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Neither gastric topological distribution nor principle virulence genes of *Helicobacter pylori* contributes to clinical outcomes

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Abstract

AIM: Studies on *Helicobacter pylori* (*H pylori*) and gastroduodenal diseases have focused mainly on the distal sites of the stomach, but relationship with the gastric cardia is lacking. The aim of this study is to determine if the gastric topology and genotypic distribution of *H pylori* were associated with different upper gastrointestinal pathologies in a multi-ethnic Asian population.

METHODS: Gastric biopsies from the cardia, body/corpus and antrum were endoscoped from a total of 155 patients with dyspepsia and/or reflux symptoms, with informed consent. *H pylori* isolates obtained were tested for the presence of *26kDa*, *ureC*, *cagA*, *vacA*, *iceA1*, *iceA2* and *babA2* genes using PCR while DNA fingerprints were generated using random amplification polymorphic DNA (RAPD).

RESULTS: *H pylori* was present in 51/155 (33%) of patients studied. Of these, 16, 15 and 20 were isolated from patients with peptic ulcer diseases, gastroesophageal reflux diseases and non-ulcer dyspepsia, respectively. Of the *H pylori* positive patients, 75% (38/51) had *H pylori* in all three gastric sites. The prevalence of various genes in the *H pylori* isolates was shown to be similar irrespective of their colonization sites as well as among the same site of different patients. The RAPD profiles of *H pylori* isolates from different gastric sites were highly similar among intra-patients but varied greatly between different patients.

CONCLUSION: Topographic colonization of *H pylori* and the virulence genes harboured by these isolates have no direct bearing to the clinical state of the patients. In multi-ethnic Singapore, the stomach of each patient is colonized by a predominant strain of *H pylori*, irrespective of the clinical diagnosis.

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INTRODUCTION

Helicobacter pylori (*H pylori*) is a common gastric pathogen that has infected more than 50% of the world's population[1]. It is the major aetiological agent of chronic active gastritis and is generally accepted as being the primary cause of peptic ulcer disease and a carcinogenic factor for gastric cancer (GC)[2]. However, only a minority of *H pylori* infected subjects develops these diseases. This has led to the suggestion that clinical sequelae that develop may be dependent upon differentially expressed bacterial determinants, e.g., bacterial virulence genes: *cagA*, *vacA*, *iceA1*, *iceA2*, *babA2*[3-5]. In many parts of Asia, the prevalence of *cagA* and *vacA* strains is high regardless of the presence or absence of disease states[6]. This limits the usefulness of using these genes as markers to predict clinical outcome. Instead, other factors like host susceptibility as well as specific interactions between a particular strain and its host that occur during decades of coexistence might have contributed to an increased risk of developing certain clinical manifestations[7].

Our current knowledge on the epidemiology of the organism is predominantly based on data obtained from serologic studies. It has also been reported that a single strain of *H pylori* predominates the gastric antrum and corpus of infected patients in Singapore[8]. However, there is a need to study whether the topographic distribution of *H pylori* genotypes in various gastric sites in patients of different ethnic origins affects the disease state. Being an Asian country with a multi-ethnic population, Singapore is suitable for this investigation.

MATERIALS AND METHODS

Patients

Consecutive patients with dyspepsia and/or reflux symptoms presenting for upper gastrointestinal endoscopic examination to one of the authors (KYH) and who have not been exposed to antibiotics, proton pump inhibitors (PPI) or bismuth compounds within the past four week were invited to participate in the study. Patients who previously had been treated for *H pylori* infection, patients who refused gastric biopsies, patients who were unable to give consent because of age or mental illness and patients in whom gastric and oesophageal biopsies were contraindicated (*e.g.*, coagulopathy, oesophageal varices and severe co-morbidity), subjects who were pregnant and those who were <18 years old, were excluded. Informed consent was obtained from each patient.

A total of 155 patients were included in the study. The patient population comprised 122 (79%) Chinese, 17 (11%) Indians, 8 (5%) Malays and 8 (5%) subjects of other ethnicities. Of these, 95 (61%) were males and 60 (39%) females. The mean age was 48.4±15.5 (range, 24-88) years. Based on clinical history and endoscopic examination, patients were classified into the following groups: gastroesophageal reflux disease (GERD) (n = 50), peptic ulcer disease (PUD) (n = 36) and non-ulcer dyspepsia (NUD) (n = 69). GERD was defined as the presence of predominant symptoms of reflux, e.g., heartburn, acid regurgitation and/or the presence of any length of mucosal break in the oesophagus due to gastroesophageal reflux. NUD was defined as patients with neither a history of GERD nor endoscopic evidence of organic pathologies. PUD refers to patients who were either

Table 1 Primer sequences of genes of interest

Region	Primer	Nucleotide sequence (5'→3')	PCR product (bp)	Reference
26kDa	26kDa-F	TGGCGTGTCTATTGACAGCGAGC	298	9
	26kDa-R	CCTGCTGGGCATACTTCACCAAG		
ureC	*ureC*-F	AAGCTTTTAGGGGTGTTAGGGGTTT	294	10
	ureC-R	AAGCTTACTTTCTAACACTAACGC		
cagA	*cagA*-F	AATACACCAACGCCTCCAAG	400	11
	cagA-R	TTGTTGCCGCTTTTGCTCTC		
vacA	*vacA*-F	GCTTCTCTTACCACCAATGC	1160	12
	vacA-R	TGTCAGGGTTGTTCACCATG		
	m2-R	CATAACTAGCGCCTTGCAC		
iceA1	*iceA1*-F	GTGTTTTTAACCAAAGTATC	246	5
	iceA1-R	CTATAGCCAGTCTCTTTGCA		5
iceA2	*iceA2*-F	GTTGGGTDTDTCACAATTTAT	229/334	5
	iceA2-R	TTGCCCTATTTTCTAGTAGGT		5
babA2	*babA2*-F	AATCCAAAAAGGAGAAAAAGTATGAAA	831	4
	babA2-R	TGTTAGTGATTTCGGTGTAGGACA		4

F: forward primer R: reverse primer.

diagnosed upon endoscopy as suffering from gastric ulcers (ulcers at the corpus) or duodenal ulcers (ulcers at the antrum). A total of 465 biopsy specimens were obtained from the 155 patients.

Endoscopy

After an overnight or six hour fast, upper gastrointestinal endoscopy was performed according to standard technique. From each patient, one biopsy specimens was obtained using sterilized standard biopsy forceps from each of the three sites of the stomach: the cardia just below the z-line, the middle gastric corpus and the antrum within 2 cm of the pylorus, in that order. The biopsy forceps were thoroughly cleaned with alcohol swaps between biopsies to avoid contamination between specimens. The biopsies were transported in 0.85% sterile saline to the microbiological laboratory for processing within 6 h.

H pylori culture

Each biopsy specimen was homogenised aseptically in 500 μL of Brain Heart Infusion Broth (BHI, Oxoid Ltd., Basingstoke, UK) enriched with 4 g/L yeast extract (Oxoid Ltd., Basingstoke, UK). Approximately 100 μL homogenised specimens in BHI broth were inoculated onto *H pylori* selective chocolate blood agar plates and non-selective chocolate blood agar plates respectively. The selective blood chocolate agar was supplemented with 3 mg/mL vancomycin, 5 mg/mL trimethoprim, 10 mg/mL nalidixic acid and 2 mg/mL amphotericin B. All the antibiotics were from Sigma-Aldrich Chemie, Steinheim, Germany. The plates were incubated at 37 ℃ for up to 14 d in an incubator (Forma Scientific, USA) containing 50 mL/L CO_2.

Aliquots of 50 μL of BHI-biospy suspension were each inoculated into catalase reagent, oxidase reagent and 20 g/L urea solution for their respective testing. An isolate was identified as *H pylori* if minute (-1 mm in diameter) rounded translucent colonies with gram-negative S-shaped motile cells that exhibited positive catalase, oxidase and urease activities. For this study, a patient was considered positive for *H pylori* if the organism was isolated from any of the three gastric sites.

Genotyping of H pylori

The DNA of each 3-d old *H pylori* culture was extracted according to the method as described by Hua *et al*.[8]. A 50 ng working stock of DNA was used to amplify 26*kDa*[9], *ureC*[10], *cagA*[11], *vacA*[12], *iceA1*[5], *iceA2*[5] and *babA2*[4] genes according to the protocol as described by Zheng *et al*.[6] using the specific forward and reverse primers for each of the corresponding genes (Table 1). The DNA fingerprint of the *H pylori* was obtained by PCR using the universal primer, 5'- AACGCGCAAC-3' and amplified according to protocol as described by Hua *et al*.[13]. The PCR products obtained were electrophoresed and the ethidium bromide stained gels[13] were then photographed with filtered UV illumination on Chemi Genius^2 (SynGene, Cambridge, UK).

Statistical Calculation

The significance of the results obtained was calculated using SPSS v.10 for Windows (SPSS, Chicago IL) to determine the Pearson chi-square whereby a P value <0.05 was considered to indicate statistical significance.

RESULTS

H pylori isolates in various clinical groups

Of the 155 patients studied, 51 (33%) were found to harbour *H pylori* in at least one of the 3 gastric biopsy sites. In all, 43, 47 and 44 isolates were obtained from gastric antrum, corpus and cardia respectively, giving a total of 134 isolates. *H pylori* was present in 16/36 (44%) PUD patients as compared with 15/50 (30%) GERD patients (P = 0.169) and 20/69 (29%) NUD patients (P = 0.113) (Table 2).

Table 2 Relationship between *H pylori* status and disease states

Groups	No. of patients	No. of biopsies	No. of *H pylori* (+)	*P*
PUD	36	108	16 (44%)	-
GERD	50	150	15 (30%)	0.169
NUD	69	207	20 (29%)	0.113

PUD, peptic ulcer disease; GERD, gastroesophageal reflux disease; NUD, non-ulcer dyspepsia. All test values were calculated with respect to PUD; P<0.05 indicates statistical significance.

Relationship between topographic distribution of H pylori isolates and clinical outcomes

Of the 51 *H pylori* positive patients, 38 (75%) showed the presence of *H pylori* in all the three gastric sites while 1 (1%), 2 (4%) and 4 (8%) had *H pylori* isolated from antrum & corpus, antrum & cardia, and corpus & cardia, respectively. *H pylori*

was isolated from a single site of the stomach in 6 (12%) patients, among which 2 isolates were from the antrum and 4 were from the corpus. This topographical pattern of *H pylori* colonization was observed in all the patients irrespective of the underlying clinical diagnosis.

Relationship between topographic distribution of H pylori genes of interest and clinical outcomes

All the *H pylori* isolates possessed the 26*kDa* gene and the *ureC* genes. The prevalence of virulence genes of interest were present in equal ratios in all the *H pylori* isolates obtained from all the 3 different gastric biopsy sites: 74-81% for *cagA* and 80-86% for *vacA*; 53-59% for *iceA*1 and 36-42% for *babA*2 regardless of the underlying clinical diagnosis. However, the *iceA*2 gene was present less frequently, at 20-26% of the *H pylori* isolates. It is noted that the difference in gene frequency between the various sites was also not statistically significant (Table 3).

Similar observation was noted with respect to the distribution of virulence genes of *H pylori* isolated from the same site among the different disease groups. The prevalence for each virulence gene within the same site was highly similar. No significant association of the virulence gene was found to be associated with a particular biopsied site, regardless of the disease state, with the exception of *cagA* in isolates from the corpus of the stomach of GERD patients (Table 4).

Table 3 Anatomical location of the 134 *H pylori* isolates and their virulence genes

	Antrum (%) *n* = 43	Body / Corpus (%) *n* = 47	Cardia(%) *n* = 44
Clinical Diagnosis			
PUD	12 (33)	15 (42)	12 (33)
GERD	13 (26)	15 (30)	14 (28)
NUD	18 (25)	17 (24)	18 (26)
Genotype			
26kDa	43 (100)	47 (100)	44 (100)
ureC	43 (100)	47 (100)	44 (100)
cagA	35 (81)	35 (74)	33 (75)
vacA	37 (86)	38 (81)	35 (80)
*iceA*1	25 (58)	25 (53)	26 (59)
*iceA*2	10 (23)	12 (26)	9 (20)
*babA*2	18 (42)	20 (38)	16 (36)

PUD, peptic ulcer disease; GERD, gastroesophageal reflux disease; NUD, non-ulcer dyspepsia. All test values were calculated with respect to gastric antrum and none were significant.

Relationship between topographic distribution of H pylori strain based on RAPD fingerprinting and clinical outcomes

For comparison, differences in 2 or more bands of the RAPD profile are considered different while variations in band intensity were not taken into account. On this basis, the RAPD profiles of all the *H pylori* strains isolated showed an overall similarity in profiles within individual patients, with minor differences such as the presence or absence of a single band. However, distinct differences in the DNA profiles were observed between patients (Figure 1). In this study, no comparison could be made in 6 patients since *H pylori* was isolated from only one site of the stomach in these patients.

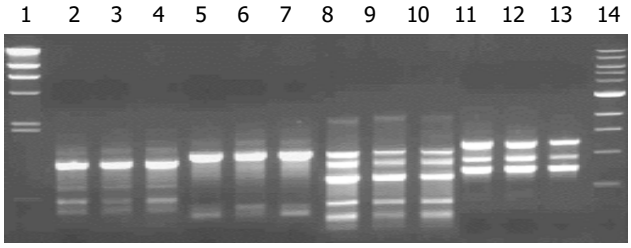

Figure 1 PCR-based RAPD patterns of *H pylori* isolated from 3 gastric sites of 4 individual patients. Lane1: λHindIII M marker, Lane 14: 1 kb M marker; Lane 2-4, 5-7, 8-10, 11-13 for patients 1, 2, 3 and 4 respectively. A: antrum; B: body; C: cardia.

DISCUSSION

It is noted that 50% of the world's population are infected with *H pylori* but only a small proportion manifest different gastroduodenal diseases[14]. One of the factors contributing to this phenomenon could be the patchy distribution of *H pylori* in different gastric sites of the stomach As most of the earlier studies focused on *H pylori* isolated from the distal sites of the stomach[8,15], the present study shows that *H pylori* isolates obtained from all the 3 gastric sites, namely cardia, corpus and antrum, in 38/51 (75%) *H pylori* patients were similar genotypically. Care was taken in cleaning and disinfecting the biopsy forceps between biopsies to avoid contamination between specimens. The results imply that *H pylori* colonises the entire stomach instead of a predominant site in three quarters of our *H pylori* positive patients, irrespective of the underlying clinical diagnosis. The finding suggests that the site of *H pylori* colonization or topographic distribution does not contribute significantly to the outcome of the infection.

Table 4 Distribution of virulence genes of 134 *H pylori* isolates from the same anatomical site of different patient groups

	Isolates	*26kDa* (%)	*ureC* (%)	*cagA* (%)	*vacA* (%)	*iceA1*(%)	*iceA2*(%)	*babA2*(%)
Antrum								
PUD	12	12 (100)	12 (100)	9 (75)	10 (83)	6 (50)	4 (33)	6 (50)
GERD	13	13 (100)	13 (100)	11 (85)	11 (85)	7 (54)	2 (15)	6 (46)
NUD	18	18 (100)	18 (100)	15 (83)	16 (89)	12 (67)	4 (22)	6 (33)
Body / Corpus								
PUD	15	15 (100)	15 (100)	9 (60)	10 (67)	6 (40)	5 (33)	7 (47)
GERD	15	15 (100)	15 (100)	14 (93)[1]	14 (93)	8 (53)	3 (20)	8 (53)
NUD	17	17 (100)	17 (100)	12 (71)	14 (82)	11 (65)	4 (24)	5 (29)
Cardia								
PUD	12	12 (100)	12 (100)	9 (75)	11 (92)	5 (42)	2 (17)	3 (25)
GERD	14	14 (100)	14 (100)	12 (86)	11 (79)	8 (57)	2 (14)	7 (50)
NUD	18	18 (100)	18 (100)	12 (67)	13 (72)	13 (72)	5 (28)	7 (39)

PUD, peptic ulcer disease; GERD, gastroesophageal reflux disease; NUD, non-ulcer dyspepsia. All test values were calculated with respect to PUD. [1]Indicates statistical significance ($P = 0.031$).

While studies in Europe suggested that virulence genes, e.g., *cagA* and *vacA* affect the clinical outcome of *H pylori* infection[3,5], the present study confirms previous studies carried out in Asian countries[6,15] that showed a high prevalence of *cagA* and *vacA* genes regardless of the clinical outcome. This study comprised Singapore patients of various ethnicities (Chinese, Malays, Indians and other races), also shows that each of the virulence genes, i.e., *cagA*, *vacA*, *iceA1*, *iceA2* and *babA2* were equally distributed in *H pylori* isolates obtained from all the three anatomical sites studied regardless of the disease states. Similarly, comparisons of these genes from the same anatomical site of different patients showed no significant presence, except for *cagA* in the corpus of GERD isolates. However, it is important to point out that the number of isolates obtained from each is relatively low ($n \leqslant 18$), regardless of the disease state. As such, the significant presence of *cagA* in the corpus of GERD patients needs further analysis with a larger pool of samples to confirm conclusively its contribution to the onset of GERD. The data therefore suggest that virulence genes and their topographic distribution do not contribute to the clinical status, at least in the Singapore population. This study supports the earlier reports[15-17] that identifying such virulence genes in order to predict clinical outcome may be of limited value in Asian *H pylori* isolates.

The finding that RAPD profiles of the *H pylori* isolates were similar from 3 different anatomical sites (antrum, corpus & cardia) of each patient further strengthens our earlier study[9] where isolates from 2 sites (antrum & corpus) were identical. This finding is complemented by the similar status of presence of various virulence genes in these isolates obtained from the respective patients. As such, the isolation of a strain from any gastric site in a single patient could be taken as representative of *H pylori* infection present in the *H pylori* infected gastric environment.

This study, which included consecutive patients with dyspepsia and/or reflux symptoms showed a lower frequency of PUD as compared with that of GERD. *H pylori* was found in only 44% of patients with PUD. This seems to run counter to the generally held view that *H pylori* occurs frequently in Asians patients with PUD[13]. However, this finding is supported by an earlier study from the same unit[18] showing the frequency of reflux oesophagitis was increasing while that of duodenal ulceration was decreasing in Singapore. The high frequency of patients with GERD in this study also relates to the fact that the endoscopist (KYH) sees most of the GERD patients in the hospital. The decreasing frequency of *H pylori* associated peptic ulcers was reported to be attributed to the increasing proportion of ulcers due to NSAID use[19].

In summary, the present study shows that in Singapore, the topographic colonization of *H pylori* and their virulence genes within the host stomach do not play a significant role in the clinical manifestations of *H pylori* infection. This study also demonstrates that in Singapore, which has a multiethnic Asian population, the stomach of each patient with dyspeptic and/or reflux symptoms is colonized by a single predominant strain of *H pylori*, irrespective of the site of isolation and the clinical diagnosis of the patient. We suggest that the pathogenesis of *H pylori* induced gastroduodenal diseases is due to a more complex mechanism possibly involving host-pathogen interaction, environmental and dietary factors.

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