**Differences in interleukin-8 expression in *Helicobacter pylori*-infected gastric mucosa
tissues from patients in Bhutan and the Dominican Republic**

Running title: IL-8 in Bhutan and Dominican Republic

Hiroyuki Nagashima MD, PhD^{1,2}, Shun Iwatani PhD^{1,2}, Modesto Cruz MD, PhD^{3,4}, José A.
Jiménez Abreu MD⁵, Lourdes Tronilo MD⁵, Eduardo Rodríguez MD⁵, Mildre Disla MD⁶, Hideo
Terao MD, PhD⁷, Tomohisa Uchida MD, PhD⁸, Varocha Mahachai MD⁹, Ratha-korn
Vilaichone MD¹⁰, Lotay Tshering MD¹¹, Takahiro Mitsui MD, PhD¹, Seiji Shiota MD, PhD¹,
David Y. Graham MD², Yoshio Yamaoka MD, PhD^{1,2,#}

¹Department of Environmental and Preventive Medicine, Oita University Faculty of Medicine,
Yufu 879-5593, Japan

²Department of Medicine-Gastroenterology, Michael E. DeBakey Veterans Affairs Medical
Center and Baylor College of Medicine, Houston, Texas 77030, USA

³Institute of Microbiology and Parasitology, Department of Science, Autonomous University of
Santo Domingo, Santo Domingo, Dominican Republic

⁴Department of Biomedical Research, School of Medicine, Santiago Technological University,
Santo Domingo, Dominican Republic

⁵Dominican-Japanese Digestive Disease Center, Dr. Luis E. Aybar Health and Hygiene City,
Santo Domingo, Dominican Republic

⁶Dominican-Japanese Friendship Medical Education Center, Dr. Luis E. Aybar Health and Hygiene City, Santo Domingo, Dominican Republic

⁷Health Science Center, Oita University, Oita 870-1192, Japan

⁸Department of Molecular Pathology, Oita University Faculty of Medicine, Yufu 879-5593, Japan

⁹Gastroenterology Unit, Department of Medicine, Thammasat University Hospital, Pathumthani, Thailand

¹⁰Department of Gastroenterology, Bangkok Hospital, Bangkok, Thailand

¹¹Department of Surgery, Jigme Dorji Wangchuk National Referral Hospital, Thimphu, Bhutan

#Address correspondence to

Yoshio Yamaoka, MD, PhD

Department of Environmental and Preventive Medicine, Oita University Faculty of Medicine,
1-1 Idaigaoka, Hasama-machi, Yufu-City, Oita 879-5593, Japan

E-mail: yyamaoka@oita-u.ac.jp, Tel.: +81-97-586-5740, Fax: +81-97-586-5749

1 **Acknowledgments**

2 This paper is based on the work supported in part by grants from the National Institutes of Health
3 (DK62813) (YY) and DK56338 (DYG), Grants-in-Aid for Scientific Research from the Ministry
4 of Education, Culture, Sports, Science and Technology (MEXT) of Japan (22390085, 22659087,
5 24406015, and 24659200) (YY), (23790798) (SS), Strategic Young Researcher Overseas Visits
6 Program for Accelerating Brain Circulation for Japan Society for the Promotion of Science
7 (JSPS), the Strategic Funds for the Promotion of Science and Technology from Japan Science
8 and Technology Agency (JST), and the National fund for Innovation and Scientific and
9 Technological development (FONDOCYT) from the Ministry of Higher Education Science and
10 Technology (MESCyT) of the Dominican Republic (MC).

11

12 **Key word**

13 *Helicobacter pylori*, *IL-8*, *cagA* genotype, Bhutan, Dominican Republic.

14 **Disclosures**

15 The authors have no financial conflicts of interest.

16

17

Abstract

The outcomes of *Helicobacter pylori* infection vary geographically. *H. pylori* strains, disease presentation, and environments differ markedly in Bhutan and Dominican Republic. The aims were to compare the strains, histology and expression of *interleukin (IL)-8* and *IL-10* from gastric mucosa from the two countries. *H. pylori* status was assessed by the combination of rapid urease test, culture and histology. Histology was evaluated using the updated Sydney System and cytokines in gastric biopsies were measured using real-time PCR. There were 138 subjects from Bhutan and 155 from Dominican Republic. The prevalence of *H. pylori* infection was 65% and 59%, respectively. The genotype of *cagA* was predominantly East-Asian type in Bhutan vs. Western type in Dominican Republic. Gastritis severity was significantly higher in *H. pylori*-infected subjects from Bhutan than those from Dominican Republic. *IL-8* expression by *H. pylori*-infection was 5.5-fold increase in Bhutan vs. 3-fold in Dominican Republic ($p < 0.001$); *IL-10* expression was similar. *IL-8* expression levels among *H. pylori*-infected cases tended to be positively correlated with polymorphonuclear leucocyte (PMN) and monocyte infiltration (MNC) scores in both countries. *IL-8* expression among those with grade 2 and 3 PMN and MNC was significantly higher in Bhutan than in Dominican Republic. The difference in *IL-8* expression in two countries is reflected in the different disease pattern between them. Whether the dominant factor is differences in *H. pylori* virulence, in host-*H. pylori*-environmental interactions, genetic factors or all remains unclear. However, severity of inflammation appears to be a critical factor in disease pathogenesis.

1. Introduction

Helicobacter pylori is a spiral, gram-negative human pathogen and a major cause of peptic ulcer disease and gastric cancer [1, 2]. *H. pylori* gastritis is characterized by infiltration of the gastric mucosa with both neutrophils and mononuclear cells and is associated with the upregulation of proinflammatory cytokines such as the potent chemotactic peptide interleukin (IL)-8 [2, 3]. The clinical manifestations of *H. pylori* infections vary both within and among populations; for example, in some areas such as Japan, where *H. pylori* infection is common, gastric cancer is an important clinical problem, whereas, in other areas such as South India, gastric cancer is rare and instead duodenal ulcer is a more common disease manifestation [4]. Whether these geographic differences correlate with the differences in the gastric mucosal cytokine levels remain unknown, because studies comparing the mucosal cytokine levels among individuals from different countries are lacking. Here, we have reported a comparison of gastric mucosal *IL-8* and *IL-10* expression and the histological findings by using gastric biopsies obtained from adults in Bhutan and the Dominican Republic. These countries were selected for the study because of the differences in their clinical outcomes of *H. pylori* infection as well as in their *cagA* genotype of the pathogenic *H. pylori* (i.e., *cagA*-negative, Western-type *cagA* in the Dominican Republic and East-Asian-type *cagA* in Bhutan). The tissue samples from both the regions were similarly processed and analyzed.

2. Materials and Methods

2.1. Study Countries

Bhutan and the Dominican Republic are relatively isolated developing countries where *H. pylori* infection is common. These countries show several differences in the age-standardized rate of gastric cancer incidence; the rate is higher in Bhutan (17.2/100,000) than in the Dominican Republic (7.3/100,000) (GLOBOCAN 2012: <http://globocan.iarc.fr/>). Bhutan is a small, landlocked mountainous country located at the eastern end of the Himalayas, and it shares borders on the south, east, and west with the Republic of India and to the north with the People's Republic of China. In contrast, the Dominican Republic is a Caribbean island it shares borders on the east with Haiti, and with a population of 73% multiracial, 16% Caucasians, and 11% Congoid. We hypothesized that the *cagA* genotype between these two countries also differs, with Bhutanese strains being East-Asian-type *cagA* and the Dominican Republic strains being Western-type-*cagA*. In addition, the presence of *cagA*-negative strains makes these regions ideal for comparing the gastric histology and mucosal cytokine levels in relation to the *cagA* genotype and its outcome.

2.2. Subjects

We recruited individuals with mild dyspeptic symptoms living in Bhutan and outpatients with mild dyspeptic symptoms living in the Dominican Republic. The surveys took place at the Jigme Dorji Wangchuk National Referral Hospital, Thimpu, Bhutan in December 2010 and at the Dr. Luís E. Aybar Health and Hygiene City (Digestive Disease Center), Santo Domingo, Dominican Republic in February 2012. Written informed consent was obtained from all the participants. The protocol was approved by the ethics committee of Oita University Faculty of Medicine (Japan), Universidad Autonoma de Santo Domingo (Dominican Republic), and by both the hospitals where sample collection was performed.

The tissue sampling procedures as well as the method of sample handling were identical at both the locations. During each endoscopy session, 4 gastric biopsy specimens were obtained from the antrum: one each for *H. pylori* culturing, rapid urease test (CLO test, Campylobacter-like organism test, Kimberly-Clark Ballard Medical Products, Roswell, GA, USA), histological examination, and cytokine examination. Rapid urease test were performed at time of endoscopy. The clinical diagnoses after endoscopy included those for gastritis, duodenal ulcer, gastric ulcer, and gastric cancer. Peptic ulcer and gastric cancer were identified by endoscopy, and gastric cancer was confirmed by histopathology. Gastritis was defined as *H. pylori* gastritis in the absence of peptic ulcer or gastric malignancy. Patients with a history of partial gastric resection were excluded. Patients who had received *H. pylori*-eradication therapy or had received treatment with antibiotics, bismuth-containing compounds, H₂-receptor blockers, or proton-pump inhibitors within 4 weeks prior to the study were also excluded.

All the biopsy specimens for culturing and RNA analyses were immediately placed on ice bath after collection and then stored in a –20°C freezer. The samples were kept frozen and dispatched on dry ice to the Oita University Faculty of Medicine, Japan via Express Mail within a week after collection. The samples received by the Oita University Faculty of Medicine were stored at –80°C until use. The biopsy specimens for histology were fixed in buffered formalin at room temperature and dispatched to the Oita University Faculty of Medicine for sectioning and analysis.

The presence of *H. pylori* was determined by using a combination of a rapid urease test, culture, serology, and histology as described by a previous study [5, 6]. Patients were considered to be *H. pylori*-negative when all the tests were negative. *H. pylori*-positive status required at least one positive test result.

2.3. *H. pylori* culture and *cagA* genotype

Antral biopsy specimens were obtained for the isolation of *H. pylori* by standard culturing technique as previously described [7]. *H. pylori* DNA was extracted from confluent plate cultures expanded from a single colony by using DNeasy Blood & Tissue Kit[®] (Qiagen, Inc., Santa Clarita, CA). The *cagA* status was determined as previously described [7].

2.4. Histology and CagA status

Biopsy specimens for histology were obtained from within 5 mm of the sites used for cytokine measurement. After arrival in Japan, the fixed specimens were embedded in paraffin wax and stained with hematoxylin and eosin and Giemsa stains, followed by evaluation by a single pathologist blinded to the patient's clinical diagnosis or the characteristics of the *H. pylori* strains. The following features were evaluated on each slide: *H. pylori* density and the degree of mononuclear cell (MNC) and polymorphonuclear leucocyte (PMN) infiltrations, with reference to the updated Sydney System. The *H. pylori* density was also evaluated by immunohistochemistry with polyclonal anti-*H. pylori* antibody, as described previously [8]. The *H. pylori* density was scored based on the average density on the surface and the foveolar epithelium. If areas with widely different scores were obtained on the same specimen, an average based on the general evaluation of the biopsy was considered. Only areas without metaplasia were evaluated for the presence of *H. pylori*. Immunohistochemistry for CagA status was performed with polyclonal α -CagA antibody (Ab) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), as described previously [8].

2.5. RNA analysis

The gastric mucosal *IL-8* mRNA and *IL-10* mRNA levels were measured. IL-10 is an anti-inflammatory cytokine [9] that is believed to be involved in the immunoregulatory response to *H. pylori* infection [10]. Gastric specimens were placed in RNAlater (Ambion[®] Carlsbad, CA, USA) and stored at -80°C until use. RNA was isolated from the gastric specimens by an experienced researcher (H.N.) blinded to the *H. pylori* status. mRNA was isolated by using a commercially available kit (Ambion[®] Carlsbad, CA, USA). Reverse-transcriptase PCR (RT-PCR) was performed by using the SuperScript[®] III Reverse Transcriptase (Invitrogen[™] Carlsbad, CA, USA). Real-time PCR was performed by TaqMan PCR. The quantification of *IL-8*, *IL-10*, and *beta-actin* mRNA levels were performed by using the ABI Prism 7300 Sequence Detection System (Life Technologies, CA, USA). Specific primers and TaqMan probes were designed by using the Gene Expression Assay Kit (Life Technologies, CA, USA). A standard curve was constructed with 7-fold serial dilutions of a partial *IL-8*, *IL-10*, and *beta-actin* cDNA levels into the pMD18-T Simple Vector (Sino Biological Inc., China). Reaction mixtures for PCR (20 μL) were prepared by mixing 4 μL of synthesized cDNA solution to 10 μL of 2X TaqMan Universal Master Mix (Life Technologies, CA, USA). The samples were placed in the analyzer and PCR was conducted according to the manufacturer's instructions. To normalize the *IL-8* and *IL-10* mRNA expression levels, the *beta-actin* mRNA level was quantified in the same reactions by using TaqMan *beta-actin* control reagents (Life Technologies, CA, USA). The expression levels of *IL-8* and *IL-10* mRNA were expressed as the ratio of *IL-8* and *IL-10* mRNA to *beta-actin* mRNA.

2.6. Statistics

Statistical analysis was performed by using Mann–Whitney U test for non-parametric data. Correlation coefficients were calculated by the Pearson product-moment correlation coefficient

1 test. Correlation coefficients of *IL-8* and *IL-10* expression and histological grade/*H. pylori*
2 density were calculated by Spearman's rank correlation coefficient. A p value of <0.05 was
3 considered statistically significant. All the analyses were performed using the JMP 10.0 software
4 (SAS, Cary, NC, USA).

5

6

3. Results

3.1. Subjects

A total of 138 subjects from Bhutan (mean age: 38.5 y, age range: 17–92 y) and 155 subjects from the Dominican Republic (mean age: 47 y, age range: 17–91 y) (Table 1) were included in this study. The subjects from Bhutan were previously reported in a survey of the prevalence of *H. pylori* in Bhutan [5]. The prevalence of *H. pylori* was 64% in Bhutan and 59% in the Dominican Republic (Table 1). All the *H. pylori*-positive subjects were offered anti-*H. pylori* therapy.

3.2. *cagA* genotyping

From among the 88 *H. pylori*-positive tissue samples from Bhutanese subjects, 73 cases were successfully cultured and were examined for the *cagA* genotype. Three samples (4.1%) showed Western-type *cagA* and 70 samples (95.9 %) showed East-Asian type *cagA* genotypes. From among the 91 *H. pylori*-positive tissues samples from Dominican subjects, 55 samples could be cultured and the *cagA* genotype were examined. Of these 55 samples, 16 (29.1%) were *cagA*-negative and 39 (70.9%) were Western-type *cagA* (Table 2).

3.3. Histological findings and *H. pylori* density

We compared the histological findings and *H. pylori* density among the *H. pylori*-positive samples with reference to the updated Sydney System (Table 3). The atrophy scores were significantly higher in the samples from Bhutanese subjects (45% for grade 2 or 3) as compared to those from the Dominican subjects (16.4% for grade 2 and 0% for grade 3) ($p < 0.001$). The *H. pylori*-density scores for grade 2 or 3 were also significantly higher ($p = 0.045$) in the biopsies from Bhutan (71.5%) than in those from the Dominican Republic (56%). Although the overall

PMN-infiltration scores were significantly higher in the biopsies from Bhutan than in those from the Dominican Republic, the proportion of biopsies from Bhutan (49%) versus that from the Dominican Republic (37%) with PMN scores of 2 or 3 did not show statistical significance ($p = 0.161$).

3.4. *IL-8* and *IL-10* mRNA expression

As expected, the *IL-8* mRNA levels of the antral mucosa from *H. pylori*-positive samples were significantly greater than those from *H. pylori*-negative samples both in the subjects from Bhutan (median: 5.4, range: 0.12–48 versus median: 0.10, range: 0.0–2.4; $p < 0.001$) and the Dominican Republic (median: 3, range: 0.08–22 versus median: 0.07, range: 0.0–11.4; $p < 0.001$) (Figure 1A). The *IL-8* mRNA levels of the antral mucosa from *H. pylori*-positive samples from Bhutanese subjects were significantly (approximately 2-fold) higher than those from the Dominican subjects ($p < 0.001$).

The *IL-10* mRNA levels of antral biopsies of *H. pylori*-positive samples were also significantly higher than those of the *H. pylori*-negative samples in both subjects from Bhutan (median: 0.30, range: 0.00–15.2 versus median: 0.01, range: 0.00–0.69; $p < 0.001$) and the Dominican Republic (median: 0.35, range: 0.0–7.6 versus median: 0.04, range: 0.0–0.6; $p < 0.0001$) (Figure 1B).

There were no significant differences between the countries, irrespective of their *H. pylori* status.

Significant positive, but weak correlations were observed between the *IL-8* and *IL-10* mRNA levels in samples from Bhutan (*H. pylori*-positive: $R^2 = 0.30$, $p < 0.001$, *H. pylori*-negative: $R^2 = 0.28$, $p = 0.006$) and the Dominican Republic (*H. pylori*-positive: $R^2 = 0.40$, $p < 0.001$, *H. pylori*-negative: $R^2 = 0.10$, $p = 0.06$), irrespective of the *H. pylori* status (Figure 1C).

3.5. Correlation between *IL-8* and *IL-10* mRNA levels and histological findings

The correlation between *IL-8/IL-10* mRNA levels and histological findings/*H. pylori* density is shown in [Supplemental Table 1](#). Significant positive correlations were noted between the *IL-8* mRNA levels and PMN grades (Bhutan: correlation coefficient = 0.259, $p = 0.015$; Dominican Republic: correlation coefficient = 0.317, $p = 0.003$) and *H. pylori* density (Bhutan: correlation coefficient = 0.247, $p = 0.020$; Dominican Republic: correlation coefficient = 0.292, $p = 0.005$) in both the countries. The *IL-10* mRNA levels of Bhutanese samples were positively correlated to the atrophy scores (correlation coefficient = 0.22, $p = 0.038$), unlike those of the Dominican samples.

The *IL-8* mRNA levels were significantly higher in biopsies from Bhutan as compared to those from the Dominican Republic on restricting the comparison to PMN infiltration scores of 1 (mild) or 2 (moderate) ($p = 0.01$ and $p = 0.03$, respectively) (Figure 2). The *IL-10* mRNA levels were not significantly different between the biopsies from Bhutan and the Dominican Republic on comparing the levels among those with the same histological grade.

3.6. Correlation between *IL-8* and *IL-10* mRNA expression levels and *H. pylori* density

As significant correlation was noted between the *IL-8* mRNA levels and *H. pylori* density, we examined the correlation of *H. pylori* density in *H. pylori*-positive samples with reference to the updated Sydney System (Figure 3). The mucosal *IL-8* mRNA levels of samples with *H. pylori*-density scores of 1 and 2 were significantly higher in samples from Bhutanese subjects (medians: 5.5 and 6.2, respectively) than in those from the Dominican subjects (medians: 3.1 and 3.0, respectively) ($p < 0.05$ for both).

3.7. *IL-8* expression in relation to *cagA* genotype and CagA status

The *IL-8* mRNA levels were significantly greater for East Asia-type *cagA* samples from Bhutan than for Western-type *cagA* samples from the Dominican Republic and *cagA*(-) *H.*

pylori-positive samples from the Dominican Republic (E-*cagA* from Bhutan; median: 7.6,

range: 0.44–48 versus W-*cagA* from Dominican Republic; median: 3.5, range: 0.08–22; $p <$

0.001 versus *cagA*(-)from Dominican Republic; median: 3.2, range: 0.3–13; $p < 0.001$,

respectively) (Figure 4). There were no significant differences between *cagA*(-) from Dominican

Republic and W-*cagA* from Dominican Republic ($p = 0.02$) genotypes in this respect.

The *IL-10* mRNA levels were not significantly different in relation to the *cagA* genotype (E-*cagA*

from Bhutan; median: 0.30, range: 0.00–5.2 versus W-*cagA* from Dominican Republic; median:

0.38, range: 0.00–7.6 versus *cagA* (-)from Dominican Republic; median: 0.30, range: 0.04–0.85)

(data not shown).

We performed immunohistochemistry to determine the CagA status. In Bhutan, 70 samples were

α -CagA positive from among 70 samples with α -*H. pylori* Ab-positive status, while in the

Dominican Republic, 28 samples were α -CagA-negative and 61 samples were α -CagA positive

from among 89 samples with α -*H. pylori* Ab-positive status. For the Dominican Republic

subjects, the *IL-8* mRNA levels of α -CagA-positive samples was significantly higher than those

of the α -CagA-negative samples (median: 3.3, range: 0.08–22 versus median: 2.00, range:

0.31–18; $p = 0.04$, respectively).

4. Discussion

To the best of our knowledge, prior reports describing inflammatory cytokine expression in *H. pylori*-infected gastric mucosa have been limited to a single region (eg. Japan, USA) [11]. In this study, samples were collected by the same team and RNA extraction and pathology diagnosis were performed by the same individuals, allowing for a comparison between areas that differed greatly in relation to gastric cancer incidence and *H. pylori cagA* genotype. CagA is a highly immunogenic protein injected into cells by a type IV secretion system [12, 13]. Most *H. pylori* isolates from the East Asian countries are *cagA*-positive as compared to 50–80% of the isolates from the Western countries [14]. The East Asia-type *cagA* differs from the Western-type *cagA* by the pattern of repeat sequences of the 3' region of *cagA* [14]. These repeat regions contain the Glu-Pro-Ile-Tyr-Ala (EPIYA) motif and is annotated according to segments (20–50 amino acids) flanking the EPIYA motifs (i.e., segments EPIYA-A, -B, -C, or -D). The East Asia-type *cagA*, containing EPIYA-D segments, exhibit a stronger binding affinity for Src homology 2 domain of Src homology 2-containing protein-tyrosine phosphatase (SHP-2), and a greater ability to induce morphological changes in the epithelial cells *in vitro* than Western-type *cagA* containing EPIYA-C segments [15]. Strains from Bhutan typically contain East Asia-type *cagA*, whereas strains from the Dominican Republic typically contain Western-type *cagA*.

East Asia-type *cagA* has been associated with a higher level of mucosal inflammation than Western-type *cagA*. Consistent with this observation, the gastric antral *IL-8* mRNA levels were significantly higher in the *H. pylori*-infected individuals from Bhutan than from the Dominican Republic. In addition, a higher density of *H. pylori* was noted in the antral specimens from Bhutan; this finding is in concordance with a previous report of close association of *H. pylori* density to the gastric mucosal *IL-8* mRNA levels [2]. Moreover, the *H. pylori* density of the antral mucosal biopsies from Bhutan was much higher than those of the gastric mucosa of infected individuals from the Dominican Republic; in both the cases, the *IL-8* mRNA level was significantly correlated with the *H. pylori* density. The mucosal *IL-8* mRNA levels among those

1 with *H. pylori*-density scores of 1 (mild) or 2 (moderate) were significantly higher in samples
2 from Bhutanese subjects (East Asia-type *cagA*) than in those from Dominican subjects
3 [Western-type *cagA* or *cagA* (-)] (Figure 6). In addition, although the number of
4 Western-type-positive strain from Bhutan was small, the mucosal *IL-8* mRNA levels were similar
5 between the samples with Western-type *cagA* from Bhutan and from Dominican Republic. These
6 data suggest that the ability of *H. pylori* to induce *IL-8* with the same density may be greater for
7 East Asia-type strains than for Western-type *cagA* or *cagA*(-) strains. This hypothesis is in
8 agreement with previous reports regarding differences in *IL-8* response to East-Asian and
9 Western type *cagA* from animal experiments [16].

10 However, more interestingly, the *IL-8* mRNA levels were also significantly higher in biopsies
11 from Bhutan compared to those from the Dominican Republic even when the comparison was
12 restricted to PMN infiltration scores of 1 (mild) or 2 (moderate). In other words, there was a
13 relatively low PMN response despite higher *IL-8* mRNA levels in Bhutanese subjects. This
14 finding is also consistent with the notion that East Asia-type *cagA*-positive strains are associated
15 with increased expression of *IL-8* mRNA as compared to Western-type *cagA*-positive strains, but
16 with lesser effectiveness in eliciting a PMN response in some patients. Future experiments are
17 needed to confirm this observation and to investigate whether host or environmental factors are
18 responsible for this discrepancy. Recently, Gil et al. [17] reported that the mucosal PMN score
19 was positively correlated with the number of Th17 cells expressing lymphocytes and negatively
20 correlated with the Th17 cells/FOX3P+Treg ratio. Serrano et al. [18] also reported that *H.*
21 *pylori*-positive children showed downregulated Th17 response in the gastric mucosa. We thus
22 speculate that the reduced PMN response, despite higher *IL-8* mRNA levels, may be related to
23 downregulation of Th17 response. The overall PMN response may therefore be the result of both
24 positive and inhibiting signals, although we assessed only the positive signal in this study.

1 The *IL-10* mRNA levels of *H. pylori*-positive samples were not significantly different between
2 the two countries, and no particular trend by histological grade and *cagA* genotype was noted.
3 IL-10 also known as human cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory
4 cytokine. Bodger et al. [19] described that *in vitro*-blocking of endogenous IL-10 secretion did
5 not significantly increase the cytokine secretion in relation to the Western-type *cagA* strain. Our
6 data confirmed that IL-10 secretion was independent of both East-Asia and Western-type *cagA*
7 genotype [2]. However, the *IL-10* mRNA levels from Bhutanese samples demonstrated a
8 significant positive correlation coefficient with atrophy score, suggesting a need to perform *in*
9 *vivo* experiments for analyzing the IL-10 expression and gastric atrophy.

11 Conclusion

12 The difference in the *IL-8* expression reflected the differences in the disease manifestation
13 patterns between the two countries.

References

- [1.] Suerbaum S, Michetti P. *Helicobacter pylori* infection. The New England journal of medicine 2002; 347, 1175-1186.
- [2.] Yamaoka Y, Kodama T, Kita M, Imanishi J, Kashima K, Graham DY. Relation between clinical presentation, *Helicobacter pylori* density, interleukin 1beta and 8 production, and *cagA* status. Gut 1999; 45, 804-811.
- [3.] Yamaoka Y, Kita M, Kodama T, Sawai N, Kashima K, Imanishi J. Induction of various cytokines and development of severe mucosal inflammation by *cagA* gene positive *Helicobacter pylori* strains. Gut 1997; 41, 442-451.
- [4.] Yamaoka Y, Kato M, Asaka M. Geographic differences in gastric cancer incidence can be explained by differences between *Helicobacter pylori* strains. Internal medicine 2008; 47, 1077-1083.
- [5.] Shiota S, Mahachai V, Vilaichone RK, Ratanachu-Ek T, Tshering L, Uchida T, et al. Seroprevalence of *Helicobacter pylori* infection and gastric mucosal atrophy in Bhutan, a country with a high prevalence of gastric cancer. Journal of medical microbiology 2013; 62, 1571-1578.
- [6.] Vilaichone RK, Mahachai V, Shiota S, Uchida T, Ratanachu-ek T, Tshering L, et al. Extremely high prevalence of *Helicobacter pylori* infection in Bhutan. World journal of gastroenterology : WJG 2013; 19, 2806-2810.
- [7.] Matsunari O, Shiota S, Suzuki R, Watada M, Kinjo N, Murakami K, et al. Association between *Helicobacter pylori* virulence factors and gastroduodenal diseases in Okinawa, Japan. Journal of clinical microbiology 2012; 50, 876-883.
- [8.] Yasuda A, Uchida T, Nguyen LT, Kawazato H, Tanigawa M, Murakami K, et al. A novel diagnostic monoclonal antibody specific for *Helicobacter pylori* CagA of East Asian type. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica 2009; 117, 893-899.
- [9.] Shiota S, Murakami K, Suzuki R, Fujioka T, Yamaoka Y. *Helicobacter pylori* infection in Japan. Expert review of gastroenterology & hepatology 2013; 7, 35-40.
- [10.] Takahashi A, Shiota S, Matsunari O, Watada M, Suzuki R, Nakachi S, et al. Intact long-type *dupA* as a marker for gastroduodenal diseases in Okinawan subpopulation, Japan. Helicobacter 2013; 18, 66-72.

- [11.] Lee KH, Cho MJ, Yamaoka Y, Graham DY, Yun YJ, Woo SY, et al. Alanine-threonine polymorphism of *Helicobacter pylori* RpoB is correlated with differential induction of interleukin-8 in MKN45 cells. *Journal of clinical microbiology* 2004; 42, 3518-3524.
- [12.] Covacci A, Censini S, Bugnoli M, Petracca R, Burrone D, Macchia G, et al. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. *Proceedings of the National Academy of Sciences of the United States of America* 1993; 90, 5791-5795.
- [13.] Tummuru MK, Cover TL, Blaser MJ. Cloning and expression of a high-molecular-mass major antigen of *Helicobacter pylori*: evidence of linkage to cytotoxin production. *Infection and immunity* 1993; 61, 1799-1809.
- [14.] Yamaoka Y. Mechanisms of disease: *Helicobacter pylori* virulence factors. *Nat Rev Gastroenterol Hepatol* 2010; 7, 629-641.
- [15.] Hatakeyama M. Oncogenic mechanisms of the *Helicobacter pylori* CagA protein. *Nat Rev Cancer* 2004; 4, 688-694.
- [16.] Fu HY, Asahi K, Hayashi Y, Eguchi H, Murata H, Tsujii M, et al. East Asian-type *Helicobacter pylori* cytotoxin-associated gene A protein has a more significant effect on growth of rat gastric mucosal cells than the Western type. *Journal of gastroenterology and hepatology* 2007; 22, 355-362.
- [17.] Gil JH, Seo JW, Cho MS, Ahn JH, Sung HY. Role of Treg and TH17 Cells of the Gastric Mucosa in Children With *Helicobacter pylori* Gastritis. *Journal of pediatric gastroenterology and nutrition* 2014; 58, 252-258.
- [18.] Serrano C, Wright SW, Bimczok D, Shaffer CL, Cover TL, Venegas A, et al. Downregulated Th17 responses are associated with reduced gastritis in *Helicobacter pylori*-infected children. *Mucosal immunology* 2013; 6, 950-959.
- [19.] Bodger K, Bromelow K, Wyatt JI, Heatley RV. Interleukin 10 in *Helicobacter pylori* associated gastritis: immunohistochemical localisation and in vitro effects on cytokine secretion. *Journal of clinical pathology* 2001; 54, 285-292.

1 **Table 1. Characteristics of the study subjects**

	Bhutan (N = 138)	Dominican Republic (N = 155)
Age (years) (age range; years)	38.5 (17–92)	47.0 (17–91)
Gender (Male: Female)	60:78	54:101
<i>H. pylori</i> -positive (%)	88 (64%)	91 (58%)
<i>H. pylori</i> -positive by culture (%)	73 (53%)	57 (37%)

2

Table 2. *cagA* genotypes among the two study countries

	Bhutan (n = 73)	Dominican Republic (n = 57)
<i>cagA</i> (-)	0	16
Western-type <i>cagA</i>	3	41
East Asia-type <i>cagA</i>	70	0

Table 3. Grading by the updated Sydney System

Grade	PMN		MNC		Atrophy		<i>H. pylori</i> density	
	Bhutan	Dominican Republic	Bhutan	Dominican Republic	Bhutan	Dominican Republic	Bhutan	Dominican Republic
0	0	5	0	0	1	11	9	8
(%)	(0)	(5.5)	(0)	(0)	(1.1)	(12.1)	(10.2)	(8.8)
1	45	52	22	26	47	65	16	32
(%)	(51.1)	(57.1)	(25.0)	(28.0)	(53.4)	(71.4)	(18.2)	(35.2)
2	37	31	56	55	36	15	26	41
(%)	(42.1)	(34.1)	(63.6)	(60.4)	(40.9)	(16.5)	(29.6)	(45.1)
3	6	3	10	10	4	0	37	10
(%)	(6.8)	(3.3)	(11.4)	(11.0)	(4.6)	(0)	(42.1)	(11.0)
mean	1.56	1.35	1.86	1.82	1.48	1.04	2.03	1.58
	[1]	[1]	[2]	[2]	[1]	[1]	[2]	[2]
P value	0.04*		0.64		< 0.001*		< 0.001*	

Abbreviations; PMN: polymorphonuclear leucocyte, MNC: mononuclear cell

Figure legends

Figure 1.

A) *IL-8* mRNA levels by *H. pylori* status and countries.

The expression levels of *IL-8* mRNA are expressed as a ratio to the *beta-actin* mRNA level.

Data are expressed by box plotting.

B) *IL-10* mRNA levels by *H. pylori* status and countries.

The expression levels of *IL-10* mRNA are expressed as a ratio to *beta-actin* mRNA level.

Data are expressed by box plotting.

C) Correlation coefficient between *IL-8* mRNA and *IL-10* mRNA levels.

Correlation coefficients were calculated by the Pearson product-moment correlation coefficient test. White circles represent *H. pylori*-negative samples and black circles represent *H. pylori*-positive samples. Dotted line and straight lines show regression line of correlation coefficient of *H. pylori*-negative cases and *H. pylori*-positive cases between the *IL-8* and *IL-10* mRNA levels, respectively.

Figure 2. *IL-8* mRNA levels by PMN infiltration.

The *IL-8* mRNA levels were significantly higher in samples from Bhutanese subjects than in those from Dominican subjects on comparing the levels among the same PMN histological grade scores of 1 and 2. Data are expressed by box plotting.

Figure 3. *IL-8* mRNA levels by *H. pylori* density.

The *IL-8* mRNA levels were significantly higher in samples from Bhutanese subjects than in those from Dominican subjects on comparing the levels among the same *H. pylori*-density scores of 1 and 2. Data are expressed by box plotting.

Figure 4. *IL-8* mRNA levels by *cagA* genotype and countries.

No samples from Bhutanese subjects were *cagA*-negative. The *IL-8* mRNA levels of E-*cagA* from samples from Bhutanese subjects was significantly higher than those of W-*cagA* and *cagA*-negative samples from Dominican subjects. No difference between the Western-type *cagA* and *cagA*-negative genotypes was noted from the samples from Dominican subjects. The *IL-8* mRNA levels of Western-type-*cagA* for samples from Bhutanese subjects were similar to those of Western-type- *cagA* for the samples from Dominican subjects. Data are expressed by box plotting.

E-*cagA*: East-Asian type *cagA*, W-*cagA*: Western-type *cagA*, *cagA*(-): *cagA*-negative

Figure 1

Figure 2

Figure 3

Figure 4