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BRIEF ARTICLE

Bacterial flora concurrent with *Helicobacter pylori* in the stomach of patients with upper gastrointestinal diseases

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Abstract

AIM: To investigate the non-*Helicobacter pylori* (*H. pylori*) bacterial flora concurrent with *H. pylori* infection.

METHODS: A total of 103 gastric biopsy specimens from *H. pylori* positive patients were selected for bacterial culture. All the non-*H. pylori* bacterial isolates were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

RESULTS: A total of 201 non-*H. pylori* bacterial isolates were cultivated from 67 (65.0%) of the 103 gastric samples, including 153 isolates identified successfully at species level and 48 at genus level by MALDI-TOF MS. The dominant species were *Streptococcus, Neisseria, Rothia* and *Staphylococcus*, which differed from the predominantly acid resistant species reported previously in healthy volunteers. The prevalence of non-*H. pylori* bacteria was higher in non-ulcer dyspepsia group than in gastric ulcer group (100% *vs* 42.9%, $P < 0.001$). Six bacterial species with urease activity (*Staphylococcus epidermidis, Staphylococcus warneri, Staphylococcus capitis, Staphylococcus aureus, Brevibacterium spp.* and *Klebsiella pneumoniae*) were also isolated.

CONCLUSION: There is a high prevalence of the non-*H. pylori* bacteria concurrent with *H. pylori* infection, and the non-*H. pylori* bacteria may also play important as-yet-undiscovered roles in the pathogenesis of stomach disorders.

Key words: Non-*Helicobacter pylori*; Bacterial flora; Gastrointestinal diseases; Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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INTRODUCTION

Stomach is generally regarded as an environment that is not conducive to bacterial colonization. A notable exception is *Helicobacter pylori* (*H. pylori*), which may cause chronic gastritis, peptic ulcer and is correlated with gastric adenocarcinoma[1,2]. However, gastritis also occurs in *H. pylori* negative human subjects or mouse models, suggesting that *H. pylori* is just one of the organisms causing gastritis.

With the reduced level of acid induced by *H. pylori* dissipates or antiulcer medications, the stomach becomes more susceptible to the colonization of other organisms[3], which may complicate the development of gastric disorders. In recent years, considerable interest has emerged in the interactions among *H. pylori*, non-*H. pylori* bacteria and acid-suppressive therapy. Some studies have shown that the co-infection of *H. pylori* and non-*H. pylori* bacteria enhances the development of atrophic corpus gastritis[4]. But up till now, there are only a limited number of studies regarding the stomach bacterial flora and all are based on a small size of a few patients or volunteers. The aim of this study was to investigate the non-*H. pylori* bacterial flora concurrent with *H. pylori* infection.

Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) is a new technology for bacterial identification[5]. Its performance has been evaluated in different studies, and a higher accuracy was obtained compared with phenotypic methods[6,7].

MATERIALS AND METHODS

Patients and materials

A total of 104 patients, who underwent upper gastrointestinal endoscopy because of dyspeptic symptoms and yield positive results in rapid urease test (RUT), were selected for the further *H. pylori* detection and bacterial examinations. Among the 104 patients, 103 were *H. pylori* positive and ultimately entered into this study, including 63 from Jiangxi Province and 40 from Beijing, China. No subject had received anti-secretory drugs, antibiotics, or probiotics two wk prior to entry into this study and no one had *H. pylori* eradication history. Diseases included gastritis (n = 23), gastric ulcer (n = 21), duodenal ulcer (n = 42), reflux esophagitis (n = 4), and non-ulcer dyspepsia (NUD) (n = 13). There were 81 (78.6%) patients aged less than 50 years. The study was reviewed and approved by the Ethics Committee of the National Institute for Communicable Disease Control and Prevention. Each subject gave informed oral consent before entering into the study.

Upper gastrointestinal endoscopy was performed after an overnight fast in all patients. Mucosal samples were taken from the greater curvature of gastric antrum and corpus using sterile disposable biopsy forceps. All the samples were placed into sterile brain-heart infusion broth for transport to the cultural laboratory.

Bacterial culture and polymerase chain reaction detection for H. pylori

After biopsies were taken, the samples were dispersed using a homogenizer. Each homogenate was inoculated onto three plates, two Columbia blood agars (CM0331, OXOID) and a Campylobacter agar (CM0935, OXOID) supplemented with 5% sheep blood, polymyxin B, vancomycin, trimethoprim, and amphotericin B. One of the Columbia blood agar was incubated under aerobic condition at 37 ℃, the other one and the Campylobacter agar were incubated under microaerophilic condition at 37 ℃.

The *H. pylori* status was assessed with culture and polymerase chain reaction (PCR) detection. Small (0.5 to 2 mm) translucent colonies were selected to gram-strain and tested for urease, catalase and oxidase activity. Microscopy of gram-stained smears with curved gram-negative rods resembling *Helicobacter,* together with positive in all the tree enzyme activity tests were identified as *H. pylori.*

H. pylori culture-negative samples were further detected by *ureA*, *vacA* and *cagA* PCR analysis, using the primers described previously[8,9]. *H. pylori* status was defined as negative only if all the three PCR detections were negative.

Mass spectrometry identification of non-H.pylori bacteria

Preparation of samples: Appropriate amount (5-10 mg) colony was scraped by inoculating loop and suspended in 300 μL distilled water, and 900 μL ethanol was added and mixed. Then the sample was centrifuged at 12 000 r/min for 2 min, and the pellets were dried. Fifty μL formic acid (70% in water) was added to the dried bacterial pellet and mixed thoroughly, and then added with 50 μL acetonitrile. After centrifugation at 12 000 r/min for 2 min, 1 μL of the supernatant containing the bacterial extract was transferred onto the MSP 96 target ground steel plate (Bruker Daltonics, Bremen, Germany) and dried, then 1 μL of matrix solution (satured solution of a cyano-4-hydroxycinnamic acid in 50% acetonitrile + 2.5% trifluoroacetic acid) was added and crystallized by air-drying at room temperature. Each isolate was analyzed on the day of isolation.

Measurement with spectrometer: Measurement was performed with Microflex LT mass spectrometer (Bruker Daltonics) equipped with a 200 Hz smart-beam laser. The parameter settings were as follows: delay 320ns; ion source 20 kV; ion source 18.5 kV; lens voltage 8.5 kV; and mass range 2-15 kDa. Each run was validated with an *E. coli* control sample where the presence of 10 specific proteins insured that the spectrometer was set properly. Raw spectra of the strains were analyzed by MALDI Biotyper 2.0 software (Bruker Daltonics) using the default settings. A list of peaks up to 100 was generated. The threshold for peak acceptance was a signal-to-noise (S/N) ratio of 3. After alignment, peaks with a mass-to charge (m/z) ratio difference of less than 250 ppm were considered to be identical. The peak lists generated were used for matches against the reference library, by directly

Table 1 Non-*Helicobacter pylori* bacterial cultures of gastric biopsies

Micro-organism	Culture condition[1]			Colonized patients (*n*)[2]	Urease activity[3]	Gram stain
	Ⅰ	Ⅱ	Ⅲ			
Streptococcus				(54)		G+
Streptococcus pneumoniae	3	10		11		
Streptococcus salivarius	10	9		16		
Streptococcus anginosus		3		3		
Streptococcus oralis		2		2		
Streptococcus cristatus	1			1		
Streptococcus gordonii	2			2		
Streptococcus vestibularis	1			1		
Streptococcus spp.	10	13		18		
Neisseria				(32)		G-
Neisseria flavescens	11	16	11	23		
Neisseria perflava	2	1	1	3		
Neisseria macacae		1		1		
Neisseria sicca	1			1		
Neisseria spp.	3	1		4		
Rothia				(29)		G+
Rothia mucilaginosa	6	10		15		
Rothia dentocariosa		3	1	4		
Rothia aeria	1			1		
Rothia spp.	4	7		9		
Staphylococcus				(21)		G+
Staphylococcus epidermidis	3	3		5	5	
Staphylococcus aureus	3	4		5	3	
Staphylococcus capitis		3		3	1	
Staphylococcus warneri	2	1		3	3	
Staphylococcus cohnii		2		2		
Staphylococcus haemolyticus		1		1		
Staphylococcus spp.		2		2		
Lactobacillus				(9)		G+
Lactobacillus salivarius			4	4		
Lactobacillus oris			2	2		
Lactobacillus fermentum			1	1		
Lactobacillus spp.		1	1	2		
Corynebacterium				(4)		G+
Corynebacterium argentoratense	2	1		3		
Corynebacterium propinquum	1	1		1		
Kingella				(3)		G-
Kingella denitrificans			2	2		
Kingella spp.			1	1		
Others						
Capnocytophaga ochracea			3	3		G-
Haemophilus parainfluenzae		1	1	2		G-
Acinetobacter lwoffii	2			2		G-
Klebsiella pneumoniae	1			1	1	G-
Cardiobacterium sp			1	1		G-
Actinomyces spp.		2		2		G+
Micrococcus luteus	1			1		G+
Weissella confusa			1	1		G+
Aerococcus spp.	1			1		G+
Bacillus spp.	1			1		G+
Brevibacterium spp.		1		1	1	G+
Total	72	99	30	168		

[1]The number of bacterial strains isolated in Ⅰ: Columbia blood agar incubated under aerobic condition; Ⅱ: Columbia blood agar incubated under microaerophilic condition; Ⅲ: Campylobacter agar under microaerophilic condition; [2]The number of patients colonized by the same genus is shown in parentheses; [3]The number of bacterial isolates with urease activity.

using the integrated pattern matching algorithm of the software. All parameters were the same regardless of the bacteria analyzed. Spectra were obtained in the positive linear mode after 500 shots. A score was attributed to each identification. When this score was > 2.00, the identification was considered correct at the species level; between 1.7 and 1.999, the identification was considered correct at the genus level; and < 1.7, the identification was not similar enough to draw a conclusion.

Urease activity test of non-H. pylori bacteria

Each non-*H. pylori* bacterial culture was subjected to urease test. The medium used for the test was urea broth containing dipotassium hydrogen phosphate, 9.5 g/L; potassium dihydrogen phosphate, 9.1 g/L; urea 20 g/L; phenol red, 0.01 g/L; and yeast extract, 0.1 g/L, final pH 6.8. Any change in the indicator from pale yellow to pink in 24 h was taken as positive.

Statistical analysis

Continuous data were described with mean (minimum, maximum), categorical data with number and proportions. Difference between the groups for age and sex was analyzed using analysis of variance and χ^2 test. Fisher exact test was conducted to compare non-*H. pylori* prevalence data. *Post hoc* tests were conducted by the Bonferroni method. $P < 0.05$ was considered as statistically significant. All analyses were performed with SPSS 12.0 software.

RESULTS

A total of 92 (88.5%) samples were *H. pylori* culture positive. Only one of the 12 culture-negative cases was PCR negative. As result, 103 *H. pylori* positive patients were eventually enrolled into the analysis.

Non-H. pylori bacteria isolation

A total of 201 non-*H. pylori* bacterial isolates were cultivated from gastric samples of 67 (65.0%) of the 103 *H. pylori* positive patients. All the isolates were identified by mass spectrometry, including 153 identified at species level and 48 at genus level (Table 1). Some species isolated from more than two culture plates were shown in Table 1. A total of 168 isolates was obtained with the exception of the repeat identification of the same patient, and 39 patients harbored more than 2 non-*H. pylori* species. Overall, a total of 18 non-*H. pylori* bacterial genera (43 species) were isolated from the stomach biopsy specimen (Table 1), most of them were gram-positive bacteria (73.8%). The dominant bacterial species were *Neisseria flavescens* (13.7%), *Streptococcus salivarius* (9.5%), *Rothia mucilaginosa* (8.9%) and *Streptococcus pneumonia* (6.6%). Twelve gram-negative bacilli isolates (7.14%) belonged to 6 genera obtained in this study, which were discovered from 10 patients (9.7% of all the patients and 14.9% of the non-*H. pylori* positive patients). Concurrent colonization by gram-positive and/or gram-negative

Table 2 Demographic, non-*Helicobacter pylori* bacterial colonization and clinical characteristics of 103 patients

Diseases	Enrolled patients (*n*)	Age (yr)[1]	Gender (male:female)	Colonized patients
Duodenal ulcer	42	39 (20-56)	2:1	28 (66.7%)
Gastric ulcer	21	42 (20-62)	0.75:1	9 (42.9%)
Gastritis	23	39 (10-75)	0.44:1	13 (56.5%)
Non-ulcer dyspepsia	13	34 (22-57)	0.63:1	13 (100%)[a]
Reflux esophagitis	4	52 (41-63)	1:1	4 (100%)
Total	103	39 (10-75)	0.96:1	67 (65.0%)
P value		0.079[2]	0.074[3]	0.0028[4]

[1]Data are expressed as median (range); [2]By analysis of variance; [3]By χ^2 test; [4]By Fisher exact test; [a]$P = 0.00006$ *vs* gastric ulcer group by Bonferroni method.

coccus occurs in 10 of them.

The demographic and relevant clinical characters of the patients are described in Table 2. The patients were subdivided into five groups based on the clinical diseases. There were no significant intergroup differences in age and sex ratio, but the prevalence of non-*H. pylori* flora was significantly different ($P = 0.0028$) among different clinical diseases, as shown in Table 2. *Post hoc* tests were conducted and statistical difference was found between the gastric ulcer group (42.9%) and the NUD group (100%) ($P = 0.00006$). In addition, significant difference was also presented between the 50-plus age group and the younger age (< 50 years old) group (45.5% *vs* 69.1%, χ^2 test, $P = 0.048$).

Urease activity of non-H. pylori isolates

All the 201 non-*H. pylori* isolates were screened for urease activity, 14 isolates belonged to 6 species (*S. epidermidis*, *S. warneri*, *S. capitis*, *S. aureus*, *Brevibacterium spp.* and *K. pneumoniae*) and were urease positive (Table 1), which could benefit in the bacterial overgrowth in the stomach. Interestingly, not all the isolates of the 6 species were urease-positve except *S. warneri*. Especially in a 26-year-old patient with non-ulcer dyspepsia, two *Staphylococcus epidermidis* isolates were isolated under aerobic and microaerobic conditions, respectively, but only the microaerobic isolate was urease-positive.

DISCUSSION

It has been suggested that non-*H. pylori* bacteria and their by-products may act as a persistent antigenic stimulus, and thereby augment the inflammatory response induced by the *H. pylori* infection[4]. The current study documented a high prevalence (65.0%) of the non-*H. pylori* bacterial flora in the *H. pylori* positive patients, which indicate that the upper gastrointestinal disorders may enable non-*Helicobacter* bacteria to survive and colonize in the human stomach. Further studies should be done to elucidate the role of the co-infection of the stomach with *H. pylori* and non-*H. pylori* bacteria in the pathogenesis process.

Most of the non-*H. pylori* bacteria isolated in this study were upper respiratory tract microflora, which was in agreement with other researches[10]. The major species were *Streptococcus, Neisseria, Rothia* and *Staphylococcus*, that was different from the previous reports of healthy volunteers, which was predominantly acid resistant species - *Veillonella sp*, *Lactobacillus sp*, and *Clostridium sp*[11].

Gram-negative bacilli are uncommon in the upper gut of healthy individuals, but in patient populations with gastric hypochlorhydria, gram-negative bacilli are recovered in a minor proportion[12,13]. Gram-negative bacilli were discovered in 9.7% of the patients in this study. Concurrent colonization by gram-positive and/or gram-negative coccus occurs in 10 of the 12 gram-negative bacilli. *Acinetobacter lwoffii* was the only gram-negative bacilli species that was found as the only non-*H. pylori* bacteria in the patients. The two *A. lwoffii* carriers were a 42-year-old male patient with duodenal ulcer and a 25-year-old female patient with non-ulcer dyspepsia. *A. lwoffii* has been proved to cause the same histologic gastritis as *H. pylori* infection in a murine model[14]. And outer membrane protein (OmpA-like protein) of *A. lwoffii* was reported to activate interleukin 8 and gastrin promoter activity *in vitro*[15]. Moreover, a case of *A. lwoffii* bacteremia associated with acute gastroenteritis was reported recently[16], which stimulate more interest in the contribution of *A. lwoffii* to gastrointestinal diseases.

It has been well known that gastric acid secretion declines with age which may increase the sensitivity of the stomach to the bacterial colonization. Surprisingly, a higher non-*H. pylori* prevalence was found in the younger age group (less than 50 years old, $P = 0.048$). The result must be interpreted with caution.

The pathogenesis of NUD was not well understood and a number of hypotheses have been proposed. The role of *H. pylori* infection in NUD remains controversial too[17]. A high prevalence of non-*H. pylori* bacteria in NUD group observed in this study gave us a new clue that non-*H. pylori* bacteria may play a role in the pathogenesis of NUD.

Urease is a major virulent factor for *H. pylori*, which aids in neutralizing hydrochloric acid and allows *H. pylori* to colonize the gastric mucosa. Some rapid methods for *H. pylori* diagnosis, $^{[13C]}$urea breath test (UBT) and RUT, were also based on the urease activity. But *H. pylori* is not the only bacteria which can produce urease. It has been reported that the colonization and overgrowth of urease-producing bacteria other than *H. pylori* induced false-positive UBT results[18,19]. All the 104 patients were RUT-positive, but one of them was *H. pylori*-negative based on the further detection and no urease-positive non-*H. pylori* bacteria was isolated. So role of the urease-positive non-*H. pylori* bacteria to cause false-positive results in RUT was not definite in this study.

Overall, a high prevalence of non-*H. pylori* bacteria obtained in this study, the major of which were *Streptococcus, Neisseria, Rothia* and *Staphylococcus*, differed from the previous report of healthy volunteers. It should be noted that this study only examined cultured bacteria. But not all flora can be cultivated due to the harsh culture condi-

tion, so the prevalence of the non-*H. pylori* bacteria may underestimate here. The remained question is whether these non-*H. pylori* species contribute to the pathogenesis of gastric disorders. Further studies should be done to elucidate it in the future.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection has been known to trigger a variety of gastric disorders. Recent years, non-*H. pylori* bacteria have also been reported to play roles in the development of upper gastrointestinal disease, but little is known about the stomach bacterial flora. Analysis of stomach bacterial flora was performed in order to stimulate future research in this area.

Research frontiers

The pathogenesis of gastric disorders is complicated. Besides *H. pylori*, considerable interest has emerged in the co-infection in the stomach with non-*H. pylori* bacteria.

Innovations and breakthroughs

To date, there has only a limited number of studies regarding the stomach bacterial flora and all are based on a few patients or volunteers. In this study, the authors enrolled 103 *H. pylori* positive patients with upper gastrointestinal diseases and employed forefront matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) bacterial identification technique, which is more accurate and rapid than the routine methods or genotypic identification. Furthermore, the authors confirmed a high prevalence of the non-*H. pylori* bacteria concurrent with *H. pylori* in the patients.

Applications

A high prevalence of the non-*H. pylori* bacteria and the evaluation of the bacterial species found in gastric tissues from *H. pylori* infected patients would stimulate future research to elucidate the role of the mixed infection of the stomach in the pathogenesis of the upper gastrointestinal diseases.

Terminology

MALDI-TOF MS fingerprinting is a fast and reliable method for the classification and identification of micro-organisms. The BioTyper™ MALDI-TOF MS fingerprinting system allows researchers to perform this process for the unambiguous identification of bacteria, yeasts and fungi in minutes by measuring the exact sizes of peptides and small proteins, which are assumed to be characteristic for each species.

Peer review

The study provided an interesting evaluation of the bacterial species found in gastric tissues from *H. pylori* infected persons.

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