Genotypes lost during subculture passages

*H. pylori* colonies from 20 patients (10 with gastritis, two with dyspepsia, seven with peptic ulcers, and one with cancer) were cultured for over three generations. RAPD screening showed that some genotypes were lost from all samples during these passages. Only one genotype was detected in most (18/20) subcultured samples, whereas the remainder comprised two distinct geno-

**Table 3**
RAPD profiles according to primer 1254

| Disease | No. of genotypes | | | | | Total patients |
|---|---|---|---|---|---|---|
| | 1 genotype | 2 genotypes | 3 genotypes | 4 genotypes | 5 genotypes | |
| Dyspepsia | 0 | 3 | 2 | 0 | 0 | 5 |
| Gastritis | 5 | 11 | 17 | 2 | 0 | 35 |
| Gastric ulcer | 5 | 6 | 11 | 9 | 2 | 33 |
| Duodenal ulcer | 9 | 7 | 7 | 4 | 0 | 27 |
| Combined gastric and duodenal ulcer | 0 | 1 | 4 | 0 | 0 | 5 |
| Atrophy gastritis | 0 | 1 | 2 | 1 | 1 | 5 |
| Cancer | 0 | 3 | 0 | 1 | 2 | 6 |
| Total patients | 19 | 32 | 43 | 17 | 5 | 116 |

RAPD, randomly amplified polymorphic DNA.

**Table 4**
RAPD profiles according to primers 1281 and A11, in the 19 patients who harbored only one kind of genotype with the primer 1254

| Disease | Gastric ulcer | | | | | Duodenal ulcer | | | | | | | | | Gastritis | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| patient | C13 | C17 | C41 | H7 | H52 | C15 | C22 | C38 | C43 | C50 | H3 | H9 | H39 | H45 | C16 | C47 | C53 | H15 | H16 |
| No. of genotypes | 2 | 2 | 3 | 3 | 2 | 2 | 3 | 3 | 2 | 2 | 3 | 3 | 2 | 3 | 3 | 3 | 3 | 1 | 2 |

RAPD, randomly amplified polymorphic DNA.

**Table 5**
Distribution of the numbers of genotypes by 3 primers (1254, 1281 and A11) in different clinical gastric diseases

| Disease | No. of genotypes | | | | |
|---|---|---|---|---|---|
| | 1 genotype | 2 genotypes | 3 genotypes | 4 genotypes | 5 genotypes |
| Dyspepsia ($n=5$) | 0 | 3 | 2 | 0 | 0 |
| Gastritis ($n=35$) | 1 | 12 | 20 | 2 | 0 |
| Peptic ulcer ($n=65$) | 0 | 21 | 29 | 13 | 2 |
| Atrophy gastritis ($n=5$) | 0 | 1 | 2 | 1 | 1 |
| Cancer ($n=6$) | 0 | 3 | 0 | 1 | 2 |
| Total patients ($n=116$) | 1 | 40 | 53 | 17 | 5 |

RAPD, randomly amplified polymorphic DNA.
Fisher's exact test and the Chi-square test were used to assess the relationship between the number of genotypes and clinical disease; $r=0.527$, $p<0.05$.

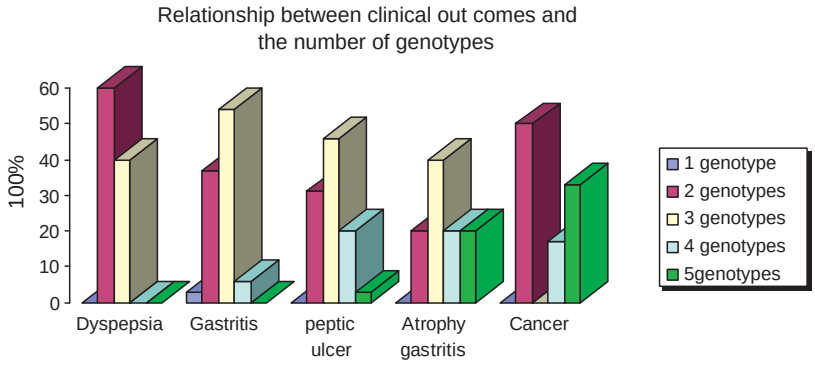


**Figure 2.** Relationship between clinical outcomes and the numbers of genotypes by RAPD analysis using the combined results of the three primers (1254, 1281, and A11).

types. Figure 3 compares the diversity of RAPD profiles from the primary isolates and subcultured colonies. Two RAPD genotypes had been lost during the passaging of cultures (Figure 3).

### 3.4. Multilocus sequence analysis

We applied multilocus sequencing to investigate variations in housekeeping genes among individuals with different RAPD profiles. Of 30 patients (17 with gastritis, 12 with peptic ulcers, and one with cancer), most of the sequencing results for six loci in the samples from each patient were essentially identical. Thus, isolates from the same patient may have contained the same housekeeping gene alleles, despite differences in RAPD genotypes. However, the *atpA* allele in three patients (two with duodenal ulcers and one with gastritis) showed slight variations, though there was no difference at the amino acid level. In particular, patient H17, who had both gastric and duodenal ulcers, harbored two alleles of *yphC*, which indicated meaningful amino acid changes. In one of them, the progress of translation was altered by a new terminator codon, due to a transversion (from UAT to UAG) (Figure 4).

## 4. Discussion

Multiple methods have been used to confirm that *H. pylori* exhibits significant diversity. Not only were polymorphisms found in the genomes of *H. pylori* from different patients, but also infection with several *H. pylori* strains was apparent in individual patients. Typically, *H. pylori* virulence markers have been studied in gastric biopsy specimens to identify *H. pylori* colonization of multiple genotypes,[14,15,18,19] which has also been found during drug resistance research.[9] However, these methods have limited value for investigating *H. pylori* diversity at the genome-wide level.

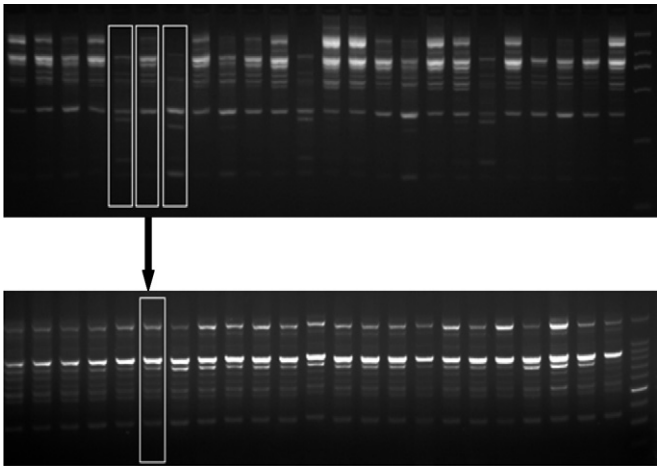

**Figure 3.** Two RAPD fingerprint patterns of *Helicobacter pylori* from the same patient (H26) using the primer 1254. Each lane represents a colony except for the last lane (marker). Three kinds of genotype were identified in the primary culture isolates (upper), while only one type of RAPD fingerprint pattern was found after three generations of subculture.

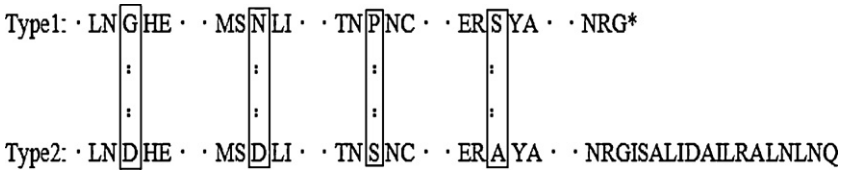


**Figure 4.** Two types of *yphC* alleles (type 1 and type 2) in patient H17 gave different amino acid translation results. *The transversion change (UAT to UAG) created a new terminator codon, which leads to loss of subsequent residues.

Microarray analysis has been widely used for such purposes, but it is expensive and information may be lost during subculturing.[11] In the present study, we focused on *H. pylori* diversity in individuals using RAPD fingerprinting, which revealed genetic changes in multiple loci caused by deletion, insertion, and mutation throughout the entire genome. Furthermore, *H. pylori* DNA samples from primary culture isolates were used for genetic analysis instead of passaged cultures, to avoid losses.

A series of primers have been used for RAPD fingerprinting, and we chose the three most frequently used random primers.[12,15,24] Initially, primer 1254 was used for RAPD screening, and the prevalence of *H. pylori* of multiple genotypes was 83.6% (97/116). Subsequently, using two further primers, A11 and 1281, only one strain was detected with a single genotype (Table 4). As a result, the rate of colonization with *H. pylori* of multiple genotypes was 99.1% (115/116), according to the combined RAPD typing. The RAPD profiles of the *H. pylori* isolates were identified as the same type using these three primers in only one patient; this strain came from a gastritis biopsy. The results therefore suggest that single *H. pylori* infections do occur, but they are very rare in the Chinese population (1/116). We determined that the combination of three primers was sufficient to characterize *H. pylori* diversity in individuals.

The indication of such a high prevalence of multiple *H. pylori* infection in China may be partly due to our improved method, i.e., extracting DNA from the primary cultured isolates rather than subsequent passages. In other related reports, bacteria were always cultivated for about three passages before DNA extraction.[12,15,24,25] However, we found that some genotypes were lost during culture passages (Figure 3). Losses of varying degrees may partly explain the inconsistency in the incidence of multiple infections. Therefore, the use of primary isolates was more practical and effective to investigate *H. pylori* diversity in individuals. Another reason may derive from the traditional customs of the Chinese population. China is a country with about 1.4 billion people, who have grown accustomed to living and eating together, as compared to Western populations; this may increase the risk of *H. pylori* transmission and infection. *H. pylori* strains can also adapt (or repair) by recombination with other strains or with colony variants of the same strain,[8] leading to greater genetic diversity in these bacteria. These findings imply that a diversity of *H. pylori* is prevalent among individuals in the Chinese population compared with reports involving Western populations.

In this study, our results showed a significant correlation between clinical diseases and RAPD profiles. The number of RAPD genotypes in individuals was associated with the process of disease development ($p < 0.05$). Typically, gastric diseases progress in a stepwise manner: gastritis, atrophy, metaplasia, and carcinoma.[26] In the present study, none of the gastritis or duodenal ulcer samples yielded five genotypes, whereas 6% of the gastric ulcer samples did so. In the gastric atrophy and cancer samples, this value rose to 20% and 33%, respectively (Table 3). These findings may infer that *H. pylori* colonization is inhibited when the gastric mucosa is atrophied, which in turn would make the intra- and inter-recombination of *H. pylori* strains more frequent. *H. pylori* can rapidly adapt and evolve in the stomach if multiple strains are present, which may lead to down-regulation of interactions with the host,[27] implying that the effects of the bacterium must be mediated relatively early in the carcinogenic process. Furthermore, *H. pylori* diversity in individuals may provide a new perspective for different clinico-pathological outcomes.

The diversity of multiple strain colonization must be considered to elucidate the resistance and recurrence of *H. pylori* infection. The diversity of *H. pylori* in individuals may arise due to genetic changes that affect the host immune responses. During long-term colonization, *H. pylori* produces effector proteins that stimulate the physico-chemical responses in the host that release mutagenic molecules, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), which increase the frequency of microbial mutants.[28] These findings raised the question of whether the strain diversity reflected actual multi-strain infection or whether it was the result of genotypic changes within one strain. In this study, multilocus sequencing methods were applied to address this quandary. There were few changes in the housekeeping genes in most individuals, which demonstrated that different genotypes may originate from a single ancestral strain. Our results are consistent with the conclusion of Colbeck et al.[29] that multiple minor variants of a strain may survive in an individual's stomach, each acting as a potential reservoir of genetic elements for their cohabitants, with the fittest dominant type being selected by environmental pressures.

Conversely, multiple (competing) strains are often present within a host, but cooperate through quorum sensing and recombination, among other processes.[27,30] Housekeeping gene analysis showed that an allele of the *yphC* locus contained amino acid substitutions in patient H17, whereas other alleles contained no variation. The presence of a premature stop codon may cause an 'off' phase variation just as it does in *oipA*.[28] The *yphC* gene encodes a GTP binding protein, which is involved in many activities such as cell communication, ribosome binding to the endoplasmic reticulum, and protein synthesis. Dysfunction of this protein could therefore impair many *H. pylori* activities, which could also explain the genotype losses during the progression of *H. pylori* culture passages. Some genetic modifications may influence the colonization and growth of *H. pylori* and eventually lead to a more consummate mechanism of microevolution.

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*Conflict of interest:* We declare that we have no financial or personal relationships with other people or organizations that could inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in this article.

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