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Heteroresistance of *Helicobacter pylori* from the same patient prior to antibiotic treatment

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ABSTRACT

Antibiotic resistance among *Helicobacter pylori* strains has been increasing worldwide and has affected the efficacy of current treatments. The aim of this study was to evaluate whether treatment failure was due to the presence of antibiotic-susceptible and -resistant *H. pylori* simultaneously within the same host before eradication. In order to discover *H. pylori* with antibiotic heteroresistance in the same patient, we examined the antibiotic susceptibility of *H. pylori* isolated from 412 patients without *H. pylori* eradication. The E-test was used to determine the minimal inhibitory concentration of these strains. The results showed 19 (4.6%) of patients harbored antibiotic heteroresistant *H. pylori*, resistant to levofloxacin (5/19), clarithromycin (1/19) and metronidazole (16/19). Among them, three patients' isolates showed heteroresistance to two antibiotics. The genetic diversity of each isolate was evaluated by random amplified polymorphic DNA PCR and the results showed that only 1 patient' isolate (5.3%) had a different pattern while the others showed identical or similar fingerprinting patterns. Mutations in the genes responsible for antibiotic resistance were investigated by direct sequencing and compared between strains within each pair. All 5 levofloxacin-resistant isolates had mutations in GyrA at the QRDR region (N87 or D91). Strain 1571R with clarithromycin resistance had a A2042G substitution in its 23S rRNA. There were 15 metronidazole-resistant strains (100%) with isogenic variation of RdxA, and 6 strains (40%) contained FrxA variation (excluded pair 1159). These results suggest that the treatment failure of heteroresistant *H. pylori* mostly develops from high genomic variation of pre-existing strains through long term evolution rather than mixed infection with different strains.

1. Introduction

Helicobacter pylori is a Gram-negative, spiral-shaped, and microaerophilic bacterium that infects 50% of the population worldwide (Pounder and Ng, 1995). Persistent infection with *H. pylori* increases the risk of developing gastroduodenal diseases, including peptic ulcer, duodenal ulcer and gastric adenocarcinoma (Ahmad et al., 2003; Graham et al., 1993; Marshall and Warren, 1984; Parsonnet et al., 1991; Rauws and Tytgat, 1990).

Although treatment regimens containing a proton pump inhibitor (ppi) and combination of two or more antibiotics (amoxicillin, clarithromycin, metronidazole or tetracycline) for 7–14 days are considered to be the most efficacious previously (Graham, 2000), successful eradication of *H. pylori* remains a challenge to clinicians due to resistance to the commonly used antibiotics for *H. pylori* eradication increasing worldwide (Chang et al., 2009; Graham and Fischbach, 2010; Megraud, 2007). Several alternative therapies, including concomitant and sequential therapy, are suggested to treat patients initially with PPI triple therapy failure or infected with clarithromycin-resistance strain (Graham and Fischbach, 2010; Lin et al., 2002; Vaira et al., 2007).

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Sequential therapy is a new treatment, instead of administering the antimicrobials all at once, they are administered in sequence. It begins with amoxicillin and a PPI followed by clarithromycin and metronidazole, again with a PPI or the four drugs prescribed concomitantly. Several studies in various countries have proven that concomitant and sequential therapies seem to maintain a high level of efficacy with eradication rates above 90%. (Gatta et al., 2009; Okada et al., 1999; Park et al., 2012; Vaira et al., 2007).

Resistance to quinolones is mainly due to mutations in the quinolone-resistance-determining region (QRDR) at codons 86, 87, 88 and 91 of the *gyrA* gene, coding for the A subunit of the DNA gyrase (Gerrits et al., 2006). Mutations in *gyrB* are also reported in quinolone-resistant strains, but often occurred together with *gyrA* mutations (Miyachi et al., 2006). The bacteriostatic activity of clarithromycin depends on its capacity to inhibit protein synthesis by binding to the 50S bacterial ribosomal subunit. Clarithromycin resistance is mainly due to point mutations in the 23S ribosomal RNA (rRNA) gene, and nucleotides A2142G and A2143G are the most frequent mutations (Taylor et al., 1997). Metronidazole resistance among *H. pylori* strains has been related to alterations in gene products having metronidazole nitroreductase activities, mainly including oxygen-insensitive NAD(P)H nitroreductase (RdxA) and NAD(P)H flavin oxidoreductase (FrxA) (Aldana et al., 2005; Jenks et al., 1999; Jeong and Berg, 2000; Jeong et al., 2000).

Although *H. pylori* from individual patients typically have either an antibiotic-susceptible or -resistant phenotype, both antibiotic-susceptible and -resistant *H. pylori* can be present simultaneously (Arents et al., 2001; van der Ende et al., 2001). Heteroresistance can represent infection with a single strain or mixed-infection with several different *H. pylori* strains (Arents et al., 2001; Kwon et al., 2001; Lee et al., 2005; Matteo et al., 2006; van der Ende et al., 2001). Kennemann et al. indicated *H. pylori* genomic evolution during infection shows genome-wide recombination in *H. pylori* colonizing humans, especially with a high rate of mixed infections (Kennemann et al., 2011). These results suggest that continuing genomic diversities through long-term evolution in the same strain may play an important role in modulating antibiotic heteroresistant *H. pylori in vivo* and thus lead to treatment failure. In this study, we collected 19 pairs of *H. pylori* containing heteroresistant phenotypes from separate patients before *H. pylori* eradication and further investigated the mechanisms leading to antibiotic resistance.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The information on bacterial strains, antibiotic susceptibility and the clinical diseases of patients in this study are described in Table 2. Nineteen heteroresistant *H. pylori* pairs were identified from 412 individual patients enrolled in clinical trials at the National Cheng Kung University Hospital, Taiwan. No patients had received *H. pylori* eradication therapy before this study. *H. pylori* strains initially harvested as multiple colonies from individual gastric mucosal biopsy specimens were cultured on CDC anaerobic blood agar (BBL, Microbiology Systems, Cockeysville, MD) under microaerophilic conditions. All strains were stored at −80 °C in Brain–Heart Infusion (BHI) broth containing 30% glycerol until testing.

2.2. In vitro antibiotic MIC test

The MIC values of each clinical isolate consecutively collected to four antibiotics (amoxicillin, clarithromycin, metronidazole and levofloxacin) were determined by Epsilometer test (E-test) (AB Biodisk, Solna, Sweden) as described previously (Chang et al., 2009; Hung et al., 2009). In brief, these *H. pylori* isolates were suspended in 0.85% NaCl solution and adjusted to a 2.0 McFarland standard. A blood agar plate was streaked in three directions with a swab dipped into each bacterial suspension to produce a lawn of growth, an E-test strip was placed onto each separate plate, which was immediately incubated in a microaerophilic condition at 37 °C for 72 h. Then the MIC was interpreted according to the manufacturer's instructions. *H. pylori* American Type Culture Collection (ATCC) J99 was used as the quality control strain. Using recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), resistance to levofloxacin, metronidazole, clarithromycin and amoxicillin was defined as MIC > 1 μg/mL, >8 μg/mL, MIC > 0.5 μg/mL, and MIC > 0.12 μg/mL, respectively. The MIC values of nineteen heteroresistant *H. pylori* pairs were further confirmed by the agar dilution method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2010).

2.3. Random amplified polymorphic DNA (RAPD)–PCR amplification

Mini Qiagen columns and a QiaAmp DNA extraction kit (Qiagen, Valencia, CA) were used for chromosomal DNA extraction. Three different primers for RAPD–PCR analysis used in this study are listed in Table 1. The RAPD–PCR reaction conditions were described previously with modification (Akopyanz et al., 1992; Sheu et al., 2009). In brief, the PCR mixtures were made in a volume of 50 μL containing 100 ng of DNA, 10 pmol of primer, 0.15 mM each deoxynucleoside triphosphate, reaction buffer with $MgCl_2$, and 1 U of Taq DNA polymerase (Promega, Madison, WI). PCR was carried out in a Perkin–Elmer 2720 thermal cycler (Perkin–Elmer; Applied Biosystems, Foster City, CA, USA) through 8 cycles of low-stringency amplification and 35 cycles of high-stringency amplification. The PCR products (15 μL) were electrophoretically separated in 1% agarose gels. The classes of fingerprint patterns were (i) highly homogeneous RAPD banding patterns in the isolates of one patient were defined as single strain infection, (ii) more than one different RAPD banding pattern was defined as mixed-infection by different strains (Sheu et al., 2009).

2.4. Gene mutation analysis by direct sequencing

PCR was carried out according to the manufacturer's instructions using Taq polymerase (Promega). Primers used in this study are described in Table 1. Using these primers, a 428 base pair fragment containing the quinolone resistance-determining region of *gyrA* gene was amplified. Reaction conditions were an initial 5 min at 95 °C, and 45 s at 95 °C, 45 s at 55 °C, and 45 s at 72 °C for 26 cycles, followed by a final extension for 10 min at 72 °C.

Table 1
Oligonucleotide primers used in this study.

Primer	Sequence (5′-3′)	Reference
PCR-Sequencing		
gyrA-1	TTTAGCTTATTCAATGAGCGT	Tankovic et al. (2003)
gyrA-2	GCAGACGGCTTGGTAGAATA	
gyrB-1	TGCAAAAGCCAGAGAAGCCA	Tankovic et al. (2003)
gyrB-2	ACATGCCCTTGTTCAATCAGC	
rdxA-1	GCAGGAGCATCAGATAGTTCT	Jeong and Berg (2000)
rdxA-2	GGGATTTTATTGTATGCTACAA	
23s rRNA-1	ATGGGAGCTGTCTCAACCAG	This study
23s rRNA-2	TGTCCTGCCTGTGGATAACA	
50bf-frxA-Fw	ATTGGATATGGCAGCCGTTTATCA	This study
frxA-3-Rv	AGCGTTTTTATTCAATCACTTCATAA	
p5-1	CGAATTGGATATGGCAGCCG	Marais et al. (2003)
RAPD–PCR		
226	AAGAGCCCGT	Akopyanz et al. (1992)
227	NNNAACAGCTATGACCATG	Akopyanz et al. (1992)
228	GAGCGGCCAAAGGGAGCAGAC	Akopyanz et al. (1992)