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ORIGINAL ARTICLE

Analysis of Gastric Body Microbiota by Pyrosequencing: Possible Role of Bacteria Other Than *Helicobacter pylori* in the Gastric Carcinogenesis

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Background: Gastric microbiota along with *Helicobacter pylori* (HP) plays a key role in gastric disease. The aim of our study is to investigate the difference of human gastric microbiota between antrum and body according to disease (control vs. gastric cancer) and HP status.
Methods: Each antrum and body biopsy was collected from 12 subjects at Seoul National University Bundang Hospital. Gastric microbiota was analyzed by bar-coded 454 pyrosequencing of the 16S rRNA gene. Twelve subjects consisted of HP-negative control (n = 2), HP-negative cancer (n = 2), HP-positive control (n = 3), and HP-positive cancer (n = 5). The analysis was focused on non-HP urease-producing bacteria (UB) and non-HP nitrosating or nitroreducing bacteria (NB) between antrum and body.
Results: Gastric body samples showed higher diversity compared to gastric antrum mucosa samples but there was no significant difference. The mean of operational taxonomic units was higher in HP(−) cancer than HP(+) cancer (antrum, 273.5 vs. 228.2, P = 0.439; body, 585.5 vs. 183.2, P = 0.053). The number of non-HP UB and non-HP NB was higher in HP(−) cancer groups than the others. These differences were more pronounced in the body (P = 0.051 and P = 0.081, respectively). Analysis of overlap of non-HP UB and non-HP NB revealed the higher composition of *Streptococcus pseudopneumoniae, S. parasanguinis*, and *S. oralis* in HP(−) cancer groups than the others, only in the body (P = 0.030) but not in the antrum (P = 0.123).
Conclusions: Higher diversity and higher composition of *S. pseudopneumoniae, S. parasanguinis*, and *S. oralis* in HP(−) cancer group than the other groups in the body suggest that analysis of microbiota from body mucosa could be beneficial to identify a role of non-HP bacteria in the gastric carcinogenesis.
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Key Words: Microbiota, *Helicobacter pylori*, Antrum mucosa, Body mucosa

INTRODUCTION

Human is constantly exposed to pathogenic microorganisms, such as bacteria, viruses, and fungi. The stomach was considered as a sterile organ due to acid production. However, *Helicobacter pylori* (HP) was found to be colonized in the gastric epithelium of more than half of the world's human population.[1] HP generates large quantities of urease, an enzyme capable of transiently buffering the acidic environment by the break-down of urea to generate ammonia and carbon dioxide.[2] These two products could serve as substrates for other microbes and change the gastric microbiome.[3] Additionally, HP urease is a major inducer of innate immune response in monocytes, macrophages, and neutrophils. Accumulation and activation of these cells is

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induced by the local production of chemokines, cytokine, and NO generation.[4-6] A potent pro-inflammatory cytokines, such as interleukin (IL)-1β and TNF-α are produced during HP infection and IL-1β is also a powerful inhibitor of gastric acid secretion.[7,8] HP infection is a risk factor for gastric cancer, which causes atrophic gastritis regulating inflammatory response or N-nitroso compounds (NOC) production.[9,10] The product of NOC has been suggested to increase the risk of cancers.[10] It has been known that many urease-producing bacteria (UB) and non-HP nitrosating or nitrate-reducing bacteria (NB) other than HP exist in stomach.

Recent advances in next-generation sequencing technology have revealed a complex gastric microbiome which may contribute to the development of gastric carcinogenesis. Our previous studies revealed that gastric microbiota were different according to HP infection status and presence or absence of gastric cancer in gastric mucosa by using a pyrosequencing method.[11-13] We also conducted a research which suggested that gastric mucosa could be more effective than gastric fluid in the detection of meaningful gastric microbiome.[11] On the other hand, gastric antrum and body are different in terms of acid secretion. Acid secretion depends on activation of the gastric H, K-ATPase, termed as the acid or proton pump.[14] This enzyme was found uniquely in gastric parietal cells which are located at oxyntic gastric gland of the body. There is a close interaction or battle between this acid secretion and HP. On the contrary to the usual concept, HP is neutralophiles.[15] That it, in the case of subjects with high acid-secretion, HP escapes from body and settles in the antrum leading to antrum-predominant gastritis.[16] However, when HP succeeds in the colonization, it begins to dominant in the stomach resulting in decrease of microbiota diversity. However, when atrophy and intestinal metaplasia occur, then HP itself decreases to colonize in the stomach[17] and eventually diversity of microbiota increases due to higher pH of gastric juice. From this background, we made a hypothesis that gastric microbiota could be different between in the antrum and in the body. Although we found a minor role of non-HP bacteria in the gastric carcinogenesis in the antrum,[12] the microbiota analysis from body could be different. Thus the aim of our study is to investigate the difference of human gastric microbiota between antrum and body according to disease (control vs. gastric cancer) and HP status.

Table 1. Baseline characteristics of 12 subjects

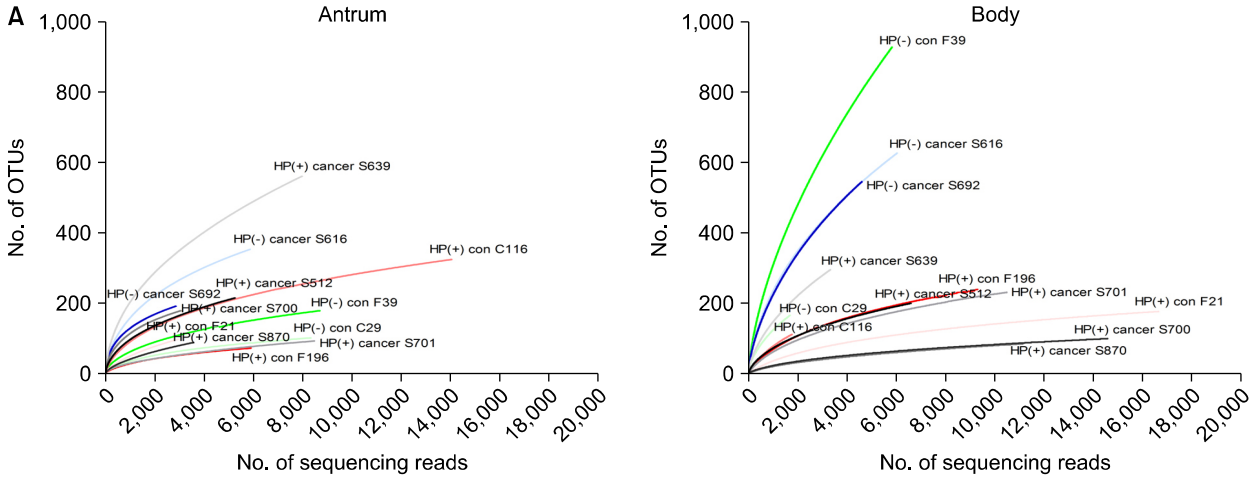
Group	Subject No.	Sex/age (yr)	Site	Intestinal metaplasia	Neutrophil infiltration	Monocyte infiltration	Atrophy	CLO	H&E	HP IgG	PG I/II ratio	Eradication history	*Helicobacter pylori* (%)
HP(−) control	C29	F/40	Antrum	Mild	No	Mild	INA	-	-	0.286	5.0	No	0.192
			Body	No	No	Mild	0	-	-				0.000
	F39	F/67	Antrum	No	No	Mild	0	-	-	0.095	4.8	No	0.023
			Body	No	No	Mild	0	-	-				0.839
HP(−) cancer	S692	F/75	Antrum	No	No	Moderate	INA	-	-	0.028	0.4	No	0.035
			Body	Mild	Mild	Moderate	INA	-	-				0.043
	S616	M/61	Antrum	Moderate	No	Mild	INA	-	-	0.029	3.3	No	0.187
			Body	Moderate	No	Mild	0	-	-				0.149
HP(+) control	F21	M/55	Antrum	Mild	Moderate	Moderate	1	+	Mild	3.338	2.0	No	82.873
			Body	No	Moderate	Moderate	0	+	Moderate				96.288
	F196	F/56	Antrum	Mild	Moderate	Moderate	INA	+	Moderate	N/A	2.3	No	94.997
			Body	No	Moderate	Moderate	0	+	Moderate				89.436
	C116	M/41	Antrum	No	Moderate	Marked	INA	+	Marked	2.341	2.0	No	81.540
			Body	No	Moderate	Marked	2	+	Moderate				83.606
HP(+) cancer	S512	F/36	Antrum	Mild	Moderate	Marked	INA	+	Moderate	2.301	3.0	No	69.842
			Body	No	Moderate	Moderate	0	+	Moderate				85.466
	S700	F/54	Antrum	Moderate	Moderate	Marked	2	+	Marked	N/A	2.9	No	43.308
			Body	No	Moderate	Marked	INA	+	Marked				98.780
	S701	M/57	Antrum	No	Moderate	Moderate	INA	+	Moderate	N/A	1.8	No	95.515
			Body	No	Moderate	Moderate	INA	+	Marked				91.245
	S870	M/53	Antrum	No	Moderate	Moderate	0	+	Mild	N/A	1.9	No	88.957
			Body	Mild	Moderate	Moderate	1	+	Marked				97.986
	S639	F/64	Antrum	Moderate	No	Mild	INA	-	-	0.059	2.8	No	3.902
			Body	No	Moderate	Mild	INA	-	Mild				6.182

CLO, Campylobacter-like organism; HP IgG, *H. pylori* immunoglobulin G; PG, pepsinogen; F, female; M, male; INA, inadequate to assess atrophy; N/A, not assessed.

MATERIALS AND METHODS

1. Study subjects and gastric tissue specimen collection

Gastric biopsies were collected from 12 subjects who underwent standard endoscopy to screen for premalignant or malignant gastric mucosal lesions or received endoscopy due to dyspepsia. Gastric mucosal (antrum and body) biopsies and blood samples were obtained from each patient during endoscopy from October 2008 to March 2013 at Seoul National University Bundang Hospital. Ten biopsy specimens per subjects were obtained to perform HP tests and pyrosequencing as our previous study.[11-13] Gastric biopsy specimens were assessed for the presence of HP and for the degree of inflammatory cell infiltration, atrophy, and intestinal metaplasia (H&E staining). Histological features of gastric mucosa were recorded as the updated Sydney scoring system (i.e., 0 = none, 1 = mild, 2 =



Antrum	Group	HP(−) con		HP(−) cancer		HP(+) con			HP(+) cancer				
	Sample name	c29	f39	S609	s616	f196	f21	c116	s701	s700	s870	s512	s639
	No. of OTUs	103	180	193	354	74	99	325	94	180	90	216	561
	Total reads	8,355	8,718	2,876	5,889	5,917	2,966	14,074	8,495	3,071	3,586	5,269	7,996

Body	Group	HP(−) con		HP(−) cancer		HP(+) con			HP(+) cancer				
	Sample name	F39	C29	S616	S692	C116	F21	F196	S512	S870	S700	S701	S639
	No. of OTUs	926	167	626	545	113	178	240	201	100	86	232	296
	Total reads	5,841	1,695	6,047	4,615	1,775	16,676	9,296	6,612	14,596	11,149	10,497	3,332

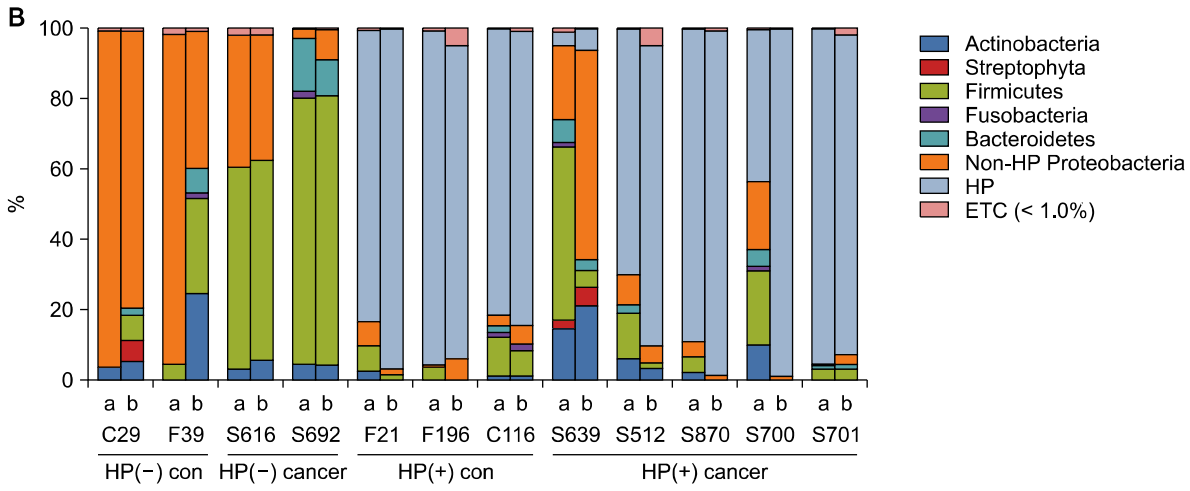


Figure 1. Bacterial diversity in gastric antrum and body mucosa samples. (A) The graph shows refraction curves indicating the number of assigned bacterial genera in relation to the number of 16S rRNA sequences, grouped by individual. (B) Taxonomic assignment of the 24 samples at the level of bacterial phylum. OTU, stands for operational taxonomic units; HP, *Helicobacter pylori*; con, control; a, antrum; b, body; ETC, et cetera.

moderate, 3 = marked).[18] To avoid contamination, the endoscopes were washed and disinfected by immersing in a detergent solution containing 7% proteolytic enzymes and 2% glutaraldehyde and sterilized gastroscopy forceps were used while gaining another biopsy from the same patient. The biopsies were stored at −80°C. This study was approved by the ethics committee of Seoul National University Bundang Hospital (B-1112/141-007). Written informed consent was obtained from all of the participants.

2. Determination of *H. pylori* infection status

To determine the presence of current HP infection according to conventional tests: 1) rapid urease test (CLO test; Delta West, Bentley, Australia), 2) histologic examination (modified Giemsa staining), 3) culture for HP. Current HP infection was positive from any of the former three tests. In order to distinguish if the infection is an existing one, the following two methods were used: Serum HP immunoglobulin G (Genedia HP ELISA; Green Cross Medical Science Co., Eumseong, Korea), and a history of HP infection eradication treatment. If all the 5 tests were negative, we would have regarded the subject as HP-negative. Besides, by using a Latex-enhanced Turbidimetric Immunoassay (Shima Laboratories, Tokyo, Japan), serum concentrations of Pepsinogen I and II were evaluated, which are known to be associated with the severity of gastric atrophy.[19]

3. Bacterial genomic DNA extraction

The antrum and body mucosal samples from 12 subjects were subjected to pyrosequencing. Bacterial genomic DNA (gDNA) was extracted with the commercial kit (iNtRON Biotechnology, Seongnam, Korea).

4. 16S rRNA sequencing

PCR amplification was done by using primers targeting the V1 to V3 regions of bacterial 16S rRNA gene with bacterial gDNA. For bacterial amplification, barcoded primers of 9F (5'-CCTATCCC-CTGTGTGCCTTGGCAGTC-TCAG-AC-AGAGTTTGATCMTGGCTC AG-3'; underlined sequence indicates the target region primer) and 541R (5'-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-X-AC-ATTACCGCGGCTGCTGG-3'; 'X' presents the unique barcode for each subject) (http://oklbb.ezbiocloud.net/content/1001) as previous study were shown. The sequencing was performed at Chunlab Inc. (Seoul, Korea) with GS Junior Sequencing system, the modified laboratory benchtop form of 454 sequencing systems (Roche, Branford, CT, USA) as stated in the manufacturer's directions.

5. Pyrosequencing data analysis

The primary analysis was conducted as described above. Reads taken from different samples were classified by unique barcodes of each PCR product. After identifying the target region in barcoded primers (9F or 541R), all of the linked sequences including adapter, barcode and linker were eliminated. Low quality sequences, such as reads containing two or more indefinite nucleotides, reads with a low quality score (average score < 25), or reads shorter than 300 bp, were eliminated. Potential chimeric sequences were confirmed by the Bellerophon formula, which compares the BLASTN search conclusions between the forward half and reverse half sequences.[16] After removing the chimeric sequences, the taxonomic sorting of each read was assigned against the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net),[17] which has the 16S rRNA gene

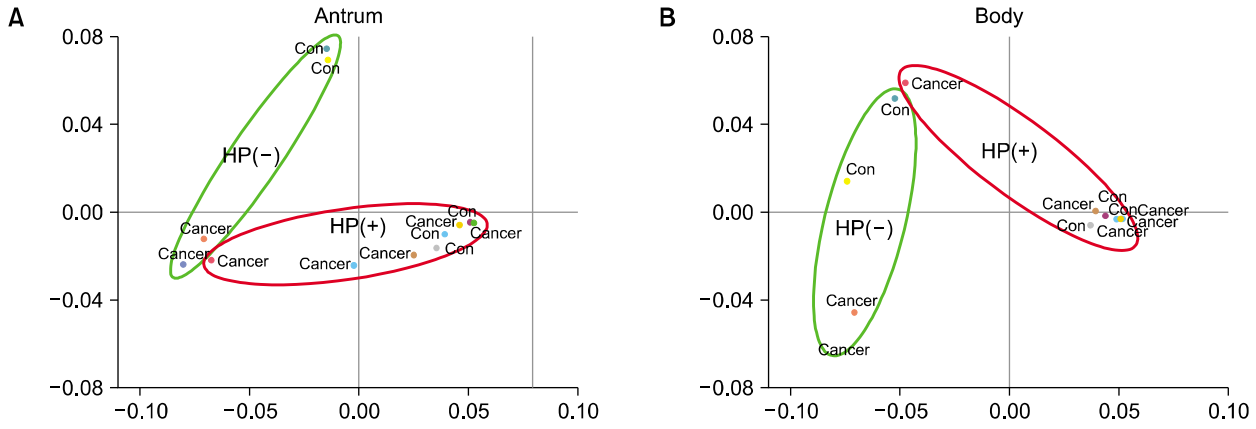


Figure 2. (A) Unweighted UniFrac-based principal coordinates analysis of gastric antrum and (B) body microbiome. There was very little separation between control and cancer groups under the same HP infection status in both of gastric antrum and body mucosa. HP, *Helicobacter pylori*; con, control.

Table 2. Comparison of species frequency of gastric antrum and body mucosal samples

Phylum	Species	Antrum (%)				Body (%)			
		HP(−) control (n = 2)	HP(−) cancer (n = 2)	HP(+) control (n = 3)	HP(+) cancer (n = 5)	HP(−) control (n = 2)	HP(−) cancer (n = 2)	HP(+) control (n = 3)	HP(+) cancer (n = 5)
Firmicutes	*Streptococcus pseudopneumoniae*	0.14	18.68[a]	3.37[a]	6.04[a]	3.80[a]	23.11[a]	0.98	0.20
	S. mitis	0.02	9.01[a]	0.51	0.82	0.45	3.09[a]	0.23	0.00
	S. salivarius	0.01	4.00[a]	0.11	0.40	0.27	6.37[a]	0.03	0.02
	S. infantis	0.32	3.39[a]	0.24	1.10[a]	3.07[a]	5.38[a]	0.08	0.12
	Veillonella atypica	0.03	2.97[a]	0.23	0.64	0.32	1.22[a]	0.03	0.01
	V. dispar	0.03	2.37[a]	0.18	0.77	0.53	1.25[a]	0.05	0.01
	Granulicatella adiacens	0.04	3.33[a]	0.08	0.71	0.51	1.98[a]	0.03	0.02
	Gemella haemolysans	0.00	2.09[a]	0.19	0.20	0.03	1.16[a]	0.05	0.01
	S. australis	0.05	2.19[a]	0.14	0.68	0.59	2.01[a]	0.10	0.01
	S. parasanguinis	0.14	1.51[a]	0.27	0.60	0.86	3.36[a]	0.14	0.04
	S._uc	0.10	1.14[a]	0.20	0.65	0.76	2.96[a]	0.06	0.06
	GQ130066_s	0.01	0.90	0.21	0.54	0.00	0.00	0.00	0.00
	S. oralis	0.02	1.72[a]	0.14	0.30	0.50	2.07[a]	0.08	0.01
	Lactobacillus salivarius	0.00	1.93[a]	0.00	0.05	0.01	0.67	0.00	0.00
	S. tigurinus	0.01	1.06[a]	0.03	0.17	0.25	0.43	0.00	0.00
	Solobacterium moorei	0.02	0.17	0.03	0.54	0.01	0.13	0.00	0.02
	S. lactarius	0.02	0.88	0.05	0.08	0.14	0.65	0.02	0.00
	Megasphaera micronuciformis	0.00	0.70	0.02	0.15	0.03	0.04	0.00	0.00
	Streptococcaceae_uc_s	0.02	0.41	0.10	0.11	0.57	1.90[a]	0.12	0.05
	Lactobacillales_uc_s	0.01	0.19	0.02	0.08	0.82	2.09[a]	0.07	0.05
Proteobacteria	HP	0.11	0.11	86.47[a]	60.30[a]	0.42	0.10	89.78[a]	75.93[a]
	Haemophilus parainfluenzae	0.15	3.84[a]	0.46	0.96	2.15[a]	2.41[a]	0.13	0.02
	Escherichia coli group	0.03	0.00	0.00	1.46[a]	0.00	0.01	0.00	0.05
	H. paraphrohaemolyticus	0.00	1.60[a]	0.08	0.60	0.03	1.10[a]	0.02	0.01
	Methylobacterium adhaesivum	0.06	2.22[a]	0.12	0.56	0.06	0.00	0.00	0.05
	Bradyrhizobium jicamae	0.00	0.07	0.01	0.22	8.66[a]	0.68	0.24	1.24[a]
	Neisseria perflava	0.00	4.24[a]	1.14[a]	0.17	0.04	5.12[a]	0.04	0.02
	Pseudomonas beteli	1.05[a]	0.05	0.01	0.20	0.00	0.00	0.00	0.00
	P. hibiscicola	44.87[a]	0.06	0.24	0.17	0.00	0.02	0.00	0.01
	Helicobacteraceae_uc_s	0.01	0.00	0.08	0.07	0.06	0.03	1.28[a]	0.49
	H._uc	0.00	1.00[a]	0.07	0.11	0.09	0.86	0.04	0.00
	Aggregatibacter segnis	0.02	1.07[a]	0.00	0.05	0.00	0.44	0.00	0.01
	H. haemolyticus	0.00	0.61	0.10	0.04	0.04	0.53	0.04	0.00
	Ralstonia pickettii	45.90[a]	0.05	0.02	0.04	0.03	0.00	0.00	0.00
	Pasteurellaceae_uc_s	0.01	0.19	0.02	0.03	0.36	0.69	0.02	0.01
	Pasteurellales_uc_s	0.00	0.04	0.01	0.02	0.12	0.63	0.00	0.01
	B. pachyrhizi	0.00	0.01	0.00	0.03	5.33[a]	0.49	0.11	0.70
	Klebsiella pneumoniae	0.00	0.07	0.00	1.05[a]	0.00	0.02	0.00	0.03
	Neisseriaceae_uc_s	0.00	0.22	0.00	0.02	0.05	0.60	0.00	0.00
	Neisseriales_uc_s	0.01	0.05	0.01	0.01	0.08	0.80	0.00	0.00
	FJ269053_s	0.02	0.08	0.00	0.01	14.23[a]	1.38[a]	0.46	2.94[a]
	B. denitrificans	0.00	0.07	0.00	0.00	1.14[a]	0.09	0.03	0.10
	U87765_s	0.02	0.02	0.00	0.00	2.97[a]	0.27	0.08	0.51
	Bradyrhizobiaceae_uc_s	0.00	0.00	0.00	0.01	0.98	0.20	0.04	0.13
	DQ532251_g_uc	0.00	0.00	0.00	0.00	0.79	0.09	0.03	0.18
	Pelomonas saccharophila	0.00	0.00	0.00	0.00	3.26[a]	0.47	0.22	0.95
	Rhizobium hainanense	0.00	0.00	0.00	0.00	1.10[a]	0.11	0.04	0.24
	M. longum	0.00	0.00	0.00	0.00	1.91[a]	0.09	0.06	0.40
	M. radiotolerans	0.01	0.00	0.00	0.00	3.08[a]	0.18	0.11	0.62
	B._g1_uc	0.00	0.00	0.00	0.00	0.72	0.09	0.02	0.09

Table 2. Continued

Phylum	Species	Antrum (%)				Body (%)			
		HP(−) control (n = 2)	HP(−) cancer (n = 2)	HP(+) control (n = 3)	HP(+) cancer (n = 5)	HP(−) control (n = 2)	HP(−) cancer (n = 2)	HP(+) control (n = 3)	HP(+) cancer (n = 5)
Bacteroidetes	*Prevotella histicola*	0.01	3.08[a]	0.11	0.43	0.11	1.85[a]	0.08	0.00
	P. melaninogenica	0.01	1.56[a]	0.06	0.84	0.14	0.92	0.04	0.02
	P._uc	0.01	1.07[a]	0.23	0.31	0.21	0.98	0.06	0.01
	P. pallens	0.02	0.65	0.08	0.33	0.03	0.31	0.00	0.02
	P. salivae	0.00	0.78	0.06	0.14	0.05	0.46	0.02	0.01
	EF123551_g_uc	0.00	0.00	0.00	0.00	1.45[a]	0.11	0.07	0.51
Actinobacteria	*Propionibacterium acnes*	1.43[a]	0.42	0.82	4.42[a]	8.49[a]	1.39[a]	0.27	4.34[a]
	Actinomyces odontolyticus	0.05	1.31[a]	0.17	0.89	0.44	0.95	0.12	0.03
	Rothia mucilaginosa	0.09	0.26	0.07	0.12	0.90	0.09	0.05	0.01
	Propionibacterium_uc	0.02	0.01	0.01	0.04	0.53	0.07	0.01	0.07
	Propionibacteriaceae_uc_s	0.04	0.00	0.00	0.02	0.79	0.18	0.04	0.16
Viridiplantae	*Prunus persica*	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.83
	Nicotiana tabacum	0.00	0.00	0.00	0.00	1.09[a]	0.00	0.00	0.05
	Ipomoea purpurea	0.00	0.00	0.00	0.00	1.59[a]	0.00	0.00	0.00
Fusobacteria	*Fusobacterium nucleatum*	0.09	0.92	0.26	0.11	0.33	0.16	0.08	0.03

Values are presented as mean percent. HP, *Helicobacter pylori*. [a]This means cut off > 1.0.

sequence of type strains that have valid published names and representative species level phylotypes of either cultured or uncultured entries in the GenBank database with complete hierarchical taxonomic classification from the phylum to the species. Phylogenetic trees were not created as we assigned reads into operational taxonomic units (OTUs) according to BLAST results. The raw 16S rRNA gene sequence originated from our study was deposited in NCBI's SRA (GSE61493).

6. Evaluation of species richness and diversity

The species richness of samples was determined using the CLcommunity program (Chunlab Inc.). Random subsampling was conducted to equalize the read size of samples to compare the different read size within samples. To compare the OTUs between samples, shared OTUs were obtained with the XOR analysis of the CLcommunity program (Chunlab Inc.).

7. Statistical analysis

Comparisons between continuous parameters were performed by Kruskal-Wallis test and Mann-Whitney test. Statistical analyses were done by Prism 5 (GraphicPad Software Inc., La Jolla, CA, USA) and PASW 18.0 (IBM, Somers, NY, USA). Results with a *P*values < 0.05 were considered statistically significant.

RESULTS

1. Baseline characteristics

A total of 12 subjects were enrolled in this study, two HP(−) controls, two HP(−) cancer, three HP(+) controls and five HP(+) cancer patients. Baseline characteristics of clinical results of gastric antrum and body mucosa samples are shown in Table 1. The mean age of subjects was higher in the HP(−) groups than in the HP(+) groups (60.8 years vs. 52 years; $P = 0.174$). However, there was no significant difference between the two groups. Pepsinogen I/II ratio reflecting gastric atrophy was no significant difference between the two groups. (3.4 vs. 2.3, $P = 0.173$; Table 1). The grades of neutrophils and monocytes infiltration were lower in the HP(−) groups compared to those in the HP(+) groups (antrum, $P = 0.006$, $P = 0.037$; body, $P = 0.001$, $P = 0.041$ respectively). Additionally, the grades of neutrophils and monocytes infiltration were significantly different between HP (+) cancer group and the others (antrum, $P = 0.046$, $P = 0.184$; body, $P = 0.013$, $P = 0.162$, respectively).

2. Gastric antrum versus body mucosa

The means of reads and OTUs were lower in gastric antral mucosa samples than gastric body mucosa samples (Fig. 1A). Gastric body mucosa samples showed higher diversity compared to antrum mucosa samples (Fig. 1B). The unweighted UniFrac analysis indicated that there was very little separation between control and cancer groups under the HP infection status in both of

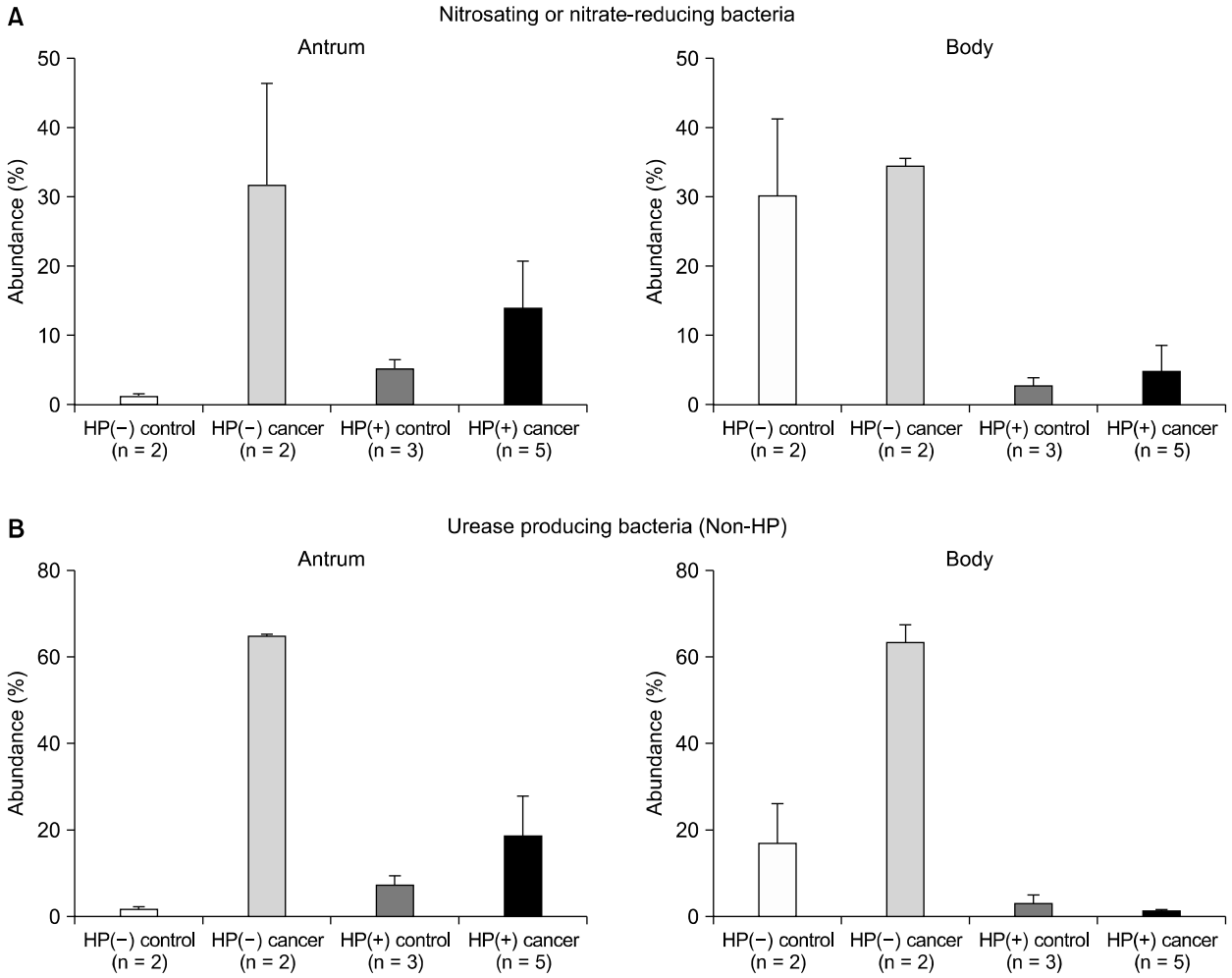
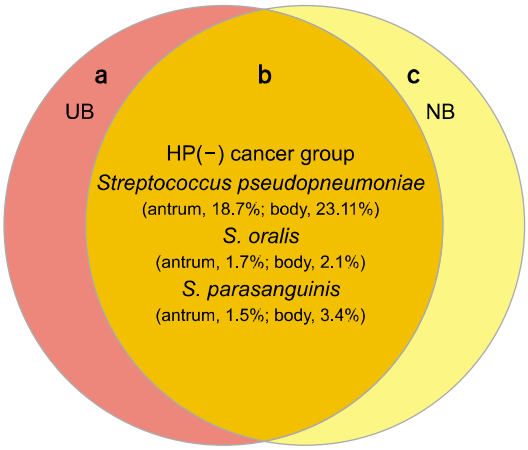


Figure 3. The comparison of gastric microbiome in gastric antrum and body mucosa. (A) The proportion of non-HP nitrosating or nitrate-reducing bacteria between the gastric antrum and body mucosa. (B) The proportion of non-HP urease-producing bacteria between the gastric antrum and body mucosa. HP, *Helicobacter pylori*.

gastric antral and body mucosa (Fig. 2). However, HP(−) groups showed a separation between control and cancer group in both of antrum and body. The bacterial communities at the phylum level among four groups showed that the proportion of Proteobacteria of the HP(+) groups was more greater than that of the HP(−) cancer group (Table 2). In HP(−) control group, *Pseudomonas hibiscicola* (44.87% vs. 0%) and *Ralstonia pickettii* (45.90% vs.0.03%) were more abundant in antrum than in body mucosa (Table 2). In HP(−) control group, *Bradyrhizobium* sp. (*Bradyrhizobium jicamae*, *B. pachyrhizi*, *B. denitrificans*, and *B.* g1 uc) were more abundant in body (8.66%, 5.33%, 1.14%, and 0.72%, respectively) than in antrum mucosa (all 0%, Table 2). Actinobacteria of HP(+) cancer group was more greater than that of the HP(-) cancer group. However, *Propionibacterium acnes* was more abundant in body than antrum mucosa (8.49% vs. 1.43%, Table 2). The proportion of Firmicutes in the HP(−) groups was more greater than the HP(+) groups. These differences were more pronounced in the antral mucosa. The bacterial communities at the species level among four groups in gastric antral and body mucosa showed that the proportion of *Streptococcus sp.* in the HP(−) cancer group was more greater than the others (Table 2). The proportion of *Streptococcus mitis* group,[20-22] such as *S. pseudopneumoniae*, *S. mitis*, *S. infantis*, *S. oralis*, and *S. tigurinus*, in the HP(−) cancer group was more higher than in that of the HP(+) cancer group (antrum, 33.9 vs. 8.4, $P = 0.076$; body, 34.1 vs. 0.33, $P = 0.009$; Table 2). These results suggest a role of *S. mitis* in the gastric carcinogenesis despite the absence of HP.

3. *H. pylori*(−) cancer vs. *H. pylori*(+) cancer

The composition of UB and NB[12] was higher in HP(−) cancer and HP(+) groups than each control group (Supplementary 1 and



	Group	HP(−) con	HP(−) cancer	HP(+) con	HP(+) cancer
Antrum	UB (a + b)	1.59	64.86	7.20	18.54
	NB (b + c)	1.19	31.65	5.01	13.81
	b	0.54	26.84	4.52	9.48
Body	UB (a + b)	16.88	63.28	2.99	1.24
	NB (b + c)	30.03	34.42	2.72	4.76
	b	7.34	31.53	1.46	0.38

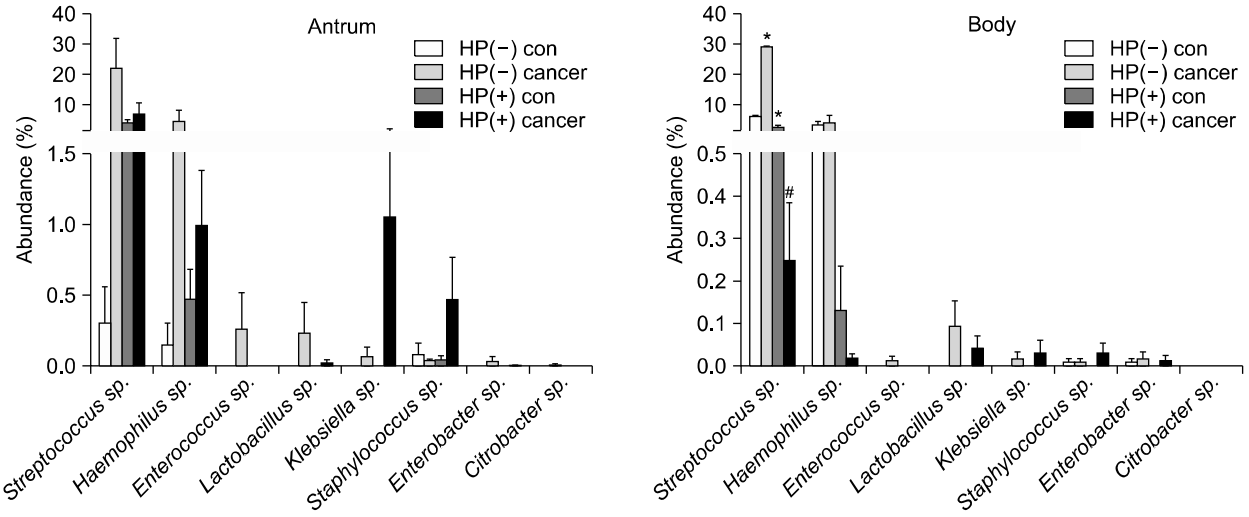


Figure 4. The comparison of urease and N-nitroso compounds-producing bacteria in gastric antrum and body mucosa. The strains of the overlap of non-HP-UB and non-HP-NB between the gastric antrum and body mucosa. Kruskal-Wallis test was used to determine statistical significance existed between the four groups. HP, *Helicobacter pylori*; NB, nitrosating or nitrate-reducing bacteria; UB, urease-producing bacteria; con, control; $^{*}P < 0.05$ compared to HP(−) control; $^{\#}P < 0.05$ compared to HP(−) cancer.

2). The proportion of non-HP-UB and non-HP-NB was higher in the HP(−) cancer group than in that of the HP(+) cancer group, especially body mucosa (antrum, $P = 0.053$, $P = 0.121$; body, $P = 0.053$, $P = 0.051$, respectively; Fig. 3). The overlap of non-HP-UB and non-HP-NB was presented at Figure 4 and Table 3. When we assessed the overlap of non-HP-UB and non-HP-NB, it revealed that *Streptococcus sp.* occupied high proportion in the HP(−) cancer group except *S. pneumoniae*. As these strains are urease and produce NOC in gastric mucosa (Fig. 4), *S. pseudopneumoniae*, *S. parasanguinis*, and *S. oralis* are pathogens (antrum, $P = 0.123$; body, $P = 0.030$, respectively; Kruskal-Wallis test).

DISCUSSION

In recent years, high throughput techniques for studying microbiome have been developed which provide more comprehensive data on microbiome. The goal of these techniques is to identify key microbial players between health and disease outcome. It has a clear potential to benefit clinical part. Bacterial infection has been linked to cancer through two mechanisms; 1) chronic inflammation and 2) production of carcinogenic metabolites such as HP infection.[23]

Gastric acidity is a barrier to bacterial overgrowth.[24,25] The bacterial colonization in stomach increases under the condition such as acid-reducing drug, atrophic gastritis, and gastric surgery.[10,25] In addition, decreased gastric acid secretion is responsible for an increased risk of infection.[20] The antrum and body of stomach are distinct niches for microbial colonization owing to differential ability to secrete gastric acid.[26] Thus, comparison of gastric microbiota between gastric antrum and body will be useful. Li et al.[27] investigated the gastric microbiota of five non-HP and non-NSAID (non-steroidal anti-inflammatory

Table 3. Comparison of urease and N-nitroso compounds-producing bacteria between antrum and body

Species	Antrum (%)				Body (%)			
	HP(−) control (n = 2)	HP(−) cancer (n = 2)	HP(+) control (n = 3)	HP(+) cancer (n = 5)	HP(−) control (n = 2)	HP(−) cancer (n = 2)	HP(+) control (n = 3)	HP(+) cancer (n = 5)
Streptococcus pseudopneumoniae	0.139	18.68[a]	3.375	6.038	3.797	23.11[a]	0.983	0.196
Haemophilus parainfluenzae	0.155	3.842	0.457	0.959	2.154	2.413	0.130	0.018
S. oralis	0.023	1.724[a]	0.145	0.297	0.500	2.073[a]	0.078	0.009
S. parasanguinis	0.138	1.506[a]	0.275	0.596	0.855	3.361[a]	0.135	0.042
H. influenzae	0.000	0.433	0.016	0.034	0.000	0.408	0.000	0.000
Enterococcus hirae	0.000	0.261	0.000	0.000	0.000	0.011	0.000	0.000
Lactobacillus fermentum	0.000	0.209	0.000	0.000	0.000	0.084	0.000	0.030
HP	0.107	0.111	86.470	60.305	0.419	0.096	89.777	75.932
Klebsiella pneumoniae	0.000	0.068	0.000	1.054	0.000	0.017	0.000	0.030
Staphylococcus epidermidis	0.084	0.043	0.047	0.468	0.009	0.008	0.000	0.030
Enterobacter aerogenes	0.000	0.035	0.000	0.004	0.000	0.000	0.000	0.012
L. gasseri	0.000	0.026	0.000	0.025	0.000	0.008	0.000	0.011
Citrobacter rodentium	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000
S. pneumoniae	0.006	0.008	0.201	0.008	0.017	0.019	0.133	0.000
E. mori	0.000	0.000	0.000	0.000	0.009	0.017	0.000	0.000

Values are expressed as mean percent. HP, *Helicobacter pylori*. [a]This means cut off > 1.0.

drug) antral gastritis individuals and five normal individuals by pyrosequencing, and they identified potential pathogens (*S. pneumonia*, *S. mitis* and *S. salivarius*) were high in antral gastritis stomach. However, there was little difference in gastric microbiota between antrum and body in normal control group except *Prevotella*.[27] Similarly, our results also showed that HP(−) cancer group showed high proportion of *Streptococcus* (phylum Firmicutes) in both gastric antrum (41.3%) and body (49.5%). However, the proportion of *Streptococcus* sp. was more pronounced in the body.

HP causes atrophic gastritis modulating inflammatory responses and making NOC.[9,10] NOC can be formed from nitrite and secondary amines by nitrosating bacteria of stomach, such as *Clostridium*, *Veillonella*, *Haemophilus*, *Staphylococcus*, *Streptococcus*, and *Neisseria*.[12,28] NOC formation has been suggested to increase the risk of gastric cancer.[12] Urease is a major inducer of innate immune response.[4-6] Urease-producing non-HP microbes including *Actinomyces, Clostridium, Corynebacterium*, member of the *Enterobacteriaceae* (*Citrobacter, Enterobacter, Klebsiella, Morganella, Providencia*, and *Proteus*), *Enterococcus, Gardnerella, Haemophilus, Lactococcus, Mycobacterium, Streptococcus, Staphylococcus, Ureaplasma*, and *Yersinia* were detected in the oral cavity, gastrointestinal tract, urethrogenital tract and skin.[29-31] Gastric pH modifications induced by UB may modify bacterial substrate availability and local immune responses by relationship between their members. NB concentrations were significantly higher when pH was > 4.[32] Nitrosating capacity was higher in a range from pH 3 to pH 6.[33] UB-NB interaction was able to produce a pro-carcinogenic inflammatory response like as HP. Surprisingly, the proportion of UB and NB was significantly higher in HP(−) cancer group, especially in the body mucosa. When we assessed the overlap of non-HP-UB and non-HP-NB, it revealed that the composition of *S. pseudopneumoniae*, *S. parasanguinis*, and *S. oralis* was higher in HP(−) cancer groups than the others. *S. mitis* group, such as *S. mitis*, *S. pseudopneumoniae*, *S. oralis*, *S. infantis*, and *S. tigurinus* strains were associated with serious invasive infections, pneumonia, and endocarditis.[34-36] In addition, *S. mitis* was significantly more prevalent within oesophageal carcinoma tissues.[37] Importantly, *S. mitis* could induce the expression of CXC chemokine genes (*IL-8* and *GROa*), which recruitment and activation of neutrophils and monocytes could be stimulated during cancer progression.[37] Additionally, *S. parasanguinis* strain was associated with cystic fibrosis.[38] Taken together, these species could be a significant human pathogen. Actually, we missed this point in the previous report using 63 samples in the antrum 12, thus, further analysis is planned in the future. Anyway, our analysis using 12 samples in the body added another clue for the role of bacteria other than HP to gastric carcinogenesis. Actually, it has been suggested by a number of researches using pyrosequencing.[39,40] However, this study has a limitation due to small sample size, and further research using more samples are needed.

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CONFLICT OF INTEREST

No potential conflicts of interest were disclosed.

SUPPLEMENTARY MATERIALS

Supplementary Materials can be found via https://doi.org/10.15430/JCP.2017.22.2.115.

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