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Characterizing *Helicobacter pylori* *cagA* in Myanmar

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Background/Aims: Differences in the *Helicobacter pylori* infection rate are not sufficient to clarify the dissimilarity of gastric cancer incidence between Myanmar and its neighboring countries. To better understand this trend, the *H. pylori* virulence gene *cagA* was characterized in Myanmar.

Methods: Glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) patterns and CagA multimerization (CM) motifs of *cagA* genotypes were examined by performing polymerase chain reactions and DNA sequencing. **Results:** Of 69 tested *H. pylori* strains, *cagA*-positive patients had significantly more severe histological scores in their antrum than *cagA*-negative patients. Sequence analysis revealed that 94.1% of strains had Western-type *cagA* containing an EPIYA motif (92.6%) or EPIYT motif (6.4%). The intestinal metaplasia scores in the antral of patients infected with the ABC and ABCC types of *cagA* were significantly higher than those of patients with AB-type *cagA*. Interestingly, in patients infected with *H. pylori*, 46.3% of strains with three EPIYA motifs contained two identical Western-typical CM motifs, and these patients showed significantly higher antrum inflammation scores than patients infected with two identical nontypical-CM motif strains ($p=0.02$). **Conclusions:** In Myanmar strains, Western-type *cagA* was predominant. The presence of CM motifs and the proportion of multiple EPIYA-C segments might partially explain the intermediate gastric cancer risk found in Myanmar.

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Key Words: *Helicobacter pylori*, *cagA*, Myanmar

INTRODUCTION

Myanmar is the largest nation in mainland Southeast Asia with the gastric cancer age-standardized incidence rate (ASR) was reported to be 12.1 every 100,000 people per year based on GLOBOCAN 2012 (<http://globocan.iarc.fr/>), which is lower than China but much higher than Thailand or India (22.7, 3.1, and 6.1 per 100,000 people, respectively). However, the prevalence of *Helicobacter pylori* infection in Thailand (54.1% to 76.1%)¹ and India (58%)² were even higher than that in Myanmar. In our previous study, we reported that the overall *H. pylori* infection rate in Myanmar was 48.0% for patients (121/252) considered to be *H. pylori*-positive in no less than one out of five tests, including rapid urease test, culture, histology, immunohistochemistry, and serology.³ Therefore, the difference in the prevalence of *H. pylori* infection is not sufficient to elucidate the gastric cancer ASR differences between Myanmar and its neighboring countries.

Besides genetic and environmental components, the incidence of gastric cancer variety were reported to be clarified by *H. pylori* virulence factors' divergences, such as CagA, instead of the prevalence of *H. pylori* infection.⁴ Every CagA repeat region conceives glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) motifs, denoted as EPIYA-A or EPIYA-B segments for the first repeat region, and EPIYA-C (Western-type *cagA*) or EPIYA-D (East Asian-type *cagA*) segments for the second repeat region. We previously showed that East Asian-type *cagA* was more virulent than Western-type *cagA*,⁵ partially due to a stronger binding affinity for Src homology 2 (SH2) containing protein-

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tyrosine phosphatase (SHP-2) and a greater ability to induce morphological changes in epithelial cells than Western-type *cagA*.⁶⁻⁸ In addition, the number of "C" sites among Western-type *cagA* varieties is directly correlated to the level of tyrosine phosphorylation and SHP-2-binding activity.⁹ We also previously reported that the number of "C" sites was associated with the severity of atrophic gastritis and gastric cancer in patients infected with Western-type *cagA*-positive strains.^{10,11}

Among the EPIYA-B motif of Western-type CagA; EPIYA was the most common sequences, followed by EPIYT^{5,12,13} and it was associated with clinical outcomes.^{13,14} Based on the analysis of 364 Western-type CagA sequences, Zhang *et al.*¹³ revealed that gastric cancer cases were more belonged to the EPIYA sequence in the EPIYA-B motif rather than the EPIYT sequence, which might be due to differences in its affinity for phosphoinositol 3-kinase and induction of protein kinase B. In addition, we also confirmed that Indonesian patients that were infected with strains containing EPIYA sequences had less histological severity in their antrum than those with EPIYT sequences. This could predispose these patients to duodenal ulcer due to an opportunity of *H. pylori* to colonize in the duodenum and induce gastric metaplasia on the duodenal mucosa.¹⁴

Sixteen amino acids conserved motif sequence in the C-terminal variable region of CagA (e.g., FPLXRXVXVXDLKSKVG), designated as CagA multimerization (CM) or CRPIA (conserved repeat responsible for phosphorylation-independent activity) motifs, is a prerequisite for the CagA-SHP-2 interaction, and it functions in CM, playing a vital role in *H. pylori* pathogenesis.^{15,16} Among them, 11 amino acids are well conserved in both *cagA* genotypes. East Asian-type *cagA* strains carries a single CM motif, located after the EPIYA-D segment, whilst two CM motifs placed within each and distal to the ending of EPIYA-C possessed by Western-type *cagA* strains.¹⁷ The interaction of CM and c-Met promotes inflammation and proliferation through nuclear factor κ B transcriptional activities and upregulation of β -catenin.¹⁸ The impact of CagA on host cells multimerization may influenced by the CM motifs frequency and variety, thus may influence the CagA competence to induce host cell disruption through SHP-2 deregulation.¹⁶

We hypothesized that characteristics of *H. pylori cagA* in Myanmar are different than those in neighboring countries and become a determinant of gastric cancer incidence rate differences between these countries.

MATERIALS AND METHODS

1. Patients and *H. pylori*

We conducted a survey in both the largest cities of Myanmar, Yangon, and the second largest city, Mandalay. Gastric biopsy specimens for histological examination were embedded in paraffin after fixed into 10% buffered formalin. Hematoxylin and eosin as well as May-Giemsa were selected as stain techniques.

The updated Sydney and the Operative Link on Gastritis Assessment (OLGA) system were used to analyze histological severity.¹⁹ Peptic ulcer and gastric cancer were identified by endoscopy, and gastric cancer was further confirmed by histopathology. Gastritis were determined based on any score of activity and inflammation (score ≥ 1) in antral and/or corpus in histological examination. Informed consent agreement was signed by all participants, and the Ethics and Research Committee of the University of Medicine, Myanmar; Mandalay General Hospital; and Oita University Faculty of Medicine, Japan approved our protocol.

2. *H. pylori* genotyping

We cultured and isolated *H. pylori* colonies from biopsy specimens as previously described.²⁰ The QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA) was used to extract *H. pylori* DNA for *H. pylori* genotyping in accordance with the manufacturer's instructions. Polymerase chain reaction were used to determine the positivity of *cagA* and the bands were detected with electrophoresis (1.5% agarose gel) in an ultraviolet transilluminator.⁵ The direct sequencing of *cagA* EPIYA repeat region and CM motif were performed using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on a ABI 3130 Genetic Analyzer according to the manufacturer's instructions. Multiple sequence alignments of the *cagA* sequences were generated using MAFFT version 7 (available at <http://mafft.cbrc.jp/alignment/server/>) and confirmed by visual inspection.

3. Data analysis

Data were analyzed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA). Discrete variables were tested using the chi-square test; continuous variables were tested using the Mann-

Table 1. Histological Scores According to *cagA* Status

	<i>cagA</i> (+)	<i>cagA</i> (-)	p-value
Number	61	8	
Antrum			
Activity	1.54 (2)	0.75 (1)	0.003
Inflammation	1.74 (2)	1.25 (1)	0.04
Atrophy	0.93 (1)	0.50 (0.5)	0.01
Intestinal metaplasia	0.16 (0)	0.00 (0)	0.32
Corpus			
Activity	0.87 (1)	0.75 (1)	0.69
Inflammation	1.10 (1)	1.25 (1)	0.25
Atrophy	0.28 (0)	0.5 (0.5)	0.13
Intestinal metaplasia	0.00 (0)	0.00 (0)	1.00
OLGA score	0.98 (1)	0.50 (0.5)	0.01

Histological scores are presented as mean (median).
OLGA, Operative Link on Gastritis Assessment system.

Whitney U test and t-test. A two-tailed p-value <0.05 was considered statistically significant.

RESULTS

We successfully isolated 69 Myanmarese strains (41 females and 28 males, mean age of 40.1 ± 11.5 years) from Yangon (n=52) and Mandalay (n=17) were examined by culture in our study. Sixty-five strains were isolated from gastritis patients, three were from duodenal ulcer patients, and one was from a gastric cancer patient. Patients from Mandalay contained slightly higher number of *cagA*-positive strains (94.1%, 16/17) compared with those from Yangon (86.5%, 45/52) (p=0.40). Table 1 described the association of histological scores and *cagA* status. Scores for atrophy in the antrum were significantly lower in patients infected with *cagA*-negative strains than those with *cagA*-positive strains (mean [median], 0.50 [0.5] vs. 0.93 [1], p=0.01). Activity and inflammation scores in the antrum of patients infected with *cagA*-positive strains were also significantly higher than in those with *cagA*-negative strains (mean [median], 1.54 [2] vs 0.75 [1], p=0.003; 1.74 [2] vs 1.25 [1], p=0.04, respectively). In contrast, these differences could not be observed in the corpus. None of the eight patients infected with *cagA*-negative strains had intestinal metaplasia in the antrum or corpus. The OLGA score in patients infected with *cagA*-positive strains was also significantly higher than in those infected with *cagA*-negative strains (mean [median], 0.98 [1] vs 0.50 [0.5], p=0.01).

1. Characterization of the EPIYA motif in the *cagA* 3' repeat region

Surprisingly, sequence analysis of the *cagA* 3' repeat region revealed that 41 strains were ABC type, 10 were ABCC, two were AB, one was ABCCC, and one was ACC. All of these strains are classified as Western-type *cagA* (55/61, 90.2%). In contrast, only five strains were ABD and one ABBD, which are considered to be East Asian-type *cagA* (6/61, 9.8%). Amino acid sequences phylogeny demonstrated that most of Western-type *cagA* was linked to the references from Asian countries (Fig. 1). There was no difference in *cagA* type between strains isolated from Yangon and Mandalay (p=0.38). Although there was no relationship between *cagA* type and clinical outcomes in the Myanmar population, all cases from peptic ulcer or gastric cancer patients were Western-type *cagA*, whilst all of East Asian-type *cagA* strains were isolated from patients with gastritis. There was no statistical difference in the histological scores between East Asian-type and Western-type *cagA* (p>0.05), possible owing to the small sample size. However, the intestinal metaplasia score in the antrum of patients infected with ABC- and ABCC-type strains were significantly higher than that of AB-type strains (p<0.0001 and p=0.02, respectively).

Four types of EPIYA or EPIYA-like sequences were obtained

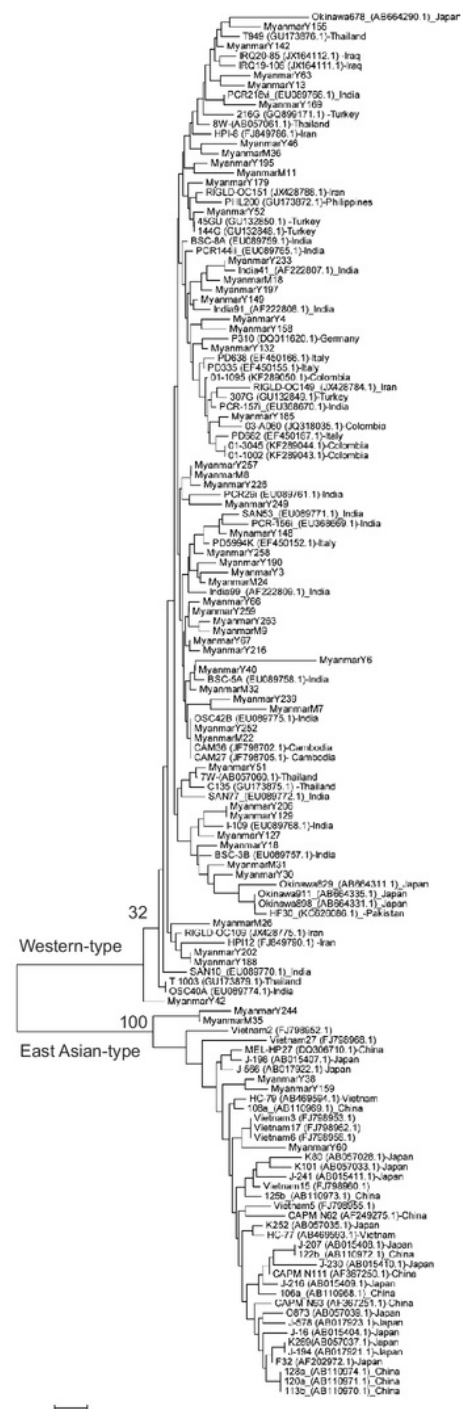


Fig. 1. Phylogeny analysis of *cagA* in Myanmar strains. Phylogenetic trees were constructed using the neighbor-joining method, and estimated genetic distances were analyzed using the six-parameter method to compare Myanmarese strains against reference strains from GenBank. Main branch bootstrap values were calculated, and horizontal bar lengths represent the number of nucleotide substitutions per site.

and the EPIYA motifs in each strain shown in Table 2. Among 61 CagA sequences, we found totally 189 EPIYA motifs. Thus, every CagA sequence contained approximately three EPIYA motifs in average. EPIYA was the most common among EPIYA motifs (175/189, 92.6%), followed by EPIYT (12/189, 6.4%). Segment B containing EPIYA was more predominant than EPIYT in Western-type *cagA* (78.3% vs 20.0%). There was no significant association between EPIYA motif type and histological score ($p>0.05$).

Table 2. Frequencies of EPIYA Motifs

Type	All motifs	Total	A motif	B motif	C or D motif
All <i>cagA</i> types	EPIYA	175	61	47	67
	EPIYT	12	0	12	0
	EPSYA	1	0	0	1
	ESIYG	1	0	1	0
	Total	189	61	60	68
Western-type <i>cagA</i>	EPIYA	160	55	44	61
	EPIYT	10	0	10	0
	EPSYA	1	0	0	1
	Total	171	55	54	62
East Asian-type <i>cagA</i>	EPIYA	15	6	3	6
	EPIYT	2	0	2	0
	ESIYG	1	0	1	0
	Total	18	6	6	6

EPIYA, glutamate-proline-isoleucine-tyrosine-alanine.

2. Characterization of the CM motif

Among 67 strains with *cagA*-positive, 25 contained the typical peptide sequence of Western countries strains (FPLKRHDKVDDLSKVG), placed within the EPIYA-C segment, immediately after and the last repeat. Only 19 of 41 strains (46.3%) with three EPIYA motifs contained the Western CM twin motifs (Fig. 2). Interestingly, 24.4% of them possessed the nontypical twin CM motifs (FPLKHKDKVDDLSKVG), while 11 strains possessed a mix of typical Western and nontypical Western CM motifs (mixed-type CM motifs). Compared with the patients infected with strains containing typical Western CM motifs, patients with strains with nontypical Western CM motifs had significantly higher scores for antrum inflammation (mean [median], 2.15 [2] vs 1.56 [2], $p=0.02$). However, we found insignificant difference in histological scores between nontypical CM motifs and mixed-type CM motifs ($p>0.05$).

3. Nucleotide sequencing

The *cagA* EPIYA repeat region and CM motif sequence data are registered with accession numbers LC209107 to LC209167 assigned by DDBJ.

DISCUSSION

Nearly all *H. pylori* isolates from East Asian countries and around three-quarter of isolates from Western countries are *cagA* positive and.^{21,22} In Western countries, the peptic ulcer or gastric cancer risk prone to individuals infected with *cagA*-positive *H. pylori* strains than those infected with *cagA*-negative strains.²³ In this study, we found that *cagA*-positive patients had

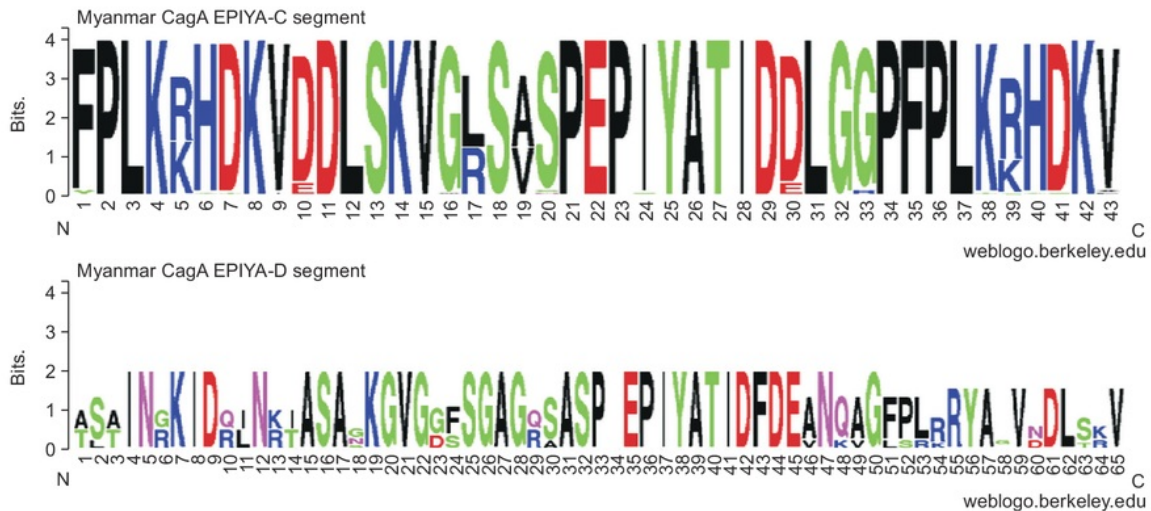


Fig. 2. CagA multimerization motifs in Western-type versus East Asian-type CagA from Myanmar strains. WebLogo, v3 software (<http://weblogo.threeplusone.com/>) was used to analyze the sequence data. EPIYA, glutamate-proline-isoleucine-tyrosine-alanine.

higher histological scores than *cagA*-negative patients. Moreover, none of gastric intestinal metaplasia observed in the eight *cagA*-negative patients suggesting the high-risk population in Myanmar are the patients infected with *cagA*-positive *H. pylori* strains as similar to Western countries.

In contrast with our prediction, most of the *cagA* sequences of the Myanmar strains were characterized as Western-type *cagA* (94.1%), similar to that from the neighboring countries India and Pakistan.^{24,25} Myanmarese ancestry includes the Tibeto-Burman (Burmese-Lolo and Karen) branches of Sino-Tibetans that moved back southwards through Yunnan to Myanmar. These tribes began their migration from the north- and the east towards the Yangtze and Yellow River basins.²⁶ Therefore, we previously believed that Myanmar would predominantly contain East Asian-type *cagA* or a minimally intermediate percentage of East Asian-type *cagA*, similar to Thailand.²⁷ Considering the current population of Myanmar, *H. pylori* would also have been acquired from intermarriage of the various races and nationalities with indigenous ethnic groups, and the prevalence of East Asian-type *cagA* could have been reduced by competition with novel strains. However, unlike the usual South Asian population that has an "Asian paradox" phenomenon, suggesting that Western-type *cagA* is likely a marker of low virulence in these populations, Myanmar is a country characterized by an intermediate risk of gastric cancer. Moreover, the ASR rate of gastric cancer in Myanmar is higher than that of a neighboring country, Thailand, where approximately half are East Asian-type and half are Western-type strains.^{1,27} Interestingly, we also found that the proportion of strains with multiple EPIYA-C segments was higher in patients from Myanmar (11/55, 20.0%) than in patients from Thailand (10/75, 13.3%)²⁸ or India (0/32, 0%).²⁹ In fact, patients infected with multiple EPIYA-C segments *H. pylori* had higher gastric cancer risk than in those infected with a single EPIYA-C segment strains, although only shown in Western countries.^{30,31} However, it is possible that the observation that strains with multiple EPIYA-C segments are less resistant to gastric acid¹¹ may have resulted from a correlation; notably, the mean age of patients with multiple EPIYA-C segments in that study was lower than in those with single EPIYA-C segments. In this study, patients with ABC and ABCC types showed significantly higher intestinal metaplasia in the antrum than in those with the AB type. This data is also compatible with a previous study showing that *H. pylori* with single or multiple EPIYA-C motifs is associated with a higher risk of gastric cancer than *H. pylori* with only AB motifs.^{32,33} However, the lack of significant histological difference between single and multiple EPIYA-C might simply be due to the small number of multiple EPIYA-C strains. Although we revealed that among Western-type *cagA*, EPIYA sequence in the EPIYA-B motif was the predominant with only a low proportion of EPIYT, similar to that found for India;²⁹ thus, it is not a determinant to explain the differing risk of gastric cancer between Myanmar and India.

Several studies have reported important proinflammatory and pro-oncogenic activities of CagA that are independent of the EPIYA motifs.^{16,17} Sicinschi *et al.*¹⁷ reported that specific Western/Western CM motif strains have more potent affinity between CagA and SHP-2 compared to that of Western/East Asian CM motif strains, suggesting that this may be as important for disease incidence. They also found that subjects from low gastric cancer risk areas were infected with Western/East Asian CM motif strains. Interestingly, in our study, almost half of strains contained a nontypical CM motif, which are associated with higher inflammation than a specific Western motif. Our unpublished data showed almost all CM motifs in Thailand have another specific Western motif. Therefore, the types of CM motifs might partly clarify the distinction in gastric cancer risk amongst Myanmar and Thailand.

Nevertheless, we also should take into account that the genetic and environmental factors may involve on gastric cancer risk determination in addition to influence of virulence factors towards gastric carcinogenesis. For example, the northern region of Thailand has the highest incidence rate of gastric cancer; in contrast, the southern region has the lowest rates.³⁴ This difference may possibly be due to consumption of foods high in nitrates and consumption of vegetables low in ascorbic acid, which was significantly higher in the northern and northeast regions than in the southern region. In Malaysia, the rate of gastric cancer was five times lower among Malay than that in Chinese or Indians.³⁵ Myanmar is subdivided into more than 100 ethnic groups; among them, the Bamar represent 68% of the population. Other common minorities are the Shan (10%), Karen (7%), Arakanese (4%), Chinese (3%), and the ethnolinguistically related Mon and Khmer (2%). These ethnic groups have diverse linguistic and cultural subgroups, religions, and different geographic locations. We isolated *H. pylori* from the two biggest cities in Myanmar, Yangon and Mandalay. In both of those cities, the Bamar are the major ethnic group present. Several ethnic groups have different ancestors than the Bamar, which might be important in distinguishing gastric cancer risk in the population of Myanmar. For example, the Karen ethnic group has a different genetic composition than the Bamar by mitochondrial DNA and also CYP2C19*3 allele analysis.^{26,36} On the other hand, Myanmar has a lower consumption of fruits and vegetables (0.19 vs 0.35 and 0.23 kg/day/capita) but lower consumption of alcohol and tobacco (0.0006 vs 0.0229 and 0.0025 L of alcohol/day/adult and 0.4 vs 2.9 and 3.8 cigarettes/day/adult, respectively) than Thailand and India, respectively.³⁷ Overall, the genetic and environmental components may be more relevant than bacterial factors for considering gastric cancer risk in Myanmar. Further studies are necessary to better elucidate the contribution of host and environmental factors for the different gastric cancer risk between Myanmar and Thailand.

The small number of strains obtained in this study was one of its primary limitations. Further studies with increased sample

numbers, balanced for each diagnosis, and including ethnogeographic region are prominent to better elucidate the influence of *H. pylori* virulence genes towards clinical outcomes.

In conclusion, to our knowledge, this is the first study that elucidated the diverse characteristics of the *cagA* region of *H. pylori* strains in Myanmar. We found that most Myanmar strains had a Western-type *cagA* genotype. The CM motifs and proportion of multiple EPIYA-C segments, which may partly explain differences in the gastric cancer rate between Myanmar and Thailand or India. However, it is possible that host genetic and environment factors may be more responsible for gastric cancer risk in Myanmar than bacterial factors.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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Author contributions: T.M., R.V., Y.Y., and V.M. designed the study; T.M., R.V., N.N., T.T.A., V.M., T.U., and Y.Y. provided the collection of samples; M.M. and P.S. performed data analysis and interpretation; M.M. and Y.Y. analyzed and interpreted data and wrote the manuscript, Y.Y. revised the manuscript to include important content. All authors read and approved the final version of the manuscript.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7
