Analysis of culturable microbiota present in the stomach of children with gastric symptoms

Changcheng Guo¹ · Fang Liu¹ · Li Zhu² · Fangcao Wu¹ · Guzhen Cui¹ · Yan Xiong² · Qiong Wang¹ · Lin Yin¹ · Caixia Wang¹ · Huan Wang¹ · Xiaojuan Wu¹ · Zhengrong Zhang¹ · Zhenghong Chen^{1,3}

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Abstract

Despite extensive studies on the gastric microbiota, including *Helicobacter pylori* and non-*H. pylori*, the bacterial composition in children remains unknown. In this study, we analyzed the culturable gastric bacteria in stomach biopsies from 346 children aged 1–15 years affected by gastric diseases. *H. pylori* and non-*H. pylori* were identified by specific PCR and 16S rDNA sequencing, respectively. Antibiotic susceptibilities of *H. pylori* and non-*H. pylori* were tested by the E-test and disk diffusion methods, respectively. Rapid diagnosis was also performed by *H. pylori*-specific PCR. Twenty-two *H. pylori* strains were obtained from culture, and 92 biopsies were positive by *H. pylori*-specific PCR. The positive rate was higher in boys (40.3%) than in girls (23.3%) ($P=0.001$). Resistance rates of 22 *H. pylori* strains were as follows: metronidazole, 86.4%; tetracycline, 22.7%; amoxicillin, 22.7%; levofloxacin, 31.8%; clarithromycin, 36.4%. Ten isolates were multidrug-resistant. Additionally, among 366 non-*H. pylori* strains, 204 exhibited urease activity. Non-*H. pylori* resistance rates were as follows: metronidazole, 94.8%; tetracycline, 26.2%; amoxicillin, 42.6%; levofloxacin, 15.3%; clarithromycin, 46.7%. Our results showed that children with gastric disorders harbor stomach bacteria with urease activity or nitrate reductase activity. Further studies will determine the effects of non-*H. pylori* bacteria in gastric diseases.

Keywords *Helicobacter pylori* · Gastric microbiota · Urease · Resistance

Introduction

A previously widely held view was that the gastric secretions of hydrochloric acid and the proteolytic enzyme pepsin ensure a sterile stomach [1, 2]. In 1983, Australian researchers Marshall and Warren first successfully isolated and cultured *Helicobacter pylori* from the human gastric mucosa [3].

H. pylori is a Gram-negative, microaerophilic bacterium that can tolerate an acidic environment and has invasion ability, although most infected individuals do not show obvious symptoms [4, 5]. This bacterium is able to circumvent the sophisticated immune defenses of its hosts and selectively colonizes the human stomach mucosa [6]. *H. pylori* can cause a wide array of gastric disorders and is one of the most important risk factors for chronic gastritis, peptic ulcers, gastric mucosa-associated lymphoid tissue lymphoma, and gastric cancer [7–10]. *H. pylori*-induced inflammation also damages wall cell function, alters pH values in the stomach, and provides conditions for the colonization of other microorganisms. Nevertheless, the colonization of some bacteria is not affected by *H. pylori* infection, and these are typical bacteria in the mouth and respiratory tract, such as *Streptococcus* and *Pseudomonas* [11, 12].

In addition to conventional culture techniques, which are still valuable for analyses of gastric microbiota, high-throughput next-generation sequencing has enabled detailed studies of the human gastric microbiome. Indeed, investigations with modern techniques have shown that the microbiota of the stomach includes hundreds of phylotypes, with a microbial density between 10^1 and 10^4 colony-forming units

Changcheng Guo, Fang Liu and Li Zhu contributed equally to this work.

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✉ Zhenghong Chen
chenzhenghong@gmc.edu.cn

¹ School of Basic Medical Science, Key Laboratory of Medical microbiology and parasitology of Education Department of Guizhou, Guizhou Medical University, No.1 South Dongqing Road, Guiyang 550025, China

² Clinical Medical School of Maternal and Child Affiliated to Guizhou Medical University, No.63 South Ruijin Road, Guiyang 550003, Guizhou, China

³ Department of Microbiology, School of Basic Medical Science, Guizhou Medical University, Guiyang, China

(CFU)/g [1, 2, 13, 14]. Current data suggest that the healthy human stomach holds a core microbiome dominated by *Prevotella*, *Streptococcus*, *Veillonella*, *Rothia*, and *Haemophilus*. However, a shift in the abundance of the phylum *Firmicutes* and the genera *Streptococcus* and *Prevotella* can be found in the *H. pylori*-infected stomach and in gastric cancer (GC) [1]. Five GC-enriched bacterial taxa identified as *Peptostreptococcus stomatis*, *Streptococcus anginosus*, *Parvimonas micra*, *Slackia exigua*, and *Dialister pneumosintes* have significant centralities in GC [15].

We previously isolated *H. pylori* from gastric mucosae of patients with gastric disease, in addition to a variety of non-*H. pylori* bacteria, and numerous strains were urease-positive, such as species of *Pseudomonas*, *Neisseria*, and *Actinomyces* [16]. Some studies have reported gastric non-*H. pylori* in adults with *H. pylori* infections. [17, 18] However, *H. pylori* infections often occur in childhood and few studies have examined the gastric flora in children; thus, the specific effects of gastric diseases on the microbiota in children are unclear. Accordingly, in this study, we isolated *H. pylori* and non-*H. pylori* taxa from gastric mucosa samples collected from children with stomach disorders and evaluated the antibiotic susceptibility of these strains. These analyses provide insight into the impact of *H. pylori* infection on the composition of the gastric microbiota and provide a basis for understanding the differences in gastric flora between adults and children with gastric diseases.

Materials and methods

Study population—samples

From August 2016 to June 2017, 346 gastric biopsy specimens were collected aseptically from children (age, 1–15 years, mean age = 7.44 ± 3.9 years, median = 7.8 years; M/F = 1.31:1) undergoing painless gastroendoscopy at Guiyang Children's Hospital. Subjects who received antibiotics, probiotics, proton pump inhibitors, or H2 receptor antagonists within 1 month of specimen collection were excluded. When the patients were anesthetized, the aseptic gastroscope goes through the teeth cushion in the oral cavity and the pharynx. Then, it enters into the esophagus via anterior lateral hypopharynx. The disposable sterile biopsy forceps then enter through the endoscope into the stomach cavity to collect the specimen. All biopsy samples were transported in brain heart infusion (BHI) broth with glycerol (10%) to the laboratory. This study was approved by the Institutional Review Board and Institutional Ethics Committees of the Children's Hospital of Guiyang. Before study entry, informed oral consent was obtained from the parents or guardians of each child.

H. pylori and non-*H. pylori* isolation

Gastric biopsy specimens were homogenized [17, 19], and 100 μ L of each homogenate was inoculated into selective BHI agar base (Oxoid Ltd., Hants, UK) containing 10% defibrinated sheep blood, vancomycin (10 mg/L), trimethoprim (5 mg/L), cefsulodin (5 mg/L), and amphotericin B (5 mg/L) (Oxoid Ltd.). Plates were incubated for 3–5 days at 37 °C in a microaerobic atmosphere (10% CO₂, 85% N₂, 5% O₂) with 95% relative humidity (Micro Aerobic Bag; Mitsubishi Chemical Corporation, Tokyo, Japan). *H. pylori* isolates were confirmed based on colony morphology, Gram staining, positive urease, oxidase, and catalase reactions. Genomic DNAs of suspected *H. pylori* strains were extracted using a kit (TIANGEN, Beijing, China) and then amplified with *H. pylori*-specific 16S rDNA primers (F: 5'-CTTGCTAGAGTGCTGA-3'; R: 5'-TCCCACACTCTAGAATAGT-3') [20]. Isolates were then subcultured for susceptibility tests. *H. pylori* NCTC 11637 was used as a control.

Another 100 μ L of biopsy homogenate was added to BHI agar base (Oxoid, Basingstoke, Hampshire, UK) containing 10% defibrinated sheep blood for non-*H. pylori* isolation. Plates were incubated for 3 days at 37 °C under 10% CO₂ and 95% relative humidity. Gram staining and urease, catalase, and oxidase tests were also performed. Genomic DNAs of these non-*H. pylori* strains were extracted using a kit (TIANGEN) and amplified and sequenced with universal primers for the bacterial 16S rRNA gene (27F: 5'-AGAGTTTGATCCTGGCTC-3'; 1492R: 5'-ACGGCTACCTTGTTACGACTT-3') [21]. A sequence analysis was performed using NCBI BLAST to identify strains.

Rapid diagnosis of *H. pylori* infection

The rest of the biopsy homogenates were then used for rapid diagnosis of *H. pylori* infection. Total DNAs were extracted from these homogenates using the tissue genomic DNA Extraction Kit (TIANGEN) and amplified with *H. pylori*-specific 16S rDNA primers (F: 5'-CTTGCTAGAGTGCTGATTA-3'; R: 5'-TCCCACACTCTAGAATAGT-3') [20]. *H. pylori* NCTC 11637 was used as a control.

Susceptibility tests for *H. pylori* strains

H. pylori susceptibility was tested using the Epsilometer test (E-test) [22]. Susceptibility tests were performed using 100 μ L of frozen isolates that were subcultured on BHI agar containing 10% defibrinated sheep blood and incubated for 3 days at 37 °C under microaerophilic conditions. The colonies were suspended in BHI broth, adjusted to approximately 1×10^8 cfu/mL, and then inoculated into Mueller–Hinton agar (Hangzhou Binhe, Hangzhou, China). Minimum inhibitory concentrations (MIC) were determined using E-test strips

(BIOMERIUX, FRANCE) with antimicrobial concentration ranges from 0.016 to 256 µg/mL for clarithromycin, tetracycline, metronidazole, and amoxicillin and 0.002 to 32 µg/mL for levofloxacin. After inoculation, the plates were dried at 37 °C under microaerophilic conditions and E-test strips containing an increasing gradient of antimicrobial concentrations were applied to the surface of the agar plate. The plates were incubated at 37 °C under microaerophilic conditions (5% O₂, 10% CO₂, and 85% N₂ at 95% humidity) for 72 h. The MIC values were determined by the intersection of the elliptical zone of inhibition with the graded strip after 72 h. The MICs for antimicrobial agents were interpreted based on the literature; resistance was determined at a MIC of ≥ 1 µg/mL for clarithromycin, ≥ 8 µg/mL for metronidazole, > 0.5 µg/mL for amoxicillin, ≥ 4 µg/mL for tetracycline, and ≥ 1 µg/mL for levofloxacin [19, 22–24].

Susceptibility tests for non-*H. pylori* strains

Susceptibility of non-*H. pylori* strains was tested by the Kirby–Bauer method. Antibiotic filter paper disks included amoxicillin (10 µg/slice; Hangzhou Binhe), clarithromycin (15 µg/slice; Hangzhou Binhe), metronidazole (5 µg/slice; Shanghai Jinshui, Shanghai, China), levofloxacin (5 µg/slice; Hangzhou), and tetracycline (30 µg/slice; Hangzhou). Pure cultures of non-*H. pylori* strains were suspended in BHI broth, adjusted to McFarland 4 turbidity standard (approximately 1×10^8 cfu/mL), and then inoculated into Mueller–Hinton agar containing 10% defibrinated sheep blood with a sterilized cotton swab. Antibiotic disks were placed on the surface of the agar. The plates were incubated at 37 °C under 10% CO₂ and 95% humidity for 16–18 h. Susceptibility was interpreted according to the Clinical Laboratory Standards Institute (CLSI) [25].

Statistical analysis

Comparisons of the *H. pylori*-positive rate between boys and girls and between school-aged children and pre-school children and comparisons of non-*H. pylori* taxa or frequency depending on *H. pylori* status or sex were evaluated using χ^2 tests. Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) and $P < 0.05$ was considered statistically significant.

Results

In 346 children, 22 *H. pylori* strains were obtained from culture, and 92 biopsies were positive according to *H. pylori*-specific PCR for rapid diagnosis. The total positive rate was 32.9% (114/346).

A total of 366 non-*H. pylori* strains were obtained from 189 biopsies, and the species were determined by 16S rDNA sequencing (Table 1); no non-*H. pylori* strains were isolated from the other 157 biopsies. A total of 247 non-*H. pylori* strains were Gram-positive and 119 strains were Gram-negative. Additionally, 204 (55.7%) strains were urease-positive. The *H. pylori*-positive rate for samples in which non-*H. pylori* isolates were obtained in culture was 43.9% (83/189), including eight samples that were *H. pylori* isolation-positive and 75 that were only positive by specific PCR. The *H. pylori*-positive rate for biopsies in which non-*H. pylori* bacteria were not obtained by the culture method was 19.7% (31/157), including 14 biopsies that were *H. pylori* isolation-positive and 17 biopsies that were only positive by specific PCR (Table 2).

H. pylori positivity in males was 40.3% (79/196), which was greater than the rate in female subjects (23.3%, 35/150) ($\chi^2 = 11.080$, $P = 0.001$, Table 3). All children were divided into two age groups, i.e., 1–4 years (pre-school age) and 5–15 years (school age). The *H. pylori* infection rate was higher in the school age group (35.1%, 105/299) than in the pre-school age group (19.1%, 9/47) ($\chi^2 = 4.688$, $P = 0.030$, Table 3).

Susceptibility of *H. pylori*

The resistance rates of 22 strains of *H. pylori* determined by the E-test were as follows: metronidazole, 86.4%; tetracycline, 22.7%; amoxicillin, 22.7%; levofloxacin, 31.8%; clarithromycin, 36.4%. Ten strains were multidrug-resistant (MDR), including nine that were resistant to three antibiotics of different classes and one strain that was resistant to four antibiotics (Table 4).

Susceptibility of non-*H. pylori* bacteria

The resistance rates of non-*H. pylori* bacteria to metronidazole, tetracycline, amoxicillin, levofloxacin, and clarithromycin were 94.8%, 26.2%, 42.6%, 15.3%, 46.7%, respectively (Table 5). A total of 63 (11.2%) isolates were MDR against these antibiotics, which are commonly used for eradication of *H. pylori* in children, and one strain identified as *Streptococcus mitis* was resistant to five antibiotics.

Discussion

Owing to sampling limitations, the gastric flora composition and its functions in healthy individuals or in gastric disease development are still unclear. In this study, we detected bacteria, including *H. pylori*, from gastric biopsies of children residing in Guiyang, the capital city of Guizhou province in southwestern China. The children had gastric disorders with symptoms consistent with *H. pylori* infection.

Table 1 Non-*H. pylori* bacteria isolated from gastric biopsies

Bacteria	N	Urease activity	<i>H. pylori</i> , N (%)		<i>P</i>	Male, N (%)	Female, N (%)	<i>P</i>
			Positive	Negative				
<i>Streptococcus</i>	159		58 (50.9)	101 (43.5)	0.198	91 (46.1)	68 (45.3)	0.839
<i>Streptococcus mitis</i>	76	–	28	48	0.414	36	40	0.065
<i>Streptococcus salivarius</i>	28	–	11	17	0.457	19	9	0.212
<i>Streptococcus pneumoniae</i>	12	–	5	7	0.513	9	3	0.192
<i>Streptococcus parasanguinis</i>	10	–	4	6	0.630	7	3	0.387
<i>Streptococcus thermophilus</i>	5	–	2	3	0.735	4	1	0.289
<i>Streptococcus sanguinis</i>	3	–	1	2	0.989	2	1	0.729
<i>Streptococcus oralis</i>	5	–	1	4	0.989	1	4	0.289
<i>Streptococcus australis</i>	3	–	0	3	0.223	2	1	0.725
<i>Streptococcus cristatus</i>	2	–	1	1	0.607	2	0	0.215
<i>Streptococcus rubneri</i>	3	–	1	2	0.989	3	0	0.059
<i>Streptococcus infantis</i>	4	–	1	3	0.734	1	3	0.199
<i>Streptococcus dentisani</i>	2	–	1	1	0.607	1	1	0.849
<i>Streptococcus pseudopneumoniae</i>	5	–	2	3	0.735	3	2	0.879
<i>Streptococcus tigurinus</i>	1	–	0	1	0.483	1	0	0.381
<i>Neisseria</i>	108		35 (30.7)	73 (31.5)	0.885	62 (31.6)	46 (30.7)	0.848
<i>Neisseria mucosa</i>	22	+	7	15	0.907	14	8	0.494
<i>Neisseria subflava</i>	17	+	6	11	0.833	14	3	0.028
<i>Neisseria perflava</i>	27	+	8	19	0.702	11	16	0.082
<i>Neisseria meningitidis</i>	14	+	5	9	0.001	9	5	0.556
<i>Neisseria macacae</i>	3	+	1	2	0.989	2	1	0.725
<i>Neisseria lactamica</i>	2	+	1	1	0.607	0	2	0.105
<i>Neisseria sicca</i>	1	+	1	0	0.153	0	1	0.252
<i>Neisseria pharyngis</i>	2	+	0	2	0.320	2	0	0.215
<i>Neisseria cinerea</i>	5	+	2	3	0.735	1	4	0.096
<i>Neisseria flavescens</i>	15	+	4	11	0.597	9	6	0.789
<i>Rothia</i>	42		16 (12.9)	26 (11.2)	0.636	25 (12.8)	17 (11.3)	0.688
<i>Rothia mucilaginosa</i>	27	+	9	18	0.965	18	9	0.274
<i>Rothia dentocariosa</i>	9	+	6	3	0.029	5	4	0.928
<i>Rothia aeria</i>	6	+	1	5	0.392	2	4	0.245
<i>Actinomyces</i>	12		3 (2.6)	9 (3.9)	0.551	6 (3.1)	6 (4.0)	0.636
<i>Actinomyces viscosus</i>	3	+	2	1	0.212	3	0	0.128
<i>Actinomyces naeslundii</i>	3	+	0	3	0.223	2	1	0.725
<i>Actinomyces graevenitzii</i>	2	+	0	2	0.320	1	1	0.849
<i>Actinomyces oris</i>	3	+	1	2	0.989	0	3	0.047
<i>Actinomyces odontolyticus</i>	1	+	0	1	0.483	0	1	0.252
<i>Staphylococcus</i>	13		4 (3.5)	9 (3.9)	0.853	7 (3.6)	6 (4.0)	0.835
<i>Staphylococcus aureus</i>	6	+	3	3	0.370	4	2	0.617
<i>Staphylococcus haemolyticus</i>	1	+	0	1	0.483	0	1	0.252
<i>Staphylococcus cohnii</i>	3	+	0	3	0.223	0	3	0.047
<i>Staphylococcus pasteurii</i>	1	+	1	0	0.153	1	0	0.381
<i>Staphylococcus epidermidis</i>	2	+	0	2	0.320	2	0	0.215
<i>Micrococcus</i>	7		2 (1.8)	5 (2.2)	0.803	4 (2.0)	3 (2.0)	0.979
<i>Micrococcus luteus</i>	3	+	0	3	0.223	2	1	0.725
<i>Micrococcus antarcticus</i>	4	+	2	2	0.466	2	2	0.787
<i>Microbacterium</i>	2		1 (0.9)	1 (0.4)	0.607	2 (1.0)	0	0.215
<i>Microbacterium oxydans</i>	1	–	1	0	0.153	1	0	0.381

Table 1 (continued)

Bacteria	N	Urease activity	<i>H. pylori</i> , N (%)		<i>P</i>	Male, N (%)	Female, N (%)	<i>P</i>
			Positive	Negative				
<i>Microbacterium maritypicum</i>	1	–	0	1	0.483	1	0	0.381
Others	23		12	11		15	8	
<i>Kytococcus sedentarius</i>	2	+	2	0	0.043	0	2	0.105
<i>Lactobacillus vaginalis</i>	2	+	1	1	0.607	2	0	0.849
<i>Haemophilus influenzae</i>	6	+	3	3	0.370	3	3	0.725
<i>Gemella haemolysans</i>	5	+	2	3	0.735	2	3	0.449
<i>Bacilli bacterium</i>	1	+	0	1	0.483	1	0	0.381
<i>Aerococcus urinaequi</i>	1	+	1	0	0.153	1	0	0.381
<i>Kocuria palustris</i>	1	+	1	0	0.153	1	0	0.381
<i>Cardiobacterium hominis</i>	1	–	0	1	0.483	1	0	0.381
<i>Acinetobacter johnsonii</i>	1	+	1	0	0.153	1	0	0.381
<i>Pseudomonas plecoglossicida</i>	1	+	0	1	0.483	1	0	0.381
<i>Moraxella catarrhalis</i>	1	+	0	1	0.153	1	0	0.381
<i>Eikenella corrodens</i>	1	+	1	0	0.153	1	0	0.381
Total	366		131	235		212	154	

N number of biopsies; % percentage depending on *H. pylori* status or sex

Many studies have reported *H. pylori* infections in children and adolescents [26–29]. Transmission of *H. pylori* is related to personal care practices and varies widely depending on geographic area, age, and socioeconomic status. *H. pylori* prevalence may exceed 70% in undeveloped regions and may be 40% or lower in developed regions [26]. In China, there is a high prevalence of *H. pylori* infection, i.e., greater than 50% [27]. Because tests for *H. pylori* infection are not regularly performed during physical checkups in children, little is known about the *H. pylori* prevalence in children. In Japanese children, the *H. pylori* infection rate is less than 2% [26, 28]. In Bhutan, the overall prevalence of *H. pylori* in 327 children between the ages of 4 and 19 years was 66% [29]. In this study, the *H. pylori* infection rate in 346 children with stomach symptoms was 32.9%, and the *H. pylori* infection rate was lower in pre-school-aged (1–4 years) children than in school-aged children (5–15 years), consistent with reports that the rate of infection with *H. pylori* increases with age.

An interesting analysis by Ibrahim et al. reveals that the prevalence of infection is twice as high in men as in women of all ages [30]. Consistent with the meta-analysis by Ibrahim

et al., this study showed that *H. pylori* infection rate in males was 40.3%, which was greater than that in female subjects (23.30%). Among children, sex-related differences in the prevalence of *H. pylori* infection may be explained by differences in health habits and exposure to antibiotics. In China, girls or girls' parents usually pay more attention to food hygiene than boys or boys' parents do. Most girls also have more personal hygiene habits than boys have. On the contrary, a higher incidence of urinary tract infections in girls may be associated with a greater use of antibiotics [31]. Even those antibiotics not specifically indicated for *H. pylori* eradication could contribute to clearance of the infection and may explain sex-related differences among children. Additionally, in adults, women may be more likely to clear *H. pylori* infection because of higher rates of incidental antibiotic use for other indications [32].

The use of anti-*H. pylori* drugs, including amoxicillin, clarithromycin, levofloxacin, metronidazole, and tetracycline, carries a risk of the emergence of resistant strains [33, 34]. Antibiotic resistance is the most crucial factor leading to the failure of eradication. The resistance rates of *H. pylori* in children were higher in this study than in developed areas of

Table 2 Comparison of *H. pylori*-positive rate for biopsies with or without non-*H. pylori* in culture

Groups	<i>Hp</i> -positive, N		<i>Hp</i> -positive rate	χ^2	<i>P</i>	Male, N (%)	Female, N (%)	χ^2	<i>P</i>
	Isolation	Specificity PCR							
Non- <i>Hp</i> -positive in culture (N = 189)	8	75	43.9%	22.678	0.000	129 (68.3)	60 (31.7)	22.848	0.000
Non- <i>Hp</i> -negative in culture (N = 157)	14	17	19.7%			67 (42.7)	90 (57.3)		

Hp *Helicobacter pylori*, N number of biopsies

Table 3 Demographic data and prevalence of *H. pylori* in 346 patients

	<i>H. pylori</i>		Total	χ^2	<i>P</i>
	Positive, <i>N</i> (%)	Negative, <i>N</i> (%)			
Sex					
Male	79 (40.3)	117 (59.7)	196	11.080	0.001
Female	35 (23.3)	115 (76.7)	150		
Age groups (years)					
1–4	9 (19.1)	38 (80.9)	47	4.688	0.030
5–15	105 (35.1)	194 (64.9)	299		
Living condition					
Village	50 (31.1)	111 (68.9)	161	0.440	0.507
City	64 (34.6)	121 (65.4)	185		

H. pylori-positive samples included culture-positive or *H. pylori*-specific PCR-positive biopsy samples. *N* number of biopsies, % percentage in the respective group

southeast China [35]. MDR strains and tetracycline-resistant strains were discovered in this study, even though this antibiotic is typically used with caution in children. These results suggest that the resistant strain might be transmitted from adults to children. It is important to continue monitoring the resistance of *H. pylori*. Individualized therapy based on the results of susceptibility testing is a good strategy.

Table 4 MIC values of 22 *H. pylori* strains determined by E-tests

Number of the strain	CLA	AMX	LEV	TET	MET
1	< 0.016 (S)	0.25 (S)	0.023 (S)	1 (S)	6 (S)
2	0.047 (S)	0.016 (S)	2 (R)	0.064 (S)	64 (R)
3	3 (R)	0.064 (S)	> 32 (R)	0.75 (S)	> 256 (R)
4	0.032 (S)	0.032 (S)	16 (R)	0.25 (S)	48 (R)
5	< 0.016 (S)	2 (R)	3 (R)	1.5 (S)	96 (R)
6	0.023 (S)	4 (R)	0.25 (S)	0.50 (S)	64 (R)
7	< 0.125 (S)	3 (R)	2 (R)	1.0 (S)	32 (R)
8	4 (R)	0.023 (S)	0.032 (S)	24 (R)	64 (R)
9	6 (R)	0.094 (S)	0.19 (S)	24 (R)	> 256 (R)
10	12 (R)	0.032 (S)	0.38 (S)	0.75 (S)	64 (R)
11	8 (R)	< 0.016 (S)	0.047 (S)	1.5 (S)	0.38 (S)
12	0.25 (S)	4 (R)	0.25 (S)	0.38 (S)	64 (R)
13	2 (R)	0.25 (S)	0.064 (S)	24 (R)	48 (R)
14	6 (R)	0.38 (S)	2 (R)	0.094 (S)	128 (R)
15	0.25 (S)	0.023 (S)	0.125 (S)	0.38 (S)	64 (R)
16	0.023 (S)	0.25 (S)	0.023 (S)	0.125 (S)	32 (R)
17	0.032 (S)	3 (R)	0.064 (S)	48 (R)	64 (R)
18	0.016 (S)	4 (R)	0.094 (S)	24 (R)	16 (R)
19	0.032 (S)	0.016 (S)	0.125 (S)	0.50 (S)	32 (R)
20	0.016 (S)	< 0.016 (S)	0.25 (S)	3 (S)	0.75 (S)
21	0.25 (S)	2 (R)	0.19 (S)	1 (S)	16 (R)
22	3 (R)	4 (R)	4 (R)	0.75 (S)	> 256 (R)

MTZ metronidazole, *TET* tetracycline, *LEV* levofloxacin, *CLA* clarithromycin, *AMX* amoxicillin, *R* resistant, *S* susceptible

Table 5 Resistance of 366 non-*H. pylori* strains

Antibiotic	Resistance (<i>N</i>)	Resistance rate (%)
CLA	171	46.7
AMX	156	42.6
LEV	56	15.3
MET	347	94.8
TET	96	26.2

MTZ metronidazole, *TET* tetracycline, *LEV* levofloxacin, *CLA* clarithromycin, *AMX* amoxicillin, *N* number of strains

Human gastric juice is mainly composed of proteolytic enzymes and hydrochloric acid; the gastric lumen has a low pH (pH 1–2), but this can increase up to pH ≥ 5 by food ingestion and the use of antibiotics, antacids, H₂ blockers, and proton pump inhibitors, enabling the survival and proliferation of microbes that are normally killed in acidic conditions. It has been suggested that non-*H. pylori* bacteria and their by-products act as a persistent antigenic stimulus or augment the inflammatory response induced by *H. pylori* infection [36]. Increasing evidence suggests that the stomach possesses a distinct and complex ecosystem inhabited by a wide variety of bacteria. Homeostasis of the stomach microecology is crucial for maintaining health; its perturbation can trigger various stomach diseases, and the

microbiota has profound effects on the regulation of the immune system [37]. Hundreds of bacterial species have been detected in the human stomach, and most of them belong to five phyla: *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Fusobacteria*, and *Bacteroidetes* [38]. The maintenance of the ecological balance of the stomach relies on *Streptococcus*, *Lactobacillus*, *Clostridium*, *Veillonella*, *Haemophilus*, *Enterococcus*, *Pseudomonas*, *Neisseria*, *Bifida*, and other taxa [39]. A disruption in the ecological balance can cause discomfort of the stomach, dysbacteria, and even immune deficiency [40, 41].

In this study, non-*H. pylori* species in the gastric mucosa were isolated, most of which were upper respiratory tract microflora, consistent with previous results [3, 17, 42]. However, the dominant species were different from those in previous reports of healthy adult volunteers, which were predominantly acid-resistant species, such as *Veillonella* and *Lactobacillus* species [43]. In this study, the dominant species were *Streptococcus*, *Neisseria*, *Rothia*, and *Staphylococcus*, similar to the non-*H. pylori* species detected in the stomach of Chinese adults with *H. pylori* infection [17], but differed from those of adults with gastric diseases in Malaysia, whose dominant flora included *Streptococcus*, *Neisseria*, *Lactobacillus*, and *Klebsiella* [18]. To avoid contamination by oral microflora, biopsies were collected by disposable sterile forceps during painless endoscopy. The gastroscope tube went into the gastric cavity only when the patients were anesthetized and gastric cavity could be observed clearly under the gastroscope. The tube went directly into the cavity to avoid damage of the mucous membrane of glottis, esophagus, and stomach. Furthermore, the gastroscope only stayed in the cavity and never touched the membrane. Only the disposable forceps went through the tube and touched the gastric mucous membrane. Even if some bacteria isolated in this study came from saliva into the stomach, only those colonized on the mucous membrane of the stomach under certain circumstance, such as the already damaged membrane by *H. pylori*, or the bacteria showing urease activity and acid stability could be alive.

Urease is a major virulence factor for *H. pylori*. It aids in neutralizing hydrochloric acid and allows *H. pylori* to colonize the gastric mucosa. Some rapid methods for *H. pylori* diagnosis are based on urease activity; for example, the ^{13}C urea breath test and rapid urease test are widely used in clinical settings. Urease-positive non-*H. pylori* species, including *Proteus mirabilis*, *Micrococcus*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas fluorescens*, *Neisseria flavescens*, and *Enterobacter* [44–48], have been found in gastric biopsies. In this study, urease-positive non-*H. pylori* bacteria were also isolated from 12 biopsy samples that were negative both by *H. pylori* culture and rapid diagnosis by *H. pylori*-specific PCR. These strains included *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas plecoglossicida*, *Acinetobacter johnsonii*, *Neisseria mucosa*, *Neisseria flavescens*, *Neisseria*

meningitidis, *Neisseria perflava*, *Rothia mucilaginosa*, and *Micrococcus luteus*, which could cause false positive results in the ^{13}C urea breath test and affect the diagnosis of *H. pylori* infection. Future studies should examine whether these urease-positive bacteria in the stomach act as pathogens or are normal components of the flora.

Some non-*H. pylori* isolated in this study were nitrate-reducing bacteria, such as *Staphylococcus haemolyticus*, *Staphylococcus epidermidis*, *Neisseria mucosa*, *Micrococcus luteus*, *Rothia dentocariosa*, and *Actinomyces naeslundii*. It has been reported that a nitrosating microbial community contributes to gastric malignant transformation by increasing intragastric concentrations of nitrite and *N*-nitroso compounds [38]. It is necessary to determine whether these strains with nitrate-reducing activity are related to gastric cancer, particularly after long-term proliferation in the stomach of children. Accordingly, further research is needed to determine the role of non-*H. pylori* nitrate-reducing activity in the stomach.

Furthermore, six strains of *Rothia aeria* with urease activity were isolated in this study. This bacterium is a newly discovered opportunistic pathogen known to colonize the human oral cavity [49] and the upper gastrointestinal tract [50]. It has also been isolated from patients with acute diarrhea and recurrent fever from blood samples [51]. When the gastric mucosa is destroyed by infection with *H. pylori*, *Rothia aeria* might enter the blood and induce bacteremia.

Few studies have examined the antibiotic resistance profiles of gastric non-*H. pylori* taxa. Zeng et al. [48] isolated a strain of urease-positive *Neisseria flavescens* and found that it was resistant to amoxicillin, streptomycin, and ampicillin, which are used as the first-line antibiotics in patients with gastritis. In this study, non-*H. pylori* in the stomach of children exhibited serious drug resistance, including MDR, to commonly used antibiotics for the treatment of clinical gastritis. MDR *H. pylori* is a serious problem in *H. pylori* eradication. Resistance mechanisms include point mutations in various genes or plasmids. The transformation of such genes or plasmids may lead to the dissemination of resistance among *H. pylori* strains [52]. A similar transformation might also occur between *H. pylori* and non-*H. pylori* in the stomach. The overgrowth of some species under antibiotic stress might cause an imbalance in the flora of the stomach ecosystem and lead to serious gastric disorders. More studies focused on the relationship between the stomach flora and gastric diseases are needed.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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References

- Nardone G, Compare D (2015) The human gastric microbiota: is it time to rethink the pathogenesis of stomach diseases? *United European Gastroenterol J* 3(3):255–260. <https://doi.org/10.1177/2050640614566846>
- Hunt RH, Yaghoobi M (2017) The esophageal and gastric microbiome in health and disease. *Gastroenterol Clin N Am* 46(1):121–141. <https://doi.org/10.1016/j.gtc.2016.09.009>
- Warren JR, Marshall B (1983) Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1(8336):1273–1275. [https://doi.org/10.1016/S0140-6736\(83\)92719-8](https://doi.org/10.1016/S0140-6736(83)92719-8)
- Wang F, Meng W, Wang B, Qiao L (2014) *Helicobacter pylori*-induced gastric inflammation and gastric cancer. *Cancer Lett* 345(2):196–202. <https://doi.org/10.1016/j.canlet.2013.08.016>
- Bugaytsova JA, Björnham O, Chernov YA, Gideonsson P, Henriksson S, Mendez M, Sjöström R, Mahdavi J, Shevtsova A, Ilver D, Moonens K, Quintana-Hayashi MP, Moskalenko R, Aisenbrey C, Bylund G, Schmidt A, Åberg A, Brännström K, Königer V, Vikström S, Rakhimova L, Hofer A, Ögren J, Liu H, Goldman MD, Whitmire JM, Ådén J, Younson J, Kelly CG, Gilman RH, Chowdhury A, Mukhopadhyay AK, Nair GB, Papadakis KS, Martinez-Gonzalez B, Sgouras DN, Engstrand L, Unemo M, Danielsson D, Suerbaum S, Oscarson S, Morozova-Roche LA, Olofsson A, Gröbner G, Holgersson J, Esberg A, Strömberg N, Landström M, Eldridge AM, Chromy BA, Hansen LM, Solnick JV, Lindén SK, Haas R, Dubois A, Merrell DS, Schedin S, Remaut H, Arnqvist A, Berg DE, Borén T (2017) *Helicobacter pylori* adapts to chronic infection and gastric disease via pH-responsive BabA-mediated adherence. *Cell Host Microbe* 21(3):376–389. <https://doi.org/10.1016/j.chom.2017.02.013>
- Li H, Yang T, Liao T, Debowski AW, Nilsson HO, Fulurija A, Haslam SM, Mulloy B, Dell A, Stubbs KA, Marshall BJ, Benghezal M (2017) The redefinition of *Helicobacter pylori* lipopolysaccharide O-antigen and core-oligosaccharide domains. *PLoS Pathog* 13(3):e1006280. <https://doi.org/10.1371/journal.ppat.1006280>
- Emara MH, Elhawari SA, Yousef S, Radwan MI, Abdel-Aziz HR (2016) Emerging role of probiotics in the management of *Helicobacter pylori* infection: histopathologic perspectives. *Helicobacter* 21(1):3–10. <https://doi.org/10.1111/hel.12237>
- Jiang J, Chen Y, Shi J, Song C, Zhang J, Wang K (2017) Population attributable burden of *Helicobacter pylori*-related gastric cancer, coronary heart disease, and ischemic stroke in China. *Eur J Clin Microbiol Infect Dis* 36(2):199–212. <https://doi.org/10.1007/s10096-016-2810-x>
- Tegtmeyer N, Wessler S, Necchi V, Rohde M, Harrer A, Rau TT, Asche CI, Boehm M, Loessner H, Figueiredo C, Naumann M, Palmisano R, Solcia E, Ricci V, Backert S (2017) *Helicobacter pylori* employs a unique basolateral type IV secretion mechanism for CagA delivery. *Cell Host Microbe* 22(4):552–560. <https://doi.org/10.1016/j.chom.2017.09.005>
- Sigal M, Logan CY, Kapalczynska M, Mollenkopf HJ, Berger H, Wiedenmann B, Nüsse R, Amieva MR, Meyer TF (2017) Stromal R-spondin orchestrates gastric epithelial stem cells and gland homeostasis. *Nature* 548(7668):451–455. <https://doi.org/10.1038/nature23642>
- Schulz C, Schütte K, Malfertheiner P (2016) *Helicobacter pylori* and other gastric microbiota in gastroduodenal pathologies. *Dig Dis* 34(3):210–216. <https://doi.org/10.1159/000443353>
- Franceschi F, Zuccalà G, Roccarina D, Gasbarrini A (2014) Clinical effects of *Helicobacter pylori* outside the stomach. *Nat Rev Gastroenterol Hepatol* 11(4):234–242. <https://doi.org/10.1038/nrgastro.2013.243>
- Hunt RH, Camilleri M, Crowe SE, el-Omar EM, Fox JG, Kuipers EJ, Malfertheiner P, McColl KEL, Pritchard DM, Rugge M, Sonnenberg A, Sugano K, Tack J (2015) The stomach in health and disease. *Gut* 64(10):1650–1668. <https://doi.org/10.1136/gutjnl-2014-307595>
- Sheh A, Fox JG (2013) The role of the gastrointestinal microbiome in *Helicobacter pylori* pathogenesis. *Gut Microbes* 4(6):505–531. <https://doi.org/10.4161/gmic.26205>
- Coker OO, Dai Z, Nie Y, Zhao G, Cao L, Nakatsu G, Wu WKK, Wong SH, Chen Z, Sung JJY, Yu J (2017) Mucosal microbiome dysbiosis in gastric carcinogenesis. *Gut* 67:1024–1032. <https://doi.org/10.1136/gutjnl-2017-314281>
- Yin L, Liu F, Guo CC et al (2016) Investigation of clinical significance of *Pseudomonas putida* isolated from gastric biopsies. *Chin J Zoonoses* 32(12):1102–1107 (In Chinese)
- Hu Y, He LH, Xiao D, Liu GD, Gu YX, Tao XX, Zhang JZ (2012) Bacterial flora concurrent with *Helicobacter pylori* in the stomach of patients with upper gastrointestinal diseases. *World J Gastroenterol* 18(11):1257–1261. <https://doi.org/10.3748/wjg.v18.i11.1257>
- Khosravi Y, Dieye Y, Poh BH et al (2014) Culturable bacterial microbiota of the stomach of *Helicobacter pylori* positive and negative gastric disease patients. *Scientific World J* 2014:610421. <https://doi.org/10.1155/2014/610421>
- Chen D, Cunningham SA, Cole NC et al (2017) Phenotypic and molecular antimicrobial susceptibility of *Helicobacter pylori*. *Antimicrob Agents Chemother* 61(4):e02530–e02516. <https://doi.org/10.1128/AAC.02530-16>
- Thoreson AC, Borre MB, Andersen LP et al (1995) Development of a PCR-based technique for detection of *Helicobacter pylori*. *Fems Immunol Med Mic* 10(3–4):325–334
- Wei G, Lu H, Zhou Z, Xie H, Wang A, Nelson K, Zhao L (2007) The microbial community in the feces of the giant panda (*Ailuropoda melanoleuca*) as determined by PCR-TGGE profiling and clone library analysis. *Microb Ecol* 54(1):194–202. <https://doi.org/10.1007/s00248-007-9225-2>
- Ogata SK, Gales AC, Kawakami E (2014) Antimicrobial susceptibility testing for *Helicobacter pylori* isolates from Brazilian children and adolescents: comparing agar dilution, E-test, and disk diffusion. *Braz J Mic* 45(4):1439–1448
- Liou JM, Chen CC, Chang CY, Chen MJ, Chen CC, Fang YJ, Lee JY, Yang TH, Luo JC, Wu JY, Liou TC, Chang WH, Hsu YC, Tseng CH, Chang CC, Bair MJ, Liu TY, Hsieh CF, Tsao FY, Shun CT, Lin JT, Lee YC, Wu MS, Taiwan Gastrointestinal Disease and Helicobacter Consortium (2016) Sequential therapy for 10 days versus triple therapy for 14 days in the eradication of *Helicobacter pylori* in the community and hospital populations: a randomised trial. *Gut* 65(11):1784–1792. <https://doi.org/10.1136/gutjnl-2015-310142>
- Liou JM, Chen CC, Chang CY, Chen MJ, Fang YJ, Lee JY, Chen CC, Hsu SJ, Hsu YC, Tseng CH, Tseng PH, Chang L, Chang WH, Wang HP, Shun CT, Wu JY, Lee YC, Lin JT, Wu MS, on behalf of the Taiwan Helicobacter Consortium (2013) Efficacy of genotypic resistance-guided sequential therapy in the third-line treatment of refractory *Helicobacter pylori* infection: a multicentre clinical trial. *J Antimicrob Chemother* 68(2):450–456. <https://doi.org/10.1093/jac/dks407>

25. CLSI (ed) (2018) Performance standards for antimicrobial susceptibility testing. 28th ed. CLSI supplement M100. Clinical and Laboratory Standards Institute, Wayne
26. Inoue M (2017) Changing epidemiology of *Helicobacter pylori* in Japan. *Gastric Cancer* 20(1):3–7. <https://doi.org/10.1007/s10120-016-0658-5>
27. Nagy P, Johansson S, Molloy-Bland M (2016) Systematic review of time trends in the prevalence of *Helicobacter pylori*, infection in China and the USA. *Gut Pathog* 8:8. <https://doi.org/10.1186/s13099-016-0091-7>
28. Ueda J, Gosho M, Inui Y, Matsuda T, Sakakibara M, Mabe K, Nakajima S, Shimoyama T, Yasuda M, Kawai T, Murakami K, Kamada T, Mizuno M, Kikuchi S, Lin Y, Kato M (2014) Prevalence of *Helicobacter pylori* infection by birth year and geographic area in Japan. *Helicobacter* 19(2):105–110. <https://doi.org/10.1111/hel.12110>
29. Wangda S, Richter JM, Kuenzang P, Wangchuk K, Choden T, Tenzin K, Malaty HM (2017) Epidemiology of *Helicobacter pylori* infection in asymptomatic school children in Bhutan. *Helicobacter* 22(6):e12439. <https://doi.org/10.1111/hel.12439>
30. Ibrahim A, Morais S, Ferro A, Lunet N, Peleteiro B (2017) Sex-differences in the prevalence of *Helicobacter pylori* infection in pediatric and adult populations: systematic review and meta-analysis of 244 studies. *Dig Liver Dis* 49(7):742–749. <https://doi.org/10.1111/hel.12439>
31. Zincir H, Kaya Erten Z, Özkan F, Sevig Ü, Baser M, Elmali F (2012) Prevalence of urinary tract infections and its risk factors in elementary school students. *Urol Int* 88(2):194–197. <https://doi.org/10.1111/hel.12439>
32. de Martel C, Parsonnet J (2006) *Helicobacter pylori* infection and gender: a meta-analysis of population-based prevalence surveys. *Dig Dis Sci* 51(12):2292–2301. <https://doi.org/10.1007/s10620-006-9210-5>
33. Shao Y, Lu R, Yang Y et al (2017) Antibiotic resistance of *Helicobacter pylori* to 16 antibiotics in clinical patients. *J Clin Lab Anal* e22339. <https://doi.org/10.1002/jcla.22339>
34. Hu Y, Zhu Y, Lu NH (2017) Primary antibiotic resistance of *Helicobacter pylori* in China. *Dig Dis Sci* 62(5):1146–1154. <https://doi.org/10.1007/s10620-017-4536-8>
35. Li L, Ke Y, Yu C, Li G, Yang N, Zhang J, Li Y (2017) Antibiotic resistance of *Helicobacter pylori* in Chinese children: a multicenter retrospective study over 7 years. *Helicobacter* 22(3):e12373. <https://doi.org/10.1111/hel.12373>
36. Rook G, Bäckhed F, Levin BR, McFall-Ngai MJ, McLean AR (2017) Evolution, human-microbe interactions, and life history plasticity. *Lancet* 390(10093):521–530. [https://doi.org/10.1016/S0140-6736\(17\)30566-4](https://doi.org/10.1016/S0140-6736(17)30566-4)
37. Sanduleanu S, Jonkers D, De Bruïne A et al (2001) Double gastric infection with *Helicobacter pylori*, and non-*Helicobacter pylori*, bacteria during acid-suppressive therapy: increase of pro-inflammatory cytokines and development of atrophic gastritis. *Aliment Pharmacol Ther* 15(8):1163–1175. <https://doi.org/10.1046/j.1365-2036.2001.01029.x>
38. Ferreira RM, Pereira-Marques J, Pinto-Ribeiro I, Costa JL, Cameiro F, Machado JC, Figueiredo C (2018) Gastric microbial community profiling reveals a dysbiotic cancer-associated microbiota. *Gut* 67(2):226–236. <https://doi.org/10.1136/gutjnl-2017-314205>
39. Tlaskalová-Hogenová H, Štěpánková R, Kozáková H, Hudcovic T, Vannucci L, Tučková L, Rossmann P, Hrnčíř T, Kverka M, Zákostelská Z, Klimešová K, Příbylová J, Bártová J, Sanchez D, Fundová P, Borovská D, Šrůtková D, Zidek Z, Schwarzer M, Drastich P, Funda DP (2011) The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. *Cell Mol Immunol* 8(2):110–120. <https://doi.org/10.1038/cmi.2010.67>
40. Wang ZK, Yang YS (2013) Upper gastrointestinal microbiota and digestive diseases. *World J Gastroenterol* 19(10):1540–1550. <https://doi.org/10.3748/wjg.v19.i10.1541>
41. He C, Yang Z, Lu N (2016) Imbalance of gastrointestinal microbiota in the pathogenesis of *Helicobacter pylori*-associated diseases. *Helicobacter* 21(5):337–348. <https://doi.org/10.1111/hel.12297>
42. Brawner KM, Morrow CD, Smith PD (2014) Gastric microbiome and gastric cancer. *Cancer J* 20(3):211–216. <https://doi.org/10.1097/PP0.0000000000000043>
43. Zilberstein B, Quintanilha AG, Santos MA et al (2007) Digestive tract microbiota in healthy volunteers. *Clinics (Sao Paulo)* 62(1):47–54
44. Osaki T, Mabe K, Hanawa T, Kamiya S (2008) Urease-positive bacteria in the stomach induce a false-positive reaction in a urea breath test for diagnosis of *Helicobacter pylori* infection. *J Med Microbiol* 57(7):814–819. <https://doi.org/10.1099/jmm.0.47768-0>
45. Michaud L, Gottrand F, Ganga-Zandzou PS et al (1998) Gastric bacterial overgrowth is a cause of false positive diagnosis of *Helicobacter pylori* infection using 13C urea breath test. *Gut* 42(4):594–595
46. Patel SK, Pratap CB, Verma AK, Jain AK, Dixit VK, Nath G (2013) *Pseudomonas fluorescens*-like bacteria from the stomach: a microbiological and molecular study. *World J Gastroenterol* 19(7):1056–1067. <https://doi.org/10.3748/wjg.v19.i7.1056>
47. Kulkarni G, Gohil K, Misra V, Kakrani AL, Misra SP, Patole M, Shouche Y, Dharne M (2017) Multilocus sequence typing of *Ochrobactrum* spp. isolated from gastric niche. *J Infect Public Health* 10(2):201–210. <https://doi.org/10.1016/j.jiph.2016.04.013>
48. Zeng B, Sun L, Chen Y, Qian Y, Cao Q, Zhang Z, Li Z (2018) *Neisseria flavescens*: a urease-expressing potential pathogen isolated from gastritis patients. *Cur Microbiol* 75(2):186–193. <https://doi.org/10.1007/s00284-017-1364-1>
49. Tsuzukibashi O, Uchibori S, Kobayashi T, Umezawa K, Mashimo C, Nambu T, Saito M, Hashizume-Takizawa T, Ochiai T (2017) Isolation and identification methods of *Rothia* species in oral cavities. *J Microbiol Methods* 134:21–26. <https://doi.org/10.1016/j.mimet.2017.01.005>
50. Zamakhchari M, Wei G, Dewhirst F, Lee J, Schuppan D, Oppenheim FG, Helmerhorst EJ (2011) Identification of *Rothia* bacteria as gluten-degrading natural colonizers of the upper gastro-intestinal tract. *PLoS One* 6(9):e24455. <https://doi.org/10.1371/journal.pone.0024455>
51. Nicodemo AC, Gonçalves LG, Odongo FC et al (2014) *Rothia aerea* endocarditis in a patient with a bicuspid aortic valve: case report. *Braz J Infect Dis* 18(5):561–564. <https://doi.org/10.1016/j.bjid.2014.05.001>
52. Geng X, Li W, Chen Z et al (2017) The bifunctional enzyme SpoT is involved in the clarithromycin tolerance of *Helicobacter pylori* by upregulating the transporters HP0939, HP1017, HP0497, and HP0471. *Antimicrob Agents Chemother* 61(5):e02011–e02016. <https://doi.org/10.1128/AAC.02011-16>