Helicobacter pylori cagA and *vacA* cytotoxin genes in Changsha, China

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Accepted: 14 June 2002

ABSTRACT

Cytotoxin-associated protein (cagA) and the vacuolating cytotoxin (vacA) encoded by *cagA* and *vacA* genes are virulence determinants of *Helicobacter pylori*. In earlier studies among Chinese patients, all *H. pylori* strains were *cagA*-positive and *vacAs*1*a*/*m*2 type. Here, we determine the *cagA*, *vacA* and allele status of *H. pylori* strains isolated from patients with upper gastrointestinal symptoms in Changsha, China. Forty strains of *H. pylori* isolated from patients with peptic ulcer disease between March 1997 and August 1999 were recovered from storage at -80°C and studied by the polymerase chain reaction (PCR) for *cagA* and *vacA* genotypes. *cagA* was positive in 75% of *H. pylori* isolates. Patients with peptic ulcer demonstrated *cagA* in 83% (15/18), compared with 68% (15/22) patients with superficial gastritis. *vacAs*1 allele was carried in 82.5% (33/40) isolates, of which 52.5% (21/40) were subtype *vacAs*1*a*/*m*2 and 17.5% (7/40) were subtype *vacAs*1*b*/*m*2.

KEY WORDS: Genes, bacterial.
Helicobacter pylori.
Peptic ulcer.

Introduction

Helicobacter pylori infection has a role in the pathogenesis of chronic gastritis, peptic ulcer, gastric adenocarcinoma and lymphoma.[1,2] It is estimated that *H. pylori* infects more than 50% of the world's population, yet only a small percentage of subjects develops peptic ulcer disease or gastric cancer. The reason for this remains unknown; however, possibilities include the presence of disease-specific strains, host genetics and environmental factors.[3,4]

Two phenotypic characteristics of *H. pylori* strains – the vacuolating cytotoxin (vacA) and the high molecular weight protein[5,6] encoded by cytotoxin-associated gene A (*cagA*) – are associated with distinct gastrointestinal disorders. Some 60-80% of *H. pylori* strains express the 120-140 kDa cagA product that is recognised by serum antibodies.[7,8]

Various studies have demonstrated a strong association between the presence of antibodies to cagA protein and peptic ulcer disease. The gene encoding the vacuolating toxin is present in nearly all strains of *H. pylori* but cytotoxin activity is positive in only 40-60 % of strains from patients with peptic ulcer and in 30% from patients with chronic gastritis.[9,10]

The *vacA* gene is present in all *H. pylori* strains and comprises two variable parts. The *s* region (encoding the signal peptide) is present as either the *s*1 or *s*2 allele. Within type *s*1, several subtypes (*s*1*a*, *s*1b and *s*1*c*) can be distinguished.[11] The *m* (middle) region occurs as either an *m*1 or *m*2 allele. Thus, the mosaic combination of *s* and *m* region allelic types determines cytotoxin production and is associated with pathogenicity of the bacterium.

Distribution of *cagA*, *vacA* and the alleles vary among *H. pylori* strains from the USA, Europe and East Asia.[10,11] In previous studies among Chinese patients, *cagA* distribution has varied from 90-100%,[12,13] and *vacA* genotype of all *H. pylori* strains has been *vacAs*1*a*/*m*2.[14]

The aim of this study is to determine the *cagA*, *vacA* and allele status of *H. pylori* strains isolated from patients with upper gastrointestinal symptoms in Changsha, a provincial city in the central south province of Hunan in China.

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Materials and methods

H. pylori strains

Forty clinical *H. pylori* isolates collected between March 1997 and August 1999, and used in previous studies,[15,16] were removed from storage at -80°C. 100 µL bacterial culture was inoculated immediately onto chocolate agar plates (Columbia agar base, Lab M, Oxoid Ltd., Bury, UK) containing 7% sheep blood, amphotericin B (2 mg/mL), vancomycin (10 mg/mL) and polymyxin B sulphate (2.5 mg/mL) for between three and five days. In a commercial gas-generator system (Yiwu Co., Zhejiang, China) the plates were incubated at 37°C under microaerobic conditions in 5% oxygen, 10% carbon dioxide and 85% nitrogen, with high humidity.

After incubation for three days the plates were examined daily for growth. If no *H. pylori* growth was observed after incubation for seven days, the plates were discarded. Small rounded colonies were subcultured twice to obtain a pure culture. Gram's stain and urease and catalase tests were used to confirm identity and to exclude the possibility of contamination. *H. pylori* was stored at -80°C in brain-heart infusion broth (Oxoid Ltd., Bury, UK), containing 7% sheep blood.

Extraction of genomic DNA

Bacterial cells from chocolate agar plates were washed (x2) in phosphate-buffered saline (PBS; pH 8.0) and centrifuged at

3000 x*g* for 20 mins. *H. pylori* DNA was extracted by a phenol/chloroform method described previously.[15] Briefly, the bacterial pellet was resuspended in Tris-HCl buffer containing ethylenediaminetetraacetate (TE; pH 8.0) and lysozyme (30 mg/mL) and incubated at 37˚C for 30 mins. The suspension was treated with sodium dodecyl sulphate (SDS; 10%), proteinase K (2.5 mg/mL) and RNase A (40 mg/mL). DNA was extracted with phenol/chloroform/isoamyl alcohol, precipitated with sodium acetate and ice-cold absolute alcohol, washed with ice-cold alcohol (70%), and the DNA pellet was resuspended in TE buffer. DNA content and purity were determined by measuring absorbance at 260 nm and 280 nm using a spectrophotometer (Beckman DU-600, USA).

Polymerase chain reaction

Amplification of *cagA* and *vacA* alleles by the polymerase chain reaction (PCR) was performed in a 50 μL volume containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5-2.5 mmol/L $MgCl_2$, 200 μmol/L deoxynucleoside triphosphate, 2-3 units *Thermus aquaticus* (*Taq*) DNA polymerase (all reagents obtained from Sangon, China) and 25 pmol of both forward and reverse primers (Table 1) as used previously.[5,17] Reactions were covered with mineral oil and PCR was performed in a Perkin Elmer 480 thermal cycler.

Amplification cycle for *cagA* consisted of an initial denaturation of target DNA at 94˚C for 5 min, denaturation at 94˚C for 1 min, primer annealing at 55˚C for 1 min and extension at 72˚C for 1 min and 30 sec. The final cycle included an extension step of 5 min at 72˚C to ensure full extension of the product. Samples were amplified through 35 consecutive cycles.

Amplification cycle for *vacA* alleles consisted of an initial denaturation of target DNA at 95˚C for 5 min and then denaturation at 95˚C for 1 min, primer annealing at 52˚C for 1 min and extension at 72˚C for 1 min. The final cycle included an extension step of 7 min at 72˚C to ensure full extension of the product. Samples were amplified through 35 consecutive cycles.

Positive and negative reagent control reactions were performed with each batch of amplifications. Amplified PCR products were resolved in 1.5% agarose gel containing 0.5% Tris/acetate/EDTA, stained with ethidium bromide and visualised under an ultraviolet light source.

Results

cagA prevalence

cagA primer revealed that the expected 297-bp PCR product of *cagA* was present in 75% (30/40) of *H. pylori* isolates (Table 2). Patients with peptic ulcers demonstrated *cagA* in 83% (15/18) of isolates, compared with 68% (15/22) in isolates from patients with superficial gastritis. *cagA* was almost equally associated with duodenal ulcer and gastric ulcer.

vacA prevalence

Complete *vacA s*- and *m*-region genotype was obtained in 33 out of 40 cases (Table 2). Repeat experiments failed to yield PCR product for the *s*1*a*/*b* region in six cases and for the *m* region in one case. However, all these cases were *cagA*-positive. The majority of isolates (82.5%; 33/40) contained the *s*1 allele, and, of these, 52.5% (21/40) were subtype *s*1*a*/*m*2 and 17.5% (7/40) were subtype *s*1*b*/*m*2 (Table 2). Of the 40 isolates studied, 70% (28/40) were *m*2 and 12.5% (5/40) were *m*1. Infection with the type *s*1 strain was found in 17/18 subjects with peptic ulceration (Table 2). Isolates did not have multiple *vacA* genotypes.

Association of cagA positivity with vacAs1 signal sequence

cagA gene was associated with the *vacAs*1 sequence in 75% (21/35) of the isolates studied (Table 2). Of these, 71.4% (15/21) were *vacAs*1*a* and 28.6% (6/21) were *vacAs*1*b*. This suggests a close association between *cagA* status and the *s*1 signal sequence.

Discussion

The gastric pathogen *H. pylori* appears to be one of the most genetically diverse bacterial species, with isolates easily distinguishable from each other by DNA fingerprinting or gene sequencing. In addition, there are geographic genetic variations among *H. pylori* strains. In terms of the *cagA* and *vacA* genes that encode potential virulence factors, East Asian and Western strains differ markedly in DNA sequence.[18,19]

In previous studies from Europe and the USA, prevalence of *cagA*-positive *H. pylori* varied between 69% and 79%,[9,20-21] whereas 90-100% of *H. pylori* isolated from Chinese patients possessed *cagA*.[12,13] Prevalence of *cagA*-positive *H. pylori* isolates among Chinese patients with peptic ulcer and chronic gastritis appears to be similar.[13]

vacA alleles of strains from the USA, Europe and Asia are mosaics that consist of any combination of the three signal sequence types (*s*1*a*, *s*1*b* or *s*2) and two mid-region types (*m*1or *m*2), with the exception of *s*2/*m*1.[5,18] In all parts of the world, the *vacAs*1/*cagA*-positive genotype is associated with peptic ulcer disease[19] and almost all Chinese *H. pylori* strains studied previously carried the *vacAs*1 allele.[22]

In the present study – carried out in Changsha, a central south provincial city in China – in which the mid-region of the *H. pylori cagA* gene was amplified, the expected PCR product was present in only 75% of the isolates from peptic ulcer disease patients. However, Yiqi *et al*.,[12] using the same primer, studied 74 clinical isolates in an eastern coastal province of Shanghai and demonstrated the *cagA* gene in 90.5 % of isolates. This suggests that *cagA* gene prevalence varies among peptic ulcer disease patients in different provinces. Another possibility is that allelic variation in the *cagA* gene is present in *H. pylori* strains among Chinese patients, and currently a larger study to examine this is in progress.

Pan *et al*. demonstrated difference in the *cagA* sequence at the complementary site to primer *cagA*1 between Dutch and Shanghai Chinese *H. pylori* strains.[13] This suggests that a specific genotype may exist within a particular geographic area. Therefore, a particular region within the *cagA* gene may differ substantially in *H. pylori* strains common to populations of different provinces of China. In cultured *H. pylori* strains from 22 gastritis patients, 15 patients were *cagA*-positive and 16 yielded *vacAs*1 products – an observation that matches those seen typically in the West.

Among Chinese *H. pylori*, most isolates from both peptic ulcer disease (17/18) and gastritis (16/22) patients were

Table 1. Oligonucleotide primers used in typing *H. pylori cagA* and *vacA* alleles

Region amplified	Primer designation	Primer sequence	Size and location of PCR product
cagA	D008 R008	5' ATAATGCTAAATTAGACAACTTGAGCGA 3' 5' TTAGAATAATCAACAAACATCACGCCAT 3'	297 bp (1751-2048[a])
*m*1	VA3-F VA3-R	5' GGTCAAAATGCGGTCATGG 3' 5' CCATTGGTACCTGTAGAAAC 3'	290 bp (2741-3030[b])
*m*2	VA4-F VA4-R	5' GGAGCCCCAGGAAACATTG 3' 5' CATAACTAGCGCCTTGCAC 3'	352 bp (976-1327[b])
s1*a*	SS1-F VA1-R	5' GTCAGCATCACACCGCAAC 3' 5' CTGCTTGAATGCGCCAAAC 3'	190 bp (866-1055[b])
s1*b*	SS3-F VA1-R	5' AGCGCCATACCGCAAGAG 3' 5' CTGCTTGAATGCGCCAAAC 3'	187 bp

a Location in published *cagA* sequence.[17]
b Location in published *vacA* sequence.[5]

Table 2. *cagA* and *vacA* status of 40 *H. pylori* strains from patients with peptic ulcer disease

Genotype	Gastritis	Duodenal ulcer	Gastric ulcer	Total
cag A				
cag A$^+$	15/22 (68%)	8/10 (80%)	7/8 (87.5%)	30/40 (75%)
cag A$^-$	7/22 (32%)	2/10 (20%)	1/8 (12.5%)	10/40 (25%)
vacA				
s1*a*/*m*1	0	1/10 (10%)	1/8 (12.5%)	2/40 (5%)
s1*a*/*m*2	12/22 (45%)	6/10 (60%)	3/8 (37.5%)	21/40 (52.5%)
s1*b*/*m*1	1/22 (4%)	1/10 (10%)	1/8 (12.5%)	3/40 (7.5%)
s1*b*/*m*2	3/22 (13%)	1/10 (10%)	3/8 (37.5%)	7/40 (17.5%)
Incomplete	6/22 (27%)	1/10 (10%)		7/40 (17.5%)

positive for *vacA*s1. The *vacA* genotype of 40 *H. pylori* strains studied here showed that 82 % were *s*1, of which 52.5% were *s*1*a*/*m*2 and 17.5% were *s*1*b*/*m*2. In Europe, a distribution gradient of the *s*1 subtype has been observed;[19] however, whether or not there is an *s*1 subtype gradient in the Chinese population remains unknown.

The present study showed that the *s*1*b*/*m*2 allele is also associated with peptic ulceration, although less frequently than is *s*1*a*/*m*2. This association has not been demonstrated before in Chinese studies. Previous study in the USA has also shown that strains with *vacA*s1*a* alleles are associated with peptic ulceration more frequently than those with *s*1*b* or *s*2 alleles.[23]

In contrast, Zhang *et al*.[14] in Shanghai, using the same primer sets as used here, investigated the *vacA* genotype of 62 *H. pylori* strains isolated from patients with peptic ulcer disease and gastric cancer. All possessed the *vacA* gene and all strains were type *s*1*a*/*m*2. Pan *et al*.,[22] using PCR and DNA sequencing, showed that 95 out of 96 isolates from patients in Shanghai carried *vacA s*1*a* alleles, 78 of which were *m*2 and 14 were *m*1. In contrast, in the present study only 21 out of 40 isolates were *s*1*a*.

Seven cases failed to yield PCR product – which may be attributed to the primers used, as has been reported recently[19] – and this is being studied further, using long-range PCR and sequencing of the *vacA s* and *m* regions.

Type *m*2 strains appear to be more prevalent in China, as only five isolates out of the 40 in the present study demonstrated the *m*1 allele. The reason for this is unclear. The finding that all combinations of *vacA* signal sequence and mid-region occur naturally strongly supports the concept of recombination between *vacA* genes *in vivo*, to create the mosaic gene structures observed.

Interaction with other races in the region, such as Koreans and Vietnamese, and mixing with other ethnic groups as a result of trade, may have provided the opportunity for DNA transfer of different genotypes between strains. Superimposed upon this *H. pylori* strain diversity are indications of differences at certain *cagA* and *vacA* loci in the DNA sequence among various ethnic groups of the Chinese population.

In conclusion, the prevalence of the *cagA* gene in *H. pylori* isolates among Chinese patients, although higher than in those in Western patients, varies in the Chinese population. In addition, *vacA s*1*b* is associated with peptic ulcer disease in China.

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