Dynamic changes in Antibiotic Resistance Genes and Gut Microbiota after H. Pylori Eradication Therapies

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Research

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Abstract

Background: Short-term antibiotics exposure is associated with alterations in microbiota and antibiotic resistance genes (ARGs) in the human gut. While antibiotics are critical in the successful eradication of *Helicobacter pylori*, the short-term and long-term impacts on the composition and quantity of antibiotics resistance genes after *H. pylori* eradication is unclear. This study used whole genome shotgun metagenomic of stool samples to characterize the gut microbiota and ARGs, before and after *H. pylori* eradication therapy.

Results: Forty-four *H. pylori*-infected patients were recruited including 21 treatment naïve patients who received clarithromycin-based triple therapy (CLA group) and 23 patients who failed previous therapies, in which 10 received levofloxacin-based quadruple therapy [LEVO group] and 13 received other combinations [OTHER group] in the current study. Stool samples were collected at baseline (before current treatment), 6-week and 6-month after eradication therapy. At baseline, there was only a slight difference among the three groups on ARGs and gut microbiota. After eradication therapy, there was a transient but significant increase in gut ARGs 6-week post-therapy, among which the LEVO group had the most significant ARGs alteration compared to other two groups. For treatment naïve patients, those with higher ARG richness and *ErmF* abundance were prone to fail CLA eradication. For gut microbiota, the bacteria richness decreased at 6-week and there was a significant difference in microbiota community among the three groups at 6-week.

Conclusions: Our findings demonstrated the dynamic alterations in gut microbiota and ARGs induced by different eradication therapies, which could influence the choices of antibiotics in eradication therapy.

Background

Successful eradication of *H. pylori* by the combination of antibiotics lowers the subsequent risk of gastric cancer and peptic ulcer disease [1, 2]. However, due to the widespread use of antibiotics, antibiotic resistance and emergence of drug-resistance bacteria has become one of the largest global health threats [3]. In particular, increasing antibiotic resistance to clarithromycin has reduced the effectiveness of standard clarithromycin-based triple therapy (STT) in *H. pylori* eradication therapy [4]. Hence, bismuth quadruple or non-bismuth quadruple, concomitant therapies with three antibiotics have been recommended in areas with background prevalence of clarithromycin resistance higher than 15% according to the Maastricht V/Florence Report, Toronto Consensus, and American College of Gastroenterology (AGG) [5–7]. In patients who have failed first-line eradication therapy, alternative therapies such as levofloxacin-containing and other combinations, including bismuth or rifabutin, are suggested.

However, a growing body of evidence suggests that even a short course of antibiotics may lead to disruption of the balance in the gut microbiota, or dysbiosis [8–11]. It is increasingly recognized that altered gut microbiome could be associated with various gastrointestinal diseases, metabolomic

disorders like diabetes mellitus and even central nervous systems (CNS) disorders[12–14]. With the wide availability of advanced DNA sequencing technologies, whole genome sequencing (WGS) could provide a deeper analysis of microbial diversity[15–17]. However, most studies on the consequences of *H. pylori* eradication on gut microbiota are based on the 16S rRNA metagenomic analysis, which was less effective in resolving microbial species.

Metagenomic studies have shown that antibiotic resistance genes (ARGs) are widespread in natural environments and the human gut serves as one of the major reservoirs[18–20]. Further analysis showed that the ARGs in human gut microbiota could differ across populations[21]. While macrolide resistance genes could overrepresent after short-term clarithromycin exposure[22], various antibiotics may exert diverse effects on microbiota composition and even antibiotic resistance[23]. It remains unclear how ARG composition changes as a result of *H. pylori* eradication therapies.

To address these questions, we have conducted shotgun metagenomic sequencing to investigate the impacts of short-term antibiotics exposure related to different *H. pylori* eradication therapies, including patients with prior treatment failures, on gut antibiotic resistance genes (ARGs) alteration. Moreover, we determined the dynamic changes in gut microbiota composition after different *H. pylori* eradication regimes.

Methods

Patient and HP eradication therapies

This was a prospective study including adult patients (> 18 years), who were diagnosed to have active *H. pylori* infection, including both treatment naïve and previous treatment failure. Patients were recruited in the Ulcer Clinic of the Queen Mary Hospital of Hong Kong. As a routine clinical practice, *H. pylori*-infected and treatment naïve patients were given conventional clarithromycin-containing triple therapy (CLA group; esomeprazole 20mg, amoxicillin 1g, clarithromycin 500mg, all given twice a day) for at least one week. For patients who had failed previous *H. pylori* eradication therapies, they were given either levofloxacin-containing therapy (LEVO group; esomeprazole 20mg twice a day, levofloxacin 750mg daily, tetracycline 500mg three times a day, and metronidazole 400mg three times a day for at least one week) or other combinations (OTHER group; esomeprazole 20mg twice a day, amoxicillin 1g twice a day and rifabutin 600mg daily for 2 weeks; esomeprazole 20mg twice a day, bismuth 262mg three times a day, metronidazole 400mg three times a day and tetracycline 500mg three times a day for at least 10 days; esomeprazole 20mg twice a day, moxifloxacin 400mg daily, nitrofurantoin 100mg three times a day and ursodeoxycholic acid 750mg daily for one week) depending on their previous treatment regimens.

Fecal sample collection, library preparation, and sequencing

Fecal samples were collected from patients at three different time points. First stool samples were collected before current *H. pylori* eradication therapy, whereas second stool samples were collected 6 weeks after the completion of anti-*H. pylori* therapy. A follow-up urea breath test (C¹³-UBT) was arranged

to document treatment outcome at 6-week. A third stool sample was collected 6 months after the eradication therapy. All fecal samples were stored in the OMNIgene-Gut collection kit (DNA Genotek, Ottawa, Canada) before transferred for storage at -20°C. Library preparation was conducted according to the protocol of the HAPA Hyper Prep Kit (KR0961-V1.14), which had been recently verified in our pilot study to have the best yield for ARG detection [24]. Subsequently, metagenomic sequencing was performed on the Illumina NovaSeq 6000 System (Illumina, USA; Paired-end; read length, 2 x 150 bp) at the Centre of PanorOmic Science (CPOS) of the University of Hong Kong.

Metagenomic data pre-processing

FastQC v0.11.9 was used for quality control of the sequencing data. Low-quality reads were removed, and low-quality bases at the 3' or 5' end of the reads were trimmed. Human sequence contaminations were filtered by BBDuk v38.86[25] with parameter `minid` (minimum alignment identity) = 0.95. After quality control and filtering, each sample contains an average of 54.2 million reads (range from 43.1 million to 68.4 million). MEGAHIT v1.2.1[26] was used to assemble the raw reads into contigs before ARG screening. An average of 129,276 contigs were obtained per sample, which had an average N50 length of 9,589 bp ranging from 1,944 to 74,745 bp.

ARG identification and abundance normalization

A command-line version of RGI v5.1.1 (The Resistance Gene Identifier) [27] together with the latest reference database were downloaded from the Comprehensive Antibiotic Resistance Database [28] (CARD) for identifying ARGs from contigs using default parameters. RGI predicted open reading frames (ORFs) from input contigs using Prodigal, and then the translated protein sequences from these identified ORFs were aligned to the known ARGs reference sequences in the CARD. RGI reported ARGs under three criteria: Perfect (totally matched to the reference sequences or mutations in the CARD), Strict (more flexible, to ensure the detection of a functional ARG), and Loose (outside cut-offs or partial hits for the detection of potential novel ARG). In this study, Perfect and Strict ARGs with identity higher than 90% were considered as detected unique ARGs. The number of ARGs detected in each sample was the number of unique ARG names generated by RGI output. The drug class, ARG subtypes, and resistance mechanisms were identified according to the antibiotic resistance ontology classification provided by the CARD.

The abundance of ARGs was represented by RPKM (reads per kilobase of reference sequence per million sample reads). For each sample, all reads are mapped to the reference protein sequences of reported ARGs by ShortBRED [29]. Read alignment software USEARCH [30] was called by ShortBRED with pair-end reads treated as separated reads. More specifically, `usearch_local` command is used with 95% identity and 95% read length coverage (by nucleotide length). ShortBred normalizes the number of reads mapped to each ARG by the total number of reads in the sample and the length of the reference sequences (all lengths are calculated based on nucleotide), and report RPKM value for each ARG in each sample. The sum of the abundance (RPKM) of each ARG resistant to the same class of drug was defined as the ARG

class's relative abundance. The number of changed ARGs was defined as the difference between the number of observed unique ARGs between two time points.

Microbiota taxonomic profiling and diversity analysis

Taxonomic profiling of the microbiome was performed on qualified FASTQ files using MetaPhlAn2.0 [31], which mapped raw reads to the database for the detection of the presence and read coverage of clade-specific makers and estimate the relative abundance. The relative abundance of taxonomic levels of species, genera, and phylum was extracted from MetaPhlAn2.0 output for downstream microbiota analysis. Alpha diversity estimated the richness and evenness of each sample while the beta diversity estimated the microbiome dissimilarity between samples. The alpha diversity indices (Shannon index, evenness) in each sample was calculated based on the relative abundance of each species using the vegan package in R [32]. Species richness was defined as the number of species detected in each sample. For estimation of microbiota community (beta diversity), Bray-Curtis distance was calculated based on phyloseq [33] package in R based on the relative abundance of species level.

Statistical analysis

All statistical analysis was performed in the R software v4.0.3 or GraphPad Prism 9.0 unless otherwise stated. Baseline characteristics were expressed as mean $\pm$ SEM for continuous data and n/N (%) for proportional data. For the comparison of change of observed number of ARGs, Student's *t*-tests were applied. For taxonomic profiling and potential ARGs, the Wilcoxon signed-rank test or Mann-Whitney U test was used for paired and unpaired samples respectively for metagenomic data, and the Benjamini-Hochberg procedure [34] was used to decrease the false discovery rate (FDR) when multiple comparisons were applied. Principal coordinates analysis (PCoA) of beta-diversity of gut microbiota between or within groups was visualized based on Bray-Curtis distance matrices. Permutational multivariate analysis of variance (PERMANOVA) was then performed to compare microbiota community dissimilarity using analysis of variance using distance matrices (ADONIS) for 999 permutations.

Results

Patient's characteristics

A total of 44 *H. pylori*-infected patients were enrolled in the study, including 21 patients in the CLA group, 10 patients in the LEVO group, and 13 patients in the OTHER group. The average number of prior failed eradication therapies in the CLA, LEVO, and OTHER group was 0, 1.1, and 2.5, respectively. Other baseline characteristics were similar among the three groups (**Table S1**). A total of 121 stool samples were collected. All patients completed the first (baseline) and second (6-week post-therapy) stool sample collection, while 14 patients (66.7%) in the CLA group, 8 patients (80%) in the LEVO group, and 11 patients (84.6%) in the OTHER group had completed the third (6-month post-therapy) stool sample collection.

Baseline ARG differences

At baseline, the abundance of *tetM* ($p = 0.005$) was more abundant in the LEVO group when compared with the CLA group. In the OTHER group, the relative abundance of *ErmF* ($p = 0.015$) was more abundant, while the abundance of *tetA(46)* ($p = 0.03$) and *Klebsiella pneumonia KpnH* ($p = 0.006$) were less abundant compared with the CLA group (Table 1). There was however no significant difference in the ARG classes and ARG richness among the three groups (Figure S1).

Table 1

Summary of the baseline abundance of ARGs and microbiota at genus level in the LEVO and OTHER groups when compared to CLA group. #FDR < 0.1

	Log2 (abundance)			P-value	
	CLA	LEVO	OTHER	CLA vs. LEVO	CLA vs. OTHER
Unique ARGs					
<i>ErmF</i>	3.9229	5.9215	6.3615	0.1763	0.0152
<i>Klebsiella pneumoniae KpnH</i>	-3.7542	-5.1295	-9.0929	0.5203	0.0062
<i>tetA(46)</i>	-3.1846	-4.6226	-6.6070	0.3229	0.0346
<i>tetM</i>	-2.2119	0.6478	-0.4854	0.0057	0.2208
Bacteria-genus					
<i>Phascolarctobacterium</i>	-8.0028	-16.6781	-9.1633	0.0013	0.2422
<i>Ruthenibacterium</i>	-9.6594	-18.2970	-9.9756	0.0125	0.2215
<i>Anaerostipes</i>	-11.8255	-10.8822	-9.6961	0.3978	0.0193
<i>Gordonibacter</i>	-18.5513	-18.8764	-14.2046	0.8961	0.0114
<i>Eggerthella</i>	-15.9396	-16.5527	-11.1776	0.8985	0.0048
<i>Parasutterella</i>	-17.4306	-17.5368	-13.0426	0.9823	0.0275

ARG changes after H. pylori eradication therapies

For ARG richness, significant change of ARG number was observed at 6-week and 6-month in the LEVO group. As shown in Fig. 1A, the LEVO group was observed to have a significant increase of ARG number compared to baseline (LEVO mean: 28.1; $p = 0.014$), but there was no significant difference change the CLA and the OTHER group (CLA mean: 1.2, $p = 0.85$; OTHER mean: 7.2, $p = 0.42$). At 6-month, the LEVO group showed increased ARG number compared with baseline (LEVO mean: 21, $p = 0.029$), while the CLA and OTHER group showed no significant difference (CLA mean: -4, $p = 0.66$; OTHER mean: 9.9, $p = 0.32$) (Fig. 1B).

In addition to the changes in ARG richness, the ARG classes were altered after eradication therapy. Dominant ARG classes were tetracycline, MLS (macrolide-lincosamide-streptogramin), beta-lactam, and multidrug resistance genes in all fecal samples (Figure S2). In the CLA group, the relative abundance of

MLS resistance genes significantly increased at 6-week ($p = 0.027$) and restored at 6-month (Fig. 1C). In the LEVO group, the relative abundance of aminocoumarin ($p = 0.037$), antiseptics ($p = 0.034$), elfamycin ($p = 0.027$), fluoroquinolone ($p = 0.003$), multidrug ($p = 0.037$), and trimethoprim ($p = 0.009$) were significantly increased at 6-week and restored at 6-month compared with baseline. However, the relative abundance of beta-lactam ($p = 0.005$) and MLS ($p = 0.003$) resistance genes significantly reduced at 6-week and then restored at 6-month (Fig. 1C). In the OTHER group, only mupirocin resistance genes were reduced at 6-week ($p = 0.018$) and restored at 6-month (Fig. 1C).

To better understand the changes in gut ARGs, we further examined the unique ARGs in different treatment groups. In the CLA group, the abundance of *ErmF* (MLS resistance genes, $p = 0.0004$) and *tetO* (tetracycline resistance genes, $p = 0.005$) were significantly increased at 6-week compared to baseline level (Fig. 2A). In the OTHER group, only *ErmB* (MLS resistance genes, $p = 0.04$) was significantly decreased at 6-week compared with baseline (Fig. 2B). The LEVO group had the highest number of significantly altered ARGs among the three groups. A total of 28 unique ARGs were significantly increased at 6-week compared to the baseline level (Fig. 2C). Over half of the altered ARGs (19/28) belonged to multidrug resistance genes, the rest of them (9/28) were aminocoumarin, aminoglycoside, beta-lactam, nitroimidazole, and rifamycin resistance genes. However, there was no significant difference in the abundance of unique ARGs at 6-month compared with baseline in all three treatment groups.

ARG difference between successfully eradicated and failed patients in the CLA group

In the CLA group, there was a significant difference in the ARG richness and unique ARGs between those who were successfully eradicated and failed eradication therapy. The number of observed unique ARGs was significantly higher in the failed patients as compared with those successfully eradicated at baseline ($p = 0.019$) and 6-month ($p = 0.015$), but not at 6-week ($p = 0.29$) after the eradication therapy (**Figure S3A**). The Shannon index also showed that the alpha diversity of ARGs was significantly higher in failed patients ($p = 0.015$) at 6-month (**Figure S3B**).

In addition to the ARG diversity difference, the expression of unique ARGs was also differentially enriched. At baseline, the abundance of *ErmF* (MLS resistance genes) was significantly higher in patients who failed eradication therapy (**Figure S4A**). At 6-month, 25 unique ARGs were highly abundant in failed patients compared with successfully eradicated patients (**Figure S4B**).

Gut microbiota changes after *H. pylori* eradication therapies

We also performed taxonomy analysis of the gut microbiota to explore the microbiota composition and diversity alteration in the three groups after eradication therapies. At baseline, the species richness was significantly lower in the OTHER group ($p = 0.021$) which had an average of two prior treatment failures, when compared with the CLA group (treatment naïve, **Figure S5**). At the phylum level, *Bacteroidetes* was the most abundant phylum, followed by *Firmicutes*, *Actinobacteria*, and *Proteobacteria* phylum, which account for over 98% of the microbiota (**Figure S6A**). At the genus level, *Ruthenibacterium* ($p = 0.012$) and

Phascolarctobacterium ($p = 0.001$) were less abundant in the LEVO group, while *Eggerthella*, *Gordonibacter*, *Anaerostipes*, and *Parasutterella* were more abundant ($p = 0.004$, $p = 0.011$, $p = 0.019$, $p = 0.027$, respectively) as compared with the CLA group (Table 1). There was however no significant difference in beta diversity among the three groups at baseline (Fig. 3A).

The diversity of microbiota was altered after eradication therapy. There was a significant difference in beta diversity among the three groups at 6-week (PERMANOVA, $p = 0.002$; Fig. 3B) but not at 6-month (PERMANOVA, $p = 0.323$; Fig. 3C). No significant difference in beta diversity was observed within treatment groups (**Figure S7**). Compared with baseline, the species richness index (alpha diversity) was significantly decreased in the CLA group ($p = 0.016$), the LEVO group ($p = 0.046$), and the OTHER group ($p = 0.001$) 6-week after the eradication therapy (Fig. 3D). The Shannon index (alpha diversity) was also significantly decreased at 6-week in the CLA group ($p = 0.042$) and the OTHER group ($p = 0.0009$), but not in the LEVO group ($p = 0.77$) (Fig. 3E). However, compared with baseline, alpha diversity (both richness and Shannon index) was restored 6-month after the eradication therapy (Fig. 3D and 3E).

At the phylum level, only *Firmicutes* was observed to significantly changed at 6-week after the eradication therapy, while other dominant phylum had no significant difference at either 6-week or 6-month (**Figure S6B**). The average relative abundance of *Firmicutes* increased in the CLA group (29.53–37.60%, $p = 0.032$), had an increasing trend in the LEVO group (27.17–37.38%, $p = 0.074$), but decreased in the OTHER group (29.22–15.96%, $p = 0.004$) at 6-week. All restored to baseline levels at 6-month (Fig. 4A).

Significant perturbations were also observed at the genus level. In the CLA group, the relative abundance of *Parasutterella* (*Proteobacteria* phylum, $p = 0.013$), *Lachnoclostridium* ($p = 0.007$), and *Roseburia* (*Firmicutes* phylum, $p = 0.002$) all significantly increased at 6-week compared with baseline. Most of the genus returned to baseline level at 6-month except for *Roseburia* ($p = 0.027$), which maintained at a high level at 6-month (Fig. 4B). In LEVO group, *Hungatella*, *Erysipelatoclostridium*, *Ruthenibacterium*, *Lachnoclostridium* and *Flavonifractor* genus from *Firmicutes* phylum ($p = 0.028$, $p = 0.015$, $p = 0.042$, $p = 0.005$, $p = 0.012$, respectively) and *Escherichia* from *Proteobacteria* phylum ($p = 0.016$) all significantly increased at 6-week. Among them, genus *Ruthenibacterium* ($p = 0.013$), *Lachnoclostridium* ($p = 0.032$) and *Flavonifractor* ($p = 0.013$) maintained at high level at 6-month (Fig. 4C). In the OTHER group, *Bacteroides* ($p = 0.033$) from *Bacteroidetes* phylum and *Erysipelatoclostridium* ($p = 0.002$) from *Firmicutes* phylum significantly increased at 6-week, while other genera (*Eubacterium*, *Phascolarctobacterium*, *Lawsonibacter*, *Anaerostipes*, and *Ordribacter*) from *Firmicutes* and *Bacteroidetes* phylum were significantly reduced at 6-week. Several genera from *Firmicutes* phylum were still significantly altered at 6-month compared with baseline in the OTHER group (Fig. 4D). **Table S2-4** showed the significant alterations at the species level in each group at 6-week and 6-month compared with baseline.

Discussion

In this study, we have performed a comprehensive review of the dynamic changes of human gut microbiota and ARGs in patients who underwent various *H. pylori* eradication therapies using the whole genome sequencing method. Overall, we observed a transient but dramatic shift in ARG richness, ARG class level, and unique ARGs at 6-week after the eradication, especially in patients treated with levofloxacin-based therapy. All of these changes on ARGs were restored at 6-month after eradication therapy.

In addition to the change of ARGs, we also found significant changes in gut microbiota. The alpha diversity significantly decreased at 6-week in all three treatment groups, which all restored at 6-month. The microbiota community structure (beta diversity) was significantly separated at 6-week after different eradication therapies among the three groups. Moreover, microbiota difference was observed at 6-week after the treatment at different taxonomy levels and partially restored at 6-month. For treatment naïve patients, those with higher ARG richness and *ErmF* gene abundance at baseline were prone to failure of clarithromycin-based triple therapy, and there was also high diversity and abundance of unique ARGs 6-month after the eradication therapy.

Our results showed that several ARGs (MLS, tetracycline, and multidrug resistance genes) were differentially present in the LEVO and the OTHER group compared to the CLA group (treatment naïve) at baseline. The species diversity was lower in the OTHER group and there were several differentially abundant genera compared with the CLA group. These findings may indicate that after previously failed *H. pylori* eradication therapies, there was emergence of antibiotic resistance, especially for clarithromycin and tetracycline, which was associated with reconstructions of gut microbiota. Moreover, we found that those who failed eradication therapy in the CLA group had higher number of unique ARGs and abundance of *ErmF* (MLS resistance genes) at baseline. In our patients, we found that the tetracycline (*tetQ*, *tetO*, and *tetW*) and MLS (*ErmF* and *ErmB*) resistance genes were highly abundant and prevalent in human fecal samples (Figure S8). Notably, it was also found that the vancomycin resistance genes (*VanRG* and *VanRA*) were also highly prevalent and abundant in some populations such as Danish, Spanish, and Chinese individuals[21].

One of the major issues related to *H. pylori* eradication therapy was antimicrobial resistance. Prior antibiotic treatment can induce multidrug resistance (MDR) [35], which is increasing worldwide and could hamper the success rate of conventional *H. pylori* eradication therapy [36]. The effect of different antibiotics used in various *H. pylori* eradication therapies on gut ARGs however remains unclear. In this study, we showed a transient increase in *ErmF* (macrolide resistance) after exposure to clarithromycin and amoxicillin. Besides, the relative abundance of the *ErmF* gene was overexpressed at baseline in patients who failed multiple previous treatments compared with those treatment naïve patients. A recent Russian study revealed that the *ErmB*, CFX group (beta-lactam), and *tetQ* genes were increased after clarithromycin-based quadruple eradication therapy [22]. Consistently, a small study reported that the macrolide resistance gene *ErmB* gene increased immediately after treatment and remained at a high level four years after clarithromycin-containing triple therapy using the 16S rRNA sequencing method [11]. It

would be interesting to explore in future studies whether baseline carriage of *ErmF* or *ErmB* gene can be a predictor of treatment failure.

Escherichia coli are commensals, commonly found in the lower part of the intestine and usually harmless, while virulent isolates are associated with diarrhea and colitis [37, 38]. A Japanese study showed that fluoroquinolone consumption was closely associated with *E. coli* resistance [39]. Our study showed that the relative abundance of *E. coli* significantly increased 6 weeks after the levofloxacin-based eradication therapy. In addition, resistant *E. coli* carried numerous genes that confer resistance to beta-lactam (*Escherichia coli* ampH beta-lactam), aminoglycoside (*acrD*), fosfomycin (*GlpT*), sulfonamide (*sul1*), phenicol (*catS*) [40], multidrug (*Escherichia coli* EF-Tu mutant, *acrA*, *mdfA*, *soxR*) [41–43]. *E. coli* could also gain high-level resistance to various antibiotics after levofloxacin and tetracycline exposure by inducing the efflux system during the biofilms formation, especially the *emrY/K* (tetracycline resistance) and *evgS/A* (multidrug resistance) pumps [44]. Consistent with the previous study, the relative abundance of those ARGs together with other multidrug resistance genes significantly increased 6-week after levofloxacin-containing therapy compared with baseline.

One recent systematic review reported a remarkable rise in the resistance rate of levofloxacin from 17–27% from 2006 to 2015 in the Asia-Pacific region and this may affect the efficacy of levofloxacin-containing therapies[45]. In our study, levofloxacin-based therapy has the lowest eradication success rate as second-line therapy. Therefore, alternative second-line therapy like bismuth quadruple therapy (PPI, bismuth subsalicylate, metronidazole, and tetracycline) should be considered. Bismuth salt has a synergistic effect with antibiotics and confer no resistance[46]. Recent data also confirmed that 14-day bismuth combining quadruple therapy is a highly effective (over 90% cure rate) and safe second-line option in patients with previous treatment failure[47]. Thus, bismuth quadruple therapy may be preferred in view of the post-treatment ARG profiles.

Previous studies have shown that the consumption of antibiotics leads to immediate[22, 48], short-term[8, 49, 50] and long-term[50–52] alterations in the human gut microbiota. However, most of the studies used 16S rRNA sequencing methods and few of them had employed the more detailed metagenomic sequencing methods[22]. In our study, we found that the relative abundance of *Firmicutes* increased at 6-week in the CLA and LEVO group but significantly decreased in the OTHER group. Notably, it was reported that the relative abundance of *Bacteroidetes* and *Firmicutes* phylum decreased significantly whereas *Proteobacteria* increased immediately after the eradication therapy. All phylum restored to baseline level at 8-week[8, 22, 50, 53, 54]. The relative abundance of *Firmicutes* phylum was also found to be decreased 4-week after clarithromycin-based triple therapy in a recent study[49]. In contrast, another study showed an opposite trend of *Firmicutes* phylum, which increased 6-week after bismuth quadruple therapy[51]. It thus appears that different antibiotics may cause distinct short-term effects on the gut microbiota. The bacteria from *Firmicutes* phylum can ferment carbohydrates into a variety of short-chain fatty acids (SCFAs), which can increase the intestinal barrier function[55]. The dramatic alteration of *Firmicutes* phylum after the eradication therapy may seriously affected gut ecosystem and the inflammation recovery process.

Significant changes at the genus level were also identified and most of which were involved in the production of SCFAs. Previous studies have shown that some butyrate-producing bacteria, the *Lachnospirillum*, *Roseburia*, *Eubacteria hallii*, *Erysipelatoclostridium*[56, 57] displayed protective effects by generating butyrate (SCFAs) to suppress chronic intestinal inflammation[58]. Besides, *Bacteroides* spp., which produce acetate and propionate (SCAFs), could also protect against gut inflammation[57]. As expected, these beneficial bacteria were found to be enriched 6-week after different eradication therapies, especially, *Lachnospirillum* genus was found to have high-level 6-month after eradication therapy, which was similar to previous findings that *Lachnospirillum* enriched 26 weeks after bismuth quadruple therapy[51]. Taken together, those data demonstrate the temporary microbiota perturbation caused by antibiotic exposure and potential long-term protection of bismuth-containing eradication therapy, which was related to the recovery of gut inflammation.

Our study has several strengths. Other than looking at the ARG richness, we demonstrated dynamic changes in antimicrobial classes and unique ARGs under different eradication antibiotics exposure. Besides, we used shotgun whole genome sequencing (WGS) which had better detection of bacterial species, deeper sequencing depth, and identification of potential ARGs compared with the 16S rRNA sequencing method[16]. This study also included both patients who were naïve to and had failed previous eradication therapies to examine difference at baseline and after treatment.

There are some limitations of this study. First, as there are three different treatment groups with samples collected at three different time points, each group had a relatively small sample size which may hinder the application of the results. Second, this study only focused on the changes of gut microbiota and ARG from the metagenomics level, further studies should evaluate the metabolomics and proteomics for more integrated analysis, which can further reveal the consequences of receiving different *H. pylori* eradication therapies. Third, gut microbiota could change with lifestyle modifications, for instance, after exercise training and dietary intervention[59]. Since no dietary intervention or physical exercise was enforced in this study, the impacts of these lifestyle changes could not be analyzed. However, as most changes restored to baseline at 6-month, suggesting these factors are unlikely to be playing a significant role. Finally, considering the regional variations in antibiotic consumption and resistance, the applicability of our findings may need further validation in a more diverse population, particularly in an area with high background antibiotics resistance.

Conclusion

In summary, this study uncovers a transient alteration in the gut microbiome and ARGs after various *H. pylori* eradication therapies, and most of which resolved after 6 months. However, the use of tetracycline and fluoroquinolone-containing therapies in retreatment was more likely to induce multidrug resistance genes and had a greater impact on the ARGs. Our findings provide new insights into the perturbations of gut microbiota and ARGs associated with various *H. pylori* eradication therapies, which may facilitate future clinical treatment strategies particularly in patients with treatment failure.

Abbreviations

ARG

antibiotic resistance genes

CARD

The Comprehensive Antibiotic Resistance Database

RPKM

reads per kilobase, per million reads

ORFs

open reading frames

PCoA

Principal Coordinates Analysis

PERMANOVA

Permutational Multivariate Analysis of Variance

SCFA

Short Chain Fatty Acids

STT

Standard Triple Therapy

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (UW 18-413). Written informed consent was obtained from each patient before participation.

Consent for publication

Not applicable.

Availability of supporting data

Metagenomic sequencing data for all samples have been deposited in NCBI Sequence Read Archive under BioProject PRJNA749138.

Competing interests

The authors declare no competing interests.

Author contributions and funding

LLW and WKL conceived and designed the study. TT and KSL contributed to the recruitment of the patients and samples collection. SYL provided the infrastructure for and supervised the metagenomic sequencing. HBY and LLW implemented the bioinformatics pipeline. LLW performed bioinformatics analysis, interpreted the data and drafted the manuscript. JWKH supervised the bioinformatics analysis and edited the manuscript. WKL critically revised the manuscript. All authors approved the final version of the manuscript.

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Figures

Figure 1

Profiling of antibiotic resistance genes (ARGs) (A-B) Boxplots showing the number of changed ARGs in each treatment group at 6-week (A) and 6-month (B) when compared to baseline. The horizontal box lines represent the first quartile, the median, and the third quartile. * $p < 0.05$ by Wilcoxon test. (C) Heatmap of significantly altered ARG class in different groups as compared with baseline. The color scale represents the relative abundance of each ARG class. * $p < 0.05$, ** $p < 0.01$ by Wilcoxon test.

Figure 2

Significantly altered ARGs 6-week after the eradication therapy in different treatment groups. Differentially enriched unique ARGs in CLA (A), OTHER (B) and LEVO (C) 6-weeks after the eradication therapy. For ARG abundance box plots, the boxes color coated by red denote baseline abundance, while those color coated by blue denote 6-week abundance. The horizontal box lines represent the median, the boxes extend from the first to the third quartile (25th to 75th percentiles). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Wilcoxon signed-rank test with false discovery rate (FDR) correction (only those ARGs with adjusted $p < 0.05$ are shown). ARGs names that are too long have been abbreviated (rpoB:

Bifidobacterium adolescentis rpoB mutants conferring resistance to rifampicin; ampH: Escherichia coli ampH bacte-lactamase; EF-Tu mutation: Escherchia coli EF-Tu mutants conferring resistance to Pulvomycin; marR: Escherchia coli marR mutant conferring antibiotic resistance; soxR: Escherchia coli soxR with mutation conferring antibiotic resistance)

Figure 3

Microbiota diversity (alpha and beta diversity) alteration after eradication therapies (A-C) Principal Coordinates Analysis (PCoA) from Bray-Curtis distance between three treatment groups at (A) baseline, (B) 6 weeks after the eradication therapy, and (C) 6 months afterward. (D-E) Boxplots showing alpha diversity indices (Richness, Shannon index) in three different treatment groups. The horizontal box lines represent the first quartile, the median, and the third quartile. Whiskers denote the range of the values within 1.5 times the interquartile range (IQR) from the first and third quartiles, respectively. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Wilcoxon signed-rank test and Mann-Whitney test for paired and unpaired samples.

Figure 4

Temporal changes in gut microbiota at phylum and genus levels after different H. pylori eradication therapies (A) Comparisons of the relative abundance of Firmicutes phylum in separated groups. The samples are shown as dots. The horizontal box lines represent the first quartile, the median, and the third quartile. Whiskers denote the range of the values within 1.5 times the interquartile range (IQR) from the first and third quartiles, respectively. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Wilcoxon signed-rank test and Mann-Whitney test for paired and unpaired samples. #FDR < 0.05. (B-D) Significantly altered microbiota at genus in (B) CLA group, (C) LEVO group, and (D) OTHER group. #FDR < 0.10.

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