Heterogeneity in the *Helicobacter pylori vacA* and *cagA* genes: association with gastroduodenal disease in South Africa?

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Abstract

Background—*Helicobacter pylori* infection is universally associated with gastritis, but only sometimes with clinically significant disease. Candidate virulence markers seem to be useful in identifying the pathogenic infections in some populations.

Aims—To investigate the association between putative virulence markers and disease in an African population.

Methods—Fifty nine *H. pylori* strains isolated from dyspeptic patients (11 with peptic ulceration, eight with gastric adenocarcinoma, and 28 with no pathology other than gastritis) were studied for differences in the genes *vacA* and *cagA*.

Results—Forty seven (80%) of 59 strains had the *vacA* signal sequence genotype s1 (one s1a, 46 s1b) and 12 (20%) had subtype s2. *vacA* mid-region analysis revealed that 40 (68%) strains were *vacA* m1 and 19 (32%) were m2. All 14 strains from patients with peptic ulceration were *vacA* s1, in contrast to 23 (66%) of 35 strains from patients with gastritis alone ($p < 0.01$). *vacA* s2 was found exclusively in patients with gastritis alone ($p < 0.01$). All strains isolated from patients with gastric adenocarcinoma were s1b/m1 ($p < 0.005$ versus gastritis alone). *cagA* was detectable in 56 (95%) of 59 isolates. Strains from patients with peptic ulceration (12/13 versus 19/30 with gastritis alone, $p = 0.05$) had the shortest fragment length in the 3' region of *cagA*, while 4/10 strains from patients with gastric cancer had the longest fragment length in this region ($p < 0.02$ versus gastritis alone).

Conclusion—In this study, the *vacA* s1 genotype, and fragment length of the 3' region of *cagA* identified isolates associated with significant clinical disease. The *vacA* s1bm1 genotype seems to be strongly associated with gastric cancer.

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Helicobacter pylori, a spiral shaped gastric organism, is the cause of chronic gastritis, and is important in the pathogenesis of peptic ulceration, mucosa associated lymphoid tissue lymphoma, and gastric adenocarcinoma.^{1–3} Although infection is universally associated with gastritis, the development of clinically sig-

nificant disease seems to depend on a number of factors, including the virulence of the infecting strain, the susceptibility of the host, and environmental cofactors.⁴ Focus on the organism has resulted in the identification of a number of non-conserved candidate virulence markers. These include in vitro production of vacuolating cytotoxin activity, the presence of certain types of the vacuolating cytotoxin gene *vacA*, possession of the gene *cagA* which is a marker for the *cag* pathogenicity island, and the ability to activate neutrophils directly.^{4–6}

vacA alleles are mosaics of any combination of signal sequence type (s1a, s1b, or s2) and mid-region (m1 or m2).⁷ The link between the ability of a strain to induce epithelial vacuolation in vitro and peptic ulceration in vivo is significant but not invariable.⁴ Infection with strains with the *vacA* s1a genotype in the USA, has been linked to gastric inflammation, duodenal ulceration, in vitro cytotoxin activity, and the presence of *cagA*, while *vacA* s2 strains seem to have the least pathogenic potential.⁸ In other studies, *vacA* s1 strains are also associated with peptic ulcer disease.^{9–12}

The gene *cagA* is present in 60–70% of *H. pylori* strains and encodes a high molecular weight antigenic protein (120–140 kDa).^{5, 13} CagA producing strains have been detected in patients with peptic ulceration more frequently than in patients with chronic gastritis alone.^{5, 11, 13} In addition, recent evidence has shown an association between infection with these strains and the development both of atrophic gastritis and of adenocarcinoma of the stomach.^{14, 15} Recent genotypic analysis has revealed the presence of substantial variability in the 3' region of *cagA*, which has been postulated to alter the immunogenicity of the protein. These alterations in the gene and its protein seem to correlate with clinical outcome in vivo.^{9, 16}

Based on historic observations, it has been postulated that clinically significant gastroduodenal disease is not common in the African setting,¹⁷ although contrasting views, based on objective parameters (prospective endoscopic studies) exist.^{18, 19} This has led some to state that there is no established correlation between the organism and any pathology in Africa apart from gastritis,¹⁷ and, even more contentiously, it has been suggested that *H. pylori* infection may be protective in the African setting, particularly against gastric cancer.²⁰ We have attempted to assess whether associations

Abbreviations used in this paper: PCR, polymerase chain reaction.

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between markers of *H pylori* virulence and disease in Western populations applied in the African setting.

In this report, we document the genotypic profile of *H pylori* isolates from the Western Cape in South Africa, as well as the relation between putative virulence markers and gastrointestinal disease in these individuals.

Material and methods

PATIENTS, BIOPSY SAMPLING, AND CULTIVATION OF *H PYLORI* STRAINS

H pylori isolates from 47 infected patients (15 black, seven white, 25 coloured) with *H pylori* infection undergoing upper gastrointestinal endoscopy were used in this study. Endoscopic and histological diagnoses were recorded for all patients. In 28 of the patients, no endoscopic pathology was evident, but histology confirmed the presence of chronic active gastritis; 11 patients had duodenal ulcers and eight had gastric adenocarcinoma. Gastric biopsy specimens obtained from the antrum of all 47 patients and additionally from the fundus of five patients were cultured as described previously.²¹ Briefly, specimens were transported in a jar under microaerobic conditions (Oxoid Gas Generating BR38, Basingstoke, Hampshire, UK). The specimen was cultured on tryptose blood agar (CM 233; Oxoid Ltd) containing lysed horse blood (10% vol/vol) at 37°C in a microaerobic atmosphere (12% CO₂, 88% air (6% CO₂), 95% humidity) for a minimum of seven days. *H pylori* was identified by colony morphology and positive urease reaction (Christensen's urea slope). The strains were numbered, and all analyses were performed without prior knowledge of the clinical diagnosis. Reference strains NCTC11639 and CCUG17135 were used in this study.

PREPARATION OF SAMPLES FOR PCR AMPLIFICATION

Genomic DNA was isolated from each colony utilising the High PCR (polymerase chain reaction) purification kit from Boehringer Mannheim (Johannesburg, South Africa).²² Briefly, bacterial cells were resuspended in phosphate buffered saline (PBS), and lysozyme (10 mg/ml) was added. Thereafter, cells were further digested by proteinase K and the DNA was isolated/purified by chaotropic salt/glass fibre methodology. Approximately 10 µg DNA was obtained from 10⁶ bacterial cells.

PCR AMPLIFICATION AND DETECTION OF AMPLIFIED DNA PRODUCTS

For *vacA*, primers VA1F and VA1R were used to amplify the signal sequence.⁷ Amplification fragments of 259 bp for genotype s1 and 286 bp for genotype s2 were expected. Further analysis of the s1 genotype was performed using SS1F and VA1R for s1a and SS3F and VA1R for s1b. The *vacA* mid-region was amplified with primers VAGF and VAGR, resulting in fragments of 570 bp for m1 and 645 bp for m2 respectively.⁷ For *cagA* identification, primers F1 and B1 were used to amplify a 349 bp region from the conserved 5' end of the gene.¹³ Differences in fragment length

(variability) of the 3' end of *cagA* were identified using primers cag2 and cag4 which resulted in amplicons of 400–757 bp.⁹

PCR amplification of *vacA* and *cagA* was performed as described previously.⁷ Following initial denaturation at 94°C for one minute, each reaction consisted of 35 cycles of denaturation at 94°C for one minute, annealing and extension for two minutes, and final extension at 72°C for 10 minutes. Annealing temperatures were set at 50°C for cag2/cag4; 55°C for VA1F, VA1R, VAGF, VAGR, F1, and B1; and 59°C for VA1R, SS1F, and SS3F. A 20 µl aliquot of each PCR mixture was subjected to gel electrophoresis on 1% agarose gels and a 100 bp DNA ladder (Boehringer Mannheim) was used as a size marker.

STATISTICAL METHODS

Data from non-identical strains (n=59) were analysed using the χ^2 test or Fisher's exact test as appropriate. Probability levels less than 0.05 were considered statistically significant.

Results

STRAIN COLLECTION

Single colonies (n=34) were obtained from 34 patients (21 with gastritis alone, eight with peptic ulceration, and five with gastric adenocarcinoma). Two or more morphologically distinct (either light/dark or large/small paired) colonies (n=26) were isolated from three patients with peptic ulceration, three with gastric adenocarcinoma, and seven with gastritis alone. Random amplified polymorphic DNA (RAPD) analysis, *vacA* and *cagA* typing showed that the morphologically distinct colonies were identical in only one patient (gastric adenocarcinoma, data not shown). Twenty five of the 26 strains were therefore available for analysis from this group. In total, 59 of the 60 strains isolated were available for analysis: 35 strains from 28 patients with gastritis only, 14 from 11 patients with peptic ulceration, and 11 from the eight patients with gastric adenocarcinoma.

DETERMINATION OF *vacA* GENOTYPES

The gene *vacA* was detectable in all *H pylori* isolates. By using the primers VA1F and VA1R to amplify the *vacA* signal sequences, the predicted PCR product of 259 bp for genotype s1 was obtained from 47 (80%) of 59 strains as well as the two reference strains. Twelve (20%) of the strains yielded the 286 bp amplicon (s2). None of the strains yielded a PCR product of any other size. Further analysis of the 47 strains with the s1 genotype revealed that 46 (98%) were *vacA* s1b and 1 (2%) was *vacA* s1a.

All 59 *H pylori* strains and the two reference strains contained DNA which was amplifiable by VAGF and VAGR, resulting in fragments of 570 bp (m1) in 40 (68%) (and in CCUG17135) and 645 bp (m2) in 19 (32%) (and in NCTC11639) respectively. None had DNA amplified by both sets of primers or gave products of sizes other than predicted. Among the 59 isolates studied, five of the six possible combinations of *vacA* signal and mid-regions were identified. The s1a/m1 combination was

Table 1 Distribution of *vacA* signal sequence and mid-region types in strains isolated from patients with different gastric diseases

No of strains	<i>vacA</i> signal sequence type			<i>vacA</i> mid-region type	
	s1a	s1b	s2	m1	m2
Gastritis alone (n=35)	1 (3%)	22 (63%)	12 (34%)†	20 (57%)	15 (42%)
Peptic ulceration (n=14)	0 (0%)	14 (100%)*	0 (0%)	10 (71%)	4 (29%)
Adenocarcinoma (n=10)	0 (0%)	10 (100%)*	0 (0%)	10 (100%)*	0 (0%)*
p Value	NS	<0.01*	<0.01†	<0.01*	<0.01*

*Compared with gastritis alone.

†Compared with either peptic ulceration or adenocarcinoma.

Table 2 Distribution of *cagA* 3' variability in 56 *cagA*⁺ strains

No of strains	Fragment length of <i>cag</i> 3' amplicon		
	<600 bp	600–700 bp	>700 bp
Gastritis alone (n=30)	19 (63%)	10 (33%)†‡	1 (3%)
Peptic ulceration (n=13)	12 (92%)*	1 (8%)	0 (0%)
Adenocarcinoma (n=10)	6 (60%)	0 (0%)	4 (4%)*†
p Value	0.05*	<0.07†	<0.05‡
		<0.05‡	<0.01*

*Compared with gastritis alone.

†Compared with peptic ulceration.

‡Compared with adenocarcinoma.

found in one (2%), s1b/m1 in 36 (61%), s1b/m2 in 9 (15%), s2/m1 in 3 (5%), and s2/m2 in 10 (17%). The s1a/m2 combination was not detected.

RELATION OF *vacA* GENOTYPES TO GASTROINTESTINAL DISEASE

All patients with peptic ulceration were infected with *vacA* s1 strains (14, 100%), compared with only 23 (66%) of 35 strains from subjects with gastritis alone ($p=0.009$; table 1). All of the 10 strains (100%) isolated from patients with gastric cancer were s1b/m1 compared with 16 (46%) strains from individuals with gastritis alone ($p=0.0013$). Thus the *vacA* s1b genotype was found more often in strains from patients with peptic ulceration and was more commonly associated with strains from gastric adenocarcinoma (100%) than gastritis alone (63%, $p=0.02$). In contrast, the *vacA* s2 genotype was isolated exclusively in strains from patients with gastritis alone (34%), and was not found in strains from patients with either peptic ulceration (0%) or gastric adenocarcinoma (0%) (both $p<0.01$ versus gastritis alone).

Analysis of the *vacA* mid-region allele types showed that they were similarly distributed in patients with peptic ulceration (10 m1:4 m2) and gastritis alone (20 m1:15 m2). In contrast, the *vacA* m1 genotype was found more often in strains from patients with gastric adenocarcinoma (100%) than in strains from gastritis alone (57%, $p=0.009$) or peptic ulceration (71%), although the latter difference was not significant ($p=0.09$).

PREVALENCE AND SIZE VARIATION OF THE GENE *cagA* IN *H. PYLORI* ISOLATES

Fifty six (95%) of 59 strains and the two reference strains were *cagA*⁺, utilising primers F1/B1 to identify a 349 bp conserved amplicon from the 5' region of the gene. The gene *cagA* was present in 13 (93%) of 14 strains isolated from patients with peptic ulceration and 33 (94%) of 35 strains from patients with gastritis

alone. All 10 isolates from patients with gastric adenocarcinoma were *cagA*⁺.

Fifty three of the 56 *cagA*⁺ strains had identifiable PCR products with primer set *cag2/cag4* differing in fragment length from 400 to 757 bp. Amplicons smaller than 600 bp were found more frequently in *H. pylori* strains isolated from patients with peptic ulceration (12/13, 92%) than in those with gastritis alone (19/30, 63%; $p=0.05$; table 2). Strains from patients with gastritis alone were also found more frequently to express amplicons of 600–700 bp (10/30, 30%; $p=0.07$). PCR fragments of >700 bp were more often found in patients with gastric adenocarcinoma (4/10, 40%) than in either patients with peptic ulceration (0/13; $p=0.02$) or patients with gastritis alone (1/30, 3%; $p<0.01$).

Discussion

The *Helicobacter pylori* vacuolating cytotoxin has been implicated in the pathogenesis of peptic ulceration.^{4 8 23} A US study showed that strains with *vacA* signal type s1a are often associated with peptic ulceration, while *vacA* s2 strains are usually found in patients with no ulcers.⁴ *vacA* s1b strains seemed to be intermediately ulcerogenic and *vacA* alleles with this signal sequence were found to be less cytotoxic in vitro than *vacA* s1a strains.^{7 8} In a preliminary study, in a small number of South African individuals, we noted the lack of the cytotoxic *vacA* s1a allele in this study group.²⁴

In this expanded study, our results show a high prevalence of the *vacA* s1b allele and confirm the low prevalence of the *vacA* s1a allele in this population. Analysis of disease groups showed that the *vacA* s1 allele was found consistently (100%) in strains from patients with peptic ulceration. In contrast to strains from the USA and Asia,⁴ no *vacA* s1a alleles were present in any of these cases. South African strains with the *vacA* s1b allele produce low levels or no in vitro vacuolating activity on cultured epithelial (HeLa) cells.²⁴ It is tempting to speculate that widespread prevalence of weakly or non-cytotoxic strains may be reflected in the apparent low frequency of *H. pylori* associated diseases noted in Africa. As has been noted, however, the assumption on which the "low prevalence" of disease in Africa is based can be questioned.^{18 19 25} In addition, a high prevalence of *vacA* s1b is not limited to South Africa; *vacA* s1b alleles are also highly prevalent in Brazil²⁶ and Portugal,²⁷ where reported prevalences of peptic ulceration and gastric adenocarcinoma are higher. Further studies are needed on the relative contributions of strain virulence, host susceptibility, and environmental cofactors to disease in all these populations. In this study, the *vacA* s2 allele was found exclusively in the group of individuals with no endoscopic pathology, in keeping with observations suggesting that *vacA* s2 strains are minimally or non-ulcerogenic.^{4 7 8} As in other studies, the *vacA* middle region types were not independently associated with the occurrence of peptic ulceration.⁹

Investigation of strains isolated from patients with gastric cancer have focused on the

presence or absence of the gene *cagA*. In this study we found the great majority of strains to be *cagA*+. Data from the rest of Africa are scarce, but suggest that *cagA* is commonly expressed.²⁸ It seems unlikely that a low population prevalence of *cagA*+ strains can be invoked to explain the apparent low prevalence of gastric adenocarcinoma in Africa. As expected *cagA*+ strains were predominant in our gastric cancer patients (100%). The association between *vacA* genotypes and gastric adenocarcinoma has not previously been studied in the African setting, and here our findings are striking. All strains in this study were *vacA* genotype s1b/m1. A US study showed that strains with *vacA* mid-region type m1 were more strongly associated with epithelial injury (epithelial degeneration, mucus depletion, and microscopic erosions) than mid-region type m2.⁸ We hypothesise that infection with *vacA* m1 strains, and the associated damage to the antral and corpus mucosa may play a role in the aetiology of gastric adenocarcinoma in our study population. The association between *vacA* m1 and gastric adenocarcinoma as well as the notable absence of the *vacA* s2 genotype needs to be followed up in other studies in Africa and elsewhere.

The nucleotide sequence of the gene *cagA* contains internal duplications of a 102 base pair fragment, encoding a proline rich region in the 3' region.⁵ Differences in this region (possibly by a mechanism to generate antigenic diversity) have been suggested to generate proteins with different sizes and immunogenicities.^{5 13 16} We evaluated whether there was a biological significance to the variability in fragment length of the 3' region of *cagA* in a population with a high prevalence (>95%) of isolates containing this gene. As in other studies,⁹ we found the length of this region to be shorter in strains isolated from patients with peptic ulceration than in patients with gastritis alone. In contrast, the fragment length was longer in strains isolated from patients with gastric cancer than in patients with peptic ulceration or gastritis alone. These results are similar to the preliminary results from a Japanese study which showed that a structural subtype of *cagA*, encoding a protein with a higher molecular weight, and detectable by its larger size, was associated with gastric carcinoma and atrophy.¹⁶ Thus, our data support the view that *cagA* variants provide an additional marker to distinguish disease associated strains of *H. pylori*.

In summary, we have shown the high prevalence of *vacA* s1 alleles in strains isolated from South African individuals with peptic ulceration and gastric cancer, and noted the consistent association of *vacA* s1b/m1 strains with patients with gastric cancer. While attention has been focused on the positive correlation with disease, the segregation of *vacA* s2 alleles with "non-pathogenic" strains may be an excellent negative marker. The presence of the gene *cagA* in the majority of isolates, irrespective of associated pathology, suggests a limited use of this as a virulence factor. However, vari-

ability noted in the fragment length of the 3' region of the gene correlates with disease in this population and may prove to be a useful virulence marker, if confirmed.

- Warren J. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1983;i:1273.
- Wotherspoon A, Ortiz-Hidalgo O, Falzon M, et al. Helicobacter pylori-associated gastritis and primary B-cell gastric lymphoma. *Lancet* 1991;338:1175-6.
- Parsonnet J, Friedman GF, Vandersteen DP, et al. Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med* 1991;325:1127-31.
- Atherton J. The clinical relevance of strain types of Helicobacter pylori. *Gut* 1997;40:701-3.
- Covacci A, Censini S, Bugnoli M, et al. Molecular characterization of the 128-kDa immunodominant antigen of Helicobacter pylori associated with cytotoxicity and duodenal ulcer. *Proc Natl Acad Sci USA* 1993;90:5791-5.
- Cover T, Dooley C, Blaser M. Characterization of and human serologic response to proteins in Helicobacter pylori broth culture supernatants with vacuolating cytotoxin activity. *Infect Immun* 1990;58:603-10.
- Atherton J, Cao P, Peek R, et al. Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. *J Biol Chem* 1995;270:17771-7.
- Atherton J, Peek R, Tham K, et al. Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of Helicobacter pylori. *Gastroenterology* 1997;112:92-9.
- Rudi J, Kolb C, Maiwald M, et al. Diversity of Helicobacter pylori vacA and cagA genes and relationship to VacA and CagA protein expression, cytotoxin production and associated diseases. *J Clin Microbiol* 1998;36:944-8.
- Stephens J, Folwell A, Swann R, et al. Helicobacter pylori cagA status, vacA genotypes and ulcer disease. *Eur J Gastroenterol Hepatol* 1998;10:381-4.
- Takata T, Fujimoto S, Anazai K, et al. Analysis of the expression of CagA and VacA and the vacuolating activity in 167 isolates from patients with either peptic ulcers or non-ulcer dyspepsia. *Am J Gastroenterol* 1998;93:30-4.
- van Doorn L, Figueiredo C, Sanna R, et al. Clinical relevance of the cagA, vacA and iceA status of Helicobacter pylori. *Gastroenterology* 1998;115:58-66.
- Tummuru M, Cover T, Blaser M. Cloning and expression of a high molecular mass major antigen of Helicobacter pylori: evidence of linkage to cytotoxin production. *Infect Immun* 1993;61:1799-809.
- Blaser M, Perez-Perez G, Kleanthous H, et al. Infection with Helicobacter pylori strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res* 1995;55:2111-15.
- Kuipers E, Perez-Perez G, Meuwissen S, et al. Helicobacter pylori and atrophic gastritis: importance of cagA status. *J Natl Cancer Inst* 1995;87:1777-80.
- Yamaoka Y, Kodama T, Kashima K, et al. Variants of the 3' region of the cagA gene in Helicobacter pylori isolates from patients with different H. pylori-associated diseases. *J Clin Microbiol* 1998;36:2258-63.
- Holcombe C. Helicobacter pylori: the African Enigma. *Gut* 1992;33:429-31.
- Jaskiewicz K, Louwrens H. Chronic atrophic gastritis in a population at risk for gastric carcinoma. *Anticancer Res* 1991;11:835-9.
- Glupczynski Y, Bordeaux L, De Prez C, et al. Prevalence of Helicobacter pylori in rural Kivu, eastern Zaire: a prospective endoscopic study. *Eur J Gastroenterol Hepatol* 1991;3:449-55.
- van der Merwe C. Helicobacter pylori and gastric cancer. *Eur J Gastroenterol Hepatol* 1995;7:589-90.
- Louw J, Lucke W, Jaskiewicz K, et al. Helicobacter pylori eradication in the African setting, with special reference to reinfection and duodenal ulcer recurrence. *Gut* 1995;36:544-7.
- Vogelstein B, Gillespie D. Preparative and analytical purification of DNA from agarose. *Proc Natl Acad Sci USA* 1979;76:615-19.
- Blaser M. Hypotheses on the pathogenesis and natural history of Helicobacter pylori-induced inflammation. *Gastroenterology* 1992;102:720-7.
- Letley D, Lastovica A, Louw J, et al. Analysis of vacA diversity among Helicobacter pylori isolates from South Africa reveals existence of a vacA s2m1 allele [abstract]. *Gastroenterology* 1998;113:A458.
- Cook G. *Tropical gastroenterology*. Oxford: Oxford University Press, 1980.
- Evans D, Queiroz D, Mendes E, et al. Helicobacter pylori cagA status and s and m alleles of vacA in isolates from individuals with a variety of H. pylori associated gastric diseases. *J Clin Microbiol* 1998;36:3435-7.
- van Doorn L, Figueiredo C, Rossau R, et al. Typing of Helicobacter pylori vacA gene and detection of cagA gene by PCR and reverse hybridization. *J Clin Microbiol* 1998;36:1271-6.
- Mandour El-Mahdi A, Patchett S, Char S, et al. Does CagA contribute to ulcer pathogenesis in a developing country such as Sudan? *Eur J Gastroenterol Hepatol* 1998;10:313-16.