Modulation of Oral Microflora during *Helicobacter Pylori* Eradication

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ABSTRACT

Objective: To analyse the saliva microbial abundance and composition by 16s rRNA sequence during *Helicobacter pylori (H.pylori)* eradication.

Study Design: Descriptive study.

Place and Duration of Study: Hebei University of Chinese Medicine, Hebei Provincial Hospital of Traditional Chinese Medicine, from March 2019 to January 2020.

Methodology: The saliva microbial were analysed before and after the bismuth-containing quadruple therapy. A total of ten saliva samples (three groups) were enrolled in the study. The authors used the linear discriminant analysis effect size (LEfSe) method and Welch's t-test for comparative analysis to identify which taxa could be significantly affected in three groups.

Results: *H.pylori* 16S rRNA gene sequence was not detected in the ten saliva samples. The abundance of *Prevotella_sp._oral_-clone_P4PB_83_P2* from healthy adults was higher than *H.pylori*-positive patients. Moreover, after the bismuth-containing quadruple therapy, the diversity and richness of saliva bacteria reduced. *Lautropia, Burkholderiales, uncultured bacterium, Burkholderiaceae,* and *Actinomyces* were enriched in *H.pylori*-positive patient samples after the bismuth-containing quadruple therapy.

Conclusion: The diversity and richness of salivary microbiome were reduced in *H.pylori*-positive patient, and bismuth-containing quadruple therapy affected oral microbiota.

Key Words: *Helicobacter pylori, Saliva, Microbiota, RNA, Bismuth.*

How to cite this article: Ji J, Zhang Z, Gao H, Zhou W, Li Y, Shi X. Modulation of Oral Microflora during *Helicobacter Pylori* Eradication. *J Coll Physicians Surg Pak* 2022; **32(03)**:308-312.

INTRODUCTION

Helicobacter pylori (H.pylori) is formally recognised bacterial carcinogen. About half of the world's population is believed to be infected with *H.pylori*.[1] *H.pylori* can lead to chronic gastritis, peptic ulcer, gastric carcinoma, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma.[2] *H.pylori* is a risk factor for some diseases as iron deficiency anemia, idiopathic thrombocytopenic purpura, and vitamin B12 deficiency.[3] These reports suggested that *H.pylori* influences the microbiota not only in the stomach but also distant.[4] But the impact of *H.pylori* infection on the oral microbiome is unclear. A study showed that both *H.pylori* infection and eradication therapy caused alterations of the oral microbiota.[5] *H.pylori* has been detected from oral saliva, dental plaque, root canals, and dorsum of tongue,[6-9] suggesting that the oral cavity as major extra-gastric reservoir for *H.pylori*. *H.pylori* in the oral cavity affected the eradication of *H.pylori* in the stomach, so, the oral cavity may be a source of transmission.[10] However, the route of transmission is not clear.

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Received: August 11, 2021; Revised: November 25, 2021; Accepted: December 29, 2021
DOI: https://doi.org/10.29271/jcpsp.2022.03.308

The guidelines recommend bismuth-containing quadruple therapy (PPI + bismuth + amoxicillin + metronidazole) as the primary treatment in China.[11] It is an effective first-line treatment for clarithromycin and metronidazole resistant *H.pylori* infection.[12]

Therefore, the aim of this study was to investigate bismuth-containing quadruple therapy-induced changes in oral salivary microbiota using 16s rRNA sequencing.

METHODOLOGY

It was a descriptive study conducted at Hebei University of Chinese Medicine, Hebei Provincial Hospital of Traditional Chinese Medicine, from March 2019 to January 2020. Informed consent was obtained from all the subjects. Ethical approval for the study was granted by the Ethics Committee of Hebei University of Chinese Medicine (Yxll2019039). The inclusion criteria were age above 18 and under 70 years; no antibiotics used in the past one month; subjects with chronic gastritis examined using the 14C-urease breath test (UBT). A total of six subjects were enrolled in the study, which consisted of three males and three females; and four as positive for gastric *H.pylori* infection, two as negative. Ten saliva samples were collected, including two samples from *H.pylori*-negative subjects (healthy adults). Eight samples from *H.pylori*-positive subjects before and after bismuth-containing quadruple therapy at different times,

before treatment, and after 28 days bismuth-containing quadruple therapy. Saliva was collected in a sterile tube until reaching two milliliters of saliva in each tube per subject. The saliva was transported to GENEWIZ Inc (Suzhou, China) with dry ices and stored at -80°C until tested.

According to the manufacturer's protocol, DNA was extracted from saliva using DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). Saliva bacterial sequencing of 16S rRNA genes was performed at GENEWIZ Inc. (Suzhou, China). V3 and V4 hypervariable regions of 16S rRNA were selected for generating amplicons and following taxonomy analysis. Amplicons were sequenced using the MiSeq platform as previously described.[13]

The final analysis used high-quality sequences classified into operational taxonomic units (OTUs) by the clustering programme VSEARCH (1.9.6) against the SILVA 132 database pre-clustered at 97% sequence identity. Samples diversity were assessed using the Shannon and Simpson indexes, Chao1, ACE indexes were for sample richness. The beta diversity was revealed by non-metric multidimensional scaling (NMDS) analysis of 10 samples. The authors used STAMP (V2.1.3) software for Welch's T-test between the two groups of samples to find significant differences in species. The authors used the linear discriminant analysis effect size (LEfSe) method to identify which taxa with differential abundance among the three groups.

RESULTS

All samples were categorised into three groups (Figure 1a). To test the saliva bacteria characteristics, the authors used high--throughput sequencing of the 16S rRNA. The results showed that 526,349 high-quality sequences were obtained from ten samples, with an average length of 456 bp. The more minor sequences were filtered by order of abundance, and 136 OTUs were clustered with 97% recognition threshold. The Venn diagram (Figure 1b) showed the number of shared and unique operational classification units (OTUs) in three groups. The shared OTUs for these groups were 119. The results of this study showed that the specific number of OTUs did not change in the three groups.

The relative abundance of salivary bacteria at both phylum and genus levels was analysed (Figures 2a-2d) in ten samples and three groups. The main phyla were illustrated respectively in ten samples (Figure 2a). It suggested that the bacterial community of the ten samples was similar. There was no significant difference in phyla among the ten samples. In addition, the three groups were compared (Figure 2c), the main phylum of healthy adults was *Bacteroidetes* (39.44%), *Firmicutes* (38.83%), *Actinobacteria* (9.09%), *Fusobacteria* (4.04%), *Proteobacteria* (3.48%). For before bismuth-containing quadruple therapy group, the main phyla were *Firmicutes* (49.93%), *Bacteroidetes* (22.20%), *Proteobacteria* (11.91%), *Actinobacteria* (11.26%), Saccharibacteria (2.11%).

The result showed that the abundance of the detected dominant phyla did not change significantly in three groups. (p=0.43, Figure 2c). *H.pylori* was not found in the studied ten samples.

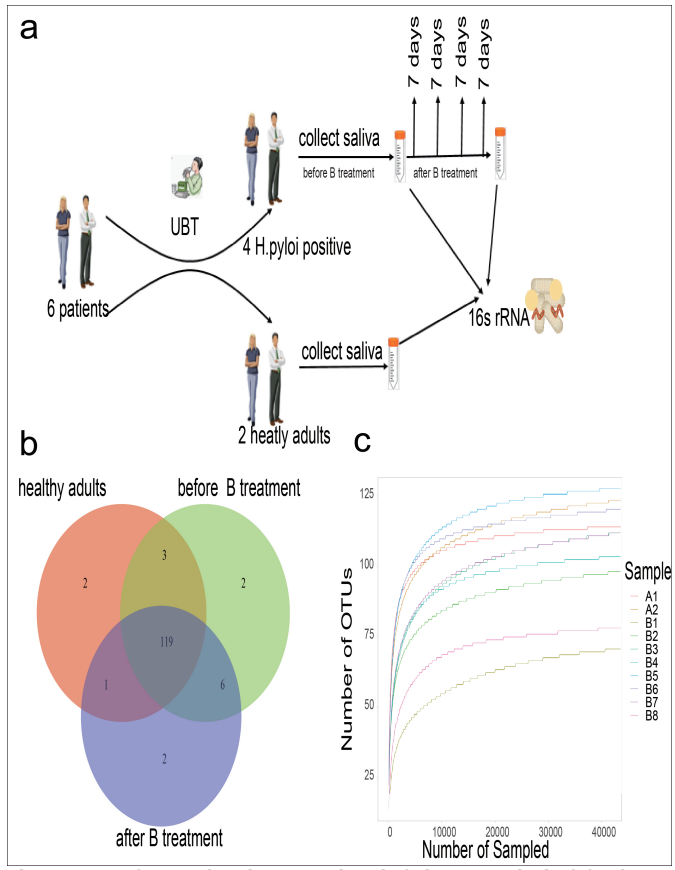

Figure 1: OTU (Operational taxonomic units) cluster analysis. (a) Schema of sample collection and analyses. (b) The sharing and unique operational taxonomic units (OTUs) numbers were shown in the Venn diagram. (c) The rarefaction curves of 10 samples based OTUs.
B: Bismuth-containing quadruple therapy.

At the genus level, there were 54 genera, including 52 genera of healthy adults, 54 genera of *H.pylori*-positive patients before treatment, and 46 genera of *H.pylori*-positive patients after treatment. Thirty-four genera were shared by all groups (Figure 2b). The genus of core bacteria in the oral saliva of healthy adults included *Streptococcus, Prevotella_7, Porphyromonas, Rothia.* Before bismuth-containing quadruple therapy, the level of core bacterial flora in the oral saliva of *H.pylori*-positive patients were *Streptococcus, Neisseria, Porphyromonas, Prevotella_7, Gemella*. After treatment, the core bacterial flora of the oral saliva of *H.pylori*-positive patients were *Streptococcus, Neisseria, Prevotella_7, Porphyromonas, Gemella*. No significant change in the abundance of dominant genes was detected (p=0.57, Figure 2d).

To find significant species differences, the authors used Welch's t-test for comparative analysis. The result showed that the abundance of *Prevotella_sp._oral_clone_P4PB_83_P2* was higher than *H.pylori*-positive patients from healthy adults (p <0.001, Figure 2e).

Linear discriminant analysis effect size (LEfSe) method was used to identify the taxa, which could be significantly affected in the three groups at the class, family, genus, order, phylum, species levels (Figure 2f, 2g). The results (Figure 2f) showed that *Actinomyces, Lautropia, Burkholderiales*, *uncultured bacterium* and *Burkholderiaceae* were considerably enriched in *H.pylori*-positive patients after bismuth-containing quadruple therapy compared to healthy adults. Genus *Lautropia* belongs to Family *Burkholderiaceae*, which belongs to the Order *Burkholderiales*. In contrast, *Absconditabacteria* was significantly increased in healthy adults. In addition, different bacteria existed in *H.pylori*-positive patients before and after treatment (Figure 2g).

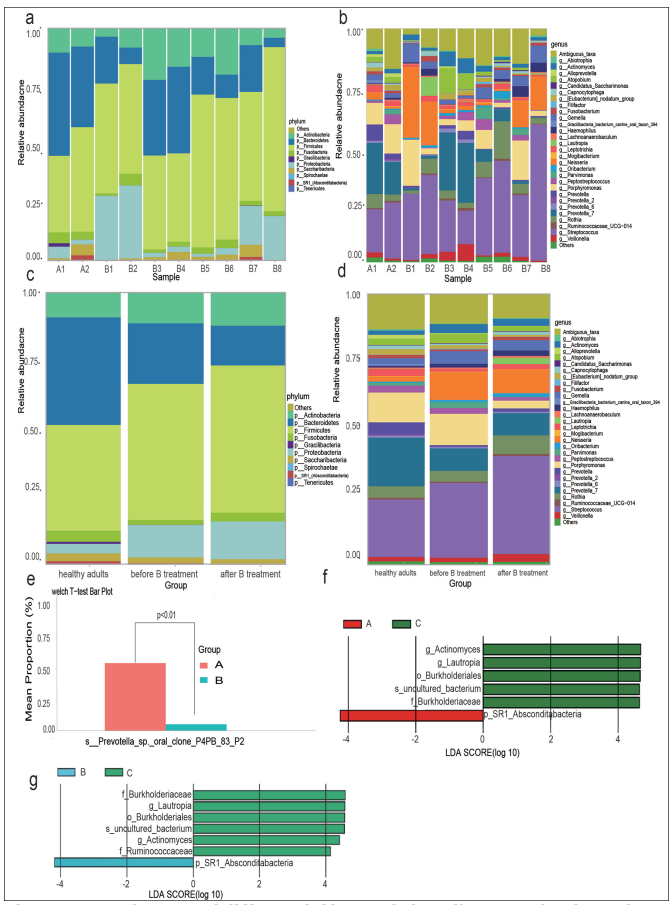

Figure 2: Dominant and differential bacteria in saliva samples from three groups. (a) Abundance and prevalence of the different bacterial phyla in 10 oral saliva samples. (b) Abundance and prevalence of the different bacterial genera in 10 oral saliva samples. (c) Abundance and prevalence of the different bacterial phyla in 3 groups. (d) Abundance and prevalence of the different bacterial genera in three groups. (e) Welch's t-test for comparative analysis. (f, g) Different taxa identified by LEfSe with LDA. The LDA score obtained by LDA analysis for significant microbial groups was calculated. The larger the LDA score, the greater the influence of species abundance on the difference effect. A, healthy adults B, before bismuth-containing quadruple therapy C, after bismuth-containing quadruple therapy.

In summary, the salivary microflora of *H.pylori*-patients was different from that of healthy adults; and differences also existed before and after treatment.

To measure the alpha diversity of the oral saliva microbial community, the authors calculated the OTUs species. Salivary microbiota richness was evaluated by measuring Chao1 and ACE. Diversity was evaluated by Shannon and Simpson indices. There were no significant differences in richness and diversity among all groups (Figure 3a-3d; $P > 0.05$). The Chao1, ACE, Shannon, and Simpson indexes showed the highest diversity in healthy adults. It gradually decreased from healthy adults to bismuth-containing quadruple therapy patients, indicating that the oral saliva bacteria may be affected with *H.pylori* and bismuth-containing quadruple therapy. The matrix heat map (Figure 3e) of ten samples (The lighter the color, the smaller difference between the two samples) showed similarity at ten samples under different colors. The authors performed the NMDS analysis (Figure 3f) at the OTU level to assess whether any group of samples shared similar bacterial communities. The closer the two points were, the smaller were the microbial differences between the two samples. The bacterial community of healthy adults had good aggregation, while the samples of *H.pylori* positive-patients showed irregular distribution patterns. Together, the richness and diversity of the salivary microflora in *H.pylori*-positive patients were changed compared with the healthy adults.

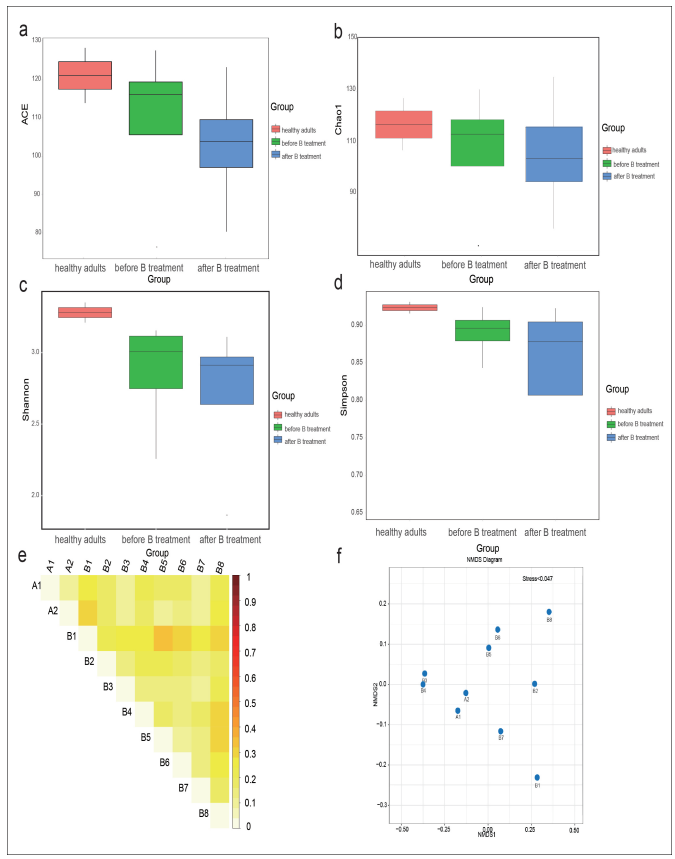

Figure 3: The diversity of oral saliva microbial community. (a) The alpha diversity (ACE index) in 3 groups. (b) The alpha diversity (Chao1 index) in 3 groups. (c) The alpha diversity (Shannon index) in 3 groups. (d) The alpha diversity (Simpson index) in 3 groups. (e) The beta diversity was revealed by Unweighted unifrac distance matrix heat map of 10 samples. (f) The beta-diversity was revealed by non-metric multi-dimensional scaling (NMDS) analysis of 10 samples. A, healthy adults B, before bismuth-containing quadruple therapy C, after bismuth-containing quadruple therapy.

DISCUSSION

Some clinical studies showed that *H.pylori* affected human health, especially stomach health. But, few people studied the influence of *H.pylori* on oral flora before and after bismuth-containing quadruple therapy. The primary function of bismuth is to improve the eradication rate of *H.pylori.*[14] Metronidazole is effective against oral microbiota. The authors characterised oral saliva microbiota from three groups, ten samples. Our analysis showed that no trace of *H.pylori* 16S rRNA gene sequence could be detected in the three groups, which may be because of the small samples. Consistently, one study suggested that *H.pylori* was *not* in any oral samples who suffer from stomach *H. pylori* infection.[15] *H.pylori* was cultured from dental plaque of a patient with gastric disease associated with *H.pylori* infection first reported in 1989.[16] Some reports indicated that *H.pylori* might colonize in the oral cavity.[17,18] The core phyla, the authors identified in healthy adults, was consistent with other reports that saliva contains five major phyla: *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria.*[19] The predominant phyla in *H.pylori*-positive patients before bismuth-containing quadruple treatment were *Firmicutes* (49.93%), *Proteobacteria* (11.91%), *Actinobacteria* (11.26%), *Saccharibacteria* (2.11%), which were higher than healthy adults. *Prevotella* were detected at higher frequencies in the children with S-ECC. Species *prevotella* was believed to be associated with SECC.[20] *Prevotella* is a Gram-negative anaerobic bacillus, which may be the primary periodontal disease pathogen.[21] In this study, *prevotella* species in the *H.pylori*-positive patients was detected in a lower frequency, indicating that the presence of *H.pylori* may be associated with species *prevotella*. In this research, Chao1, ACE, Shannon, and Simpson index revealed that diversity was highest in healthy adults and gradually decreased from healthy adults to after treatment group. This showed that bismuth-containing quadruple therapy, in particular, systemic antibiotics can influence the microbiota.

LEfSe analysis revealed that seven bacteria were different in the three groups. However, the authors did not find different bacteria between the healthy adult's group and before bismuth--containing quadruple therapy (*H.pylori* positive).

The different bacteria were in after bismuth-containing quadruple therapy patients. A previous study suggested that both *H.pylori* infection and eradication treatment cause changes in oral microflora and structure.[5] The alterations of oral microflora after bismuth-containing quadruple therapy patients may be due to *H.pylori* eradication. But the impact of systemic antibiotics, especially metronidazole, cannot be ruled out.

The genus *Burkholderia* is gram-negative bacteria, which includes metabolically diverse and adaptable. However, some members of the genus are prominent opportunistic pathogens. *Burkholderia* has significant biotechnological potential as a source of novel antibiotics and bioactive secondary metabolites.[22] Interestingly, *ruminococcaceae was* found, which belongs to gut bacteria. May be the bacteria flowed back from the stomach into the mouth. Gut microbiome *ruminococcaceae* enhanced antitumor immune responses.[23] This study tested the *H.pylori* infection, using the urease breath test (UBT) before bismuth-containing quadruple therapy, not to detect after bismuth-containing quadruple treatment.

Further research is needed with expanded sample and analysis of the oral microbiota in the same patients after eradication to show how *H.pylori* infection affects the saliva microbiome.

CONCLUSION

The abundance and diversity of the saliva microbiome were different and reduced after bismuth-containing quadruple therapy; especially, the amount of species *prevotella* in the saliva decreased in *H.pylori* infected patients; while *H.pylori* was not detected in the oral saliva from patients in this study.

FUNDING:
This work was financially supported by Science andTechnology Project of Hebei Education Department (SLRC2019043), and the Project of Undergraduate Innovation and Entrepreneurship (201714432065), Scienceand Technology Capacity Improvement Project of Hebei University of Chinese Medicine (KTY2019002), Provincial Science and Technology Plan of Hebei Province (21377757D).

ETHICAL APPROVAL:
The study was approved by the Hebei Hospital of Traditional Chinese Medicine; and ethical approval for the study was granted by the Ethics Committee of Hebei University of Chinese Medicine (Yxll2019039).

PATIENTS' CONSENT:
Informed consents were obtained from all subjects.

CONFLICT OF INTEREST:
The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:
JJ, ZZ, HG, WZ, YL: Performed the experiments.
JJ, XS: Wrote the manuscript with contributions from all authors.
XS: Designed the research.

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