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Original Research Article

Association of *Helicobacter Pylori* VacA Gene Polymorphisms and CagA Gene with Clinical Outcome in Dyspeptic Patients

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ABSTRACT

Background: Gastroduodenal diseases have been associated with *Helicobacter pylori* infection. Cytotoxin-associated gene A (*cag*A) and different isoforms of vacuolating cytotoxin (*vac*A) gene have been associated with disease severity. The prevalence and association of these genotypes with severity of disease differ geographically. The aim of this study was to determine the prevalence of the *cag*A and *vac*A variants of *Helicobacter pylori* isolated from dyspeptic patients in Kenya and to assess their association with clinical outcome.
Methods: One hundred and twenty seven dyspeptic patients were enrolled into the study. *H. pylori* positivity was determined by histology and molecular diagnostic method directly from the gastric biopsies collected. The presence of *cag*A and *vac*A variants was analyzed by polymerase chain reaction (PCR) from the eighty *H. pylori* positive samples with various gastroduodenal diseases.
Results: The *H. pylori* DNA was detected in 62.99% of the dyspeptic patients. The prevalence of *cag*A gene was 48.75% among the positive samples and was not significantly associated with the gastroduodenal diseases. The less virulent *vac*A alleles: m2, i2 and s2 were most occurring at 65%, 52% and 49%, respectively. The *vac*A allele m1, i1, and s1 were significantly associated with peptic ulcer, intestinal metaplasia and gastric cancer, all at $P < 0.05$.
Conclusion: The less toxigenic vacA isoforms s2, m2 and i2 are highly distributed in Kenya. We confirmed the significant association of the vac s1, m1 and i1 with gastric cancer and peptic ulcer. We cannot rule out the possibility of the presence of unidentified strain or certain host or environmental factors that trigger *H. pylori*-induced cancer and peptic ulceration.

Key words: *Helicobacter pylori*, vacA gene polymorphisms, Cytotoxin-associated gene A, Clinical outcome

INTRODUCTION

Helicobacter pylori (*H. pylori)* is estimated to infect the stomachs' of half the world's population and is associated with the development of gastroduodenal diseases, including peptic ulceration and gastric cancer. [1] The differences in disease outcome may be the result of host factors, environmental factors and differences in the prevalence or expression of bacterial

virulence factors. [2] The study of *H. pylori* virulence factors in populations is important, as they contribute to disease risk. *H. pylori* strains express various toxins that enable the bacteria to cause host cell. [3] Included among these toxins is cytotoxin-associated gene A (*cag*A) and vacuolating cytotoxin (*vac*A). [3]

VacA toxin is produced and secreted by all *H. pylori* strains and was previously shown to have various modes of action. [4] The *vac*A gene has been shown to contain a number of polymorphisms. [5] Currently, three polymorphic regions of *vacA* have been identified: the signal (s), intermediate (i), and middle (m) regions. [5] Each of these polymorphic regions has two main types that divide them further into type 1 and type 2. [5] The s region encodes the N-terminal signal sequence, [5] and polymorphisms in the s region affect the anion channel-forming efficiency of the toxin; the s1 type has an increased ability to form membrane channels. [5] Polymorphisms in the m region affect the cell tropism of the toxin; the m1 type of *vac*A shows toxicity toward a broader range of cells than the m2 type. [6] The i region, located between the s and m regions, also displays two main polymorphisms. [7] The i1 type of *vac*A has stronger vacuolating activity than the i2 type. Individually, the s1, i1, and m1 types have been shown to be associated with more severe forms of *H. pylori* induced. [7,8]

H. pylori strains possessing the *cag* pathogenicity island (ca*g*A) are associated with the development of gastric cancer**.** [9] The CagA protein is translocated into epithelial cells and becomes phosphorylated on tyrosine residues within EPIYA motifs, which may be repeated within the variable region of the protein. [10] Strains possessing *cag*A with greater numbers of these repeats have been more closely associated with gastric carcinogenesis. [10] Phosphorylated CagA leads to epithelial cell elongation, which is dependent on the number of variable-region EPIYA motifs. [11,12]

The geographic distribution of distinct *H. pylori* genotypes remains largely. [2] The prevalence of virulent bacterial genotypes in certain regions may have important epidemiological consequences that are linked to the presence of the certain specific genotypes and the severity of *H. pylori*-related diseases. [2] In this context we have characterized *H. pylori* strains in gastric biopsy specimens from dyspeptic patients in order to gain new insights into the population genetic structure of this important human pathogen and to learn if genotypes implicated with the disease in the West as well as Eastern Asia are similarly disease associated in Kenya.

MATERIALS AND METHODS

The study population included one hundred and twenty seven (127) patients with past and present history of dyspepsia and who had been referred for endoscopy at Kenyatta National Hospital (KNH), a national referral hospital in Kenya. Permission to carry out the study was obtained from the Kenyatta National Hospital Scientific and Ethical Review committee. It was conducted according to the ethical guidelines of the declaration of Helsinki, 2000. [13]

Patient-derived samples.

After fulfilling the inclusion criteria and obtaining a written consent the patients provided the demographic data before undergoing routine endoscopy. Patients underwent a detailed history and physical examination. Esophageal gastro duodenoscopy (EGD) was performed under conscious sedation with intravenous midazolam using a videogastroscope (Q160, Olympus). The lining of the oesophagus, stomach and duodenum and was observed. Two (2) antral and two (2) corpus biopsies about 2-3 mm in length, were obtained for

routine histology and molecular testing (DNA extraction). The biopsy specimens for molecular analysis were put in a 2ml DESS (DMSO/ EDTA/ NACl) solution and stored at $^{-}80^{0}$C at the Centre for Microbiology Research (CMR), KEMRI until use, while samples for histological analysis were immediately fixed in buffered formalin.

Histopathology

The biopsies were processed in a tissue processor and ultramicrotomy was done in a microtome to produce 4-6 µm sections. The sections were deparaffinized and hydrated to distilled water. They were then stained in fleshly prepared Giemsa working solution for 30 minutes – 1 hour; dehydrated in 3 changes of absolute alcohol and cleared in xylene for 3 changes. They were mounted with resinous medium and a cover slip placed. The slides were examined microscopically by using x400 and at least five high power fields examined by a pathologist. Histopathological findings were recorded and a histopathological classification of gastritis was used using updated Sydney system, [14] which had a scale of 0 – 3 for scoring the features of chronic gastritis, corresponding to none, mild, moderate or severe respectively.

PCR-based genotyping of the *vac*A and *cag*A genes and gel electrophoresis

The samples were removed from DESS (DMSO/ EDTA/ NaCl) solution and soaked in 2 mL TE (10 mM Tris, 1 mM EDTA) for 2 hours. The biopsies were then collected in a microcentrifuge tubes containing 120 ul of sterile phosphate buffered saline and vortexed vigorously for 2 min. The tubes were then boiled in a water bath at 95 degrees centigrade for 15 min, cooled in ice, and centrifuged at 13,000 X *g* for 1 min. The supernatant was transferred to another tube and then stored at 2-8°C. About 3 ul of the supernatant containing the DNA template was added in 25 ul volumes containing 2.5 pmol of primers VAG-F and VAG-R, 25 pmol of primers VA1-F and VA1-R, 10 pmol of primers cag5c-F and cag3c-R (Table 1), 200 µM each dNTP is in 10 mM Tris-HCl, (pH 9.0 at room temperature), ~2.5 units of puReTaq DNA polymerase (GE Healthcare UK Limited), 50 mM KCl, and 1.5 mM of MgCl2 in a reaction standard PCR buffer (GE Healthcare UK Limited). Products were amplified under the following conditions: 3 min at 94°C for initial denaturation followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final round of 10 min at 72°C, in a thermal cycler. All the PCR products were electrophoresed in 1.5% gel and visualized under UV light as described elsewhere. [15] The i region was genotyped using two independent PCRs with a universal forward primer (VacF1) and different i region type-specific reverse primers (C1R and C2R), as described previously by *Rhead et al.*, (2007). [7] C1R and C2R specifically anneal with the i1 and i2 *vacA* alleles, respectively.

Table 1 - List of primers used.

Gene	Primer	Primer Sequence (5'-3')	Product size (bp)	Reference
***vacA* s1/ s2**	VAI-F	ATGGAAATACAACAAACACAC	s1 259	Atherton *et al.*, 1995. [16]
	VAI-R	CTGCTTGAATGCGCCAAAC	s2 286	
***vacA* m1/ m2**	VAG-F	CAATCTGTCCAATCAAGCGAG	m1 567	
	VAG-R	GCGTCAAAATAATTCCAAGG	m2 645	
vac i1	C1R	TTAATTTAACGCTGTTTGAAG	426	Rhead *et al.*, 2007. [7]
	VacF1	GTTGGGATTGGGGGAATGCCG		
vacA i2	C2R	GATCAACGCTCTGATTTGA	432	
	VacF1	GTTGGGATTGGGGGAATGCCG		
cagA	cag5c-F	GTTGATAACGCTGTCGCTTC	350	Chattopadhyay *et al., 2004.* [15]
	cag3c-R	5GGGTTGTATGATATTTTCCATAA		

Data Management and Analysis.

Data analysis was done using SPSS version 17 (SPSS inc., Chicago, IL) program for windows. The association of the specific virulence-associated bacterial genotypes with the clinical outcome of *Helicobacter pylori* infection was assessed using Chi-square (χ^2) or Fisher's exact tests to compare proportions for categorical variables. A value of $p < 0.05$ was considered statistically significant. Odds ratio was estimated and corresponding 95% confidence intervals were estimated while non parametric correlations between the genotypes and severity of disease was assessed using Kendall's tau_b correlation.

RESULTS

Demographic Characteristics

A total of 127 patients were enrolled into the study having met the predetermined criteria. These included 66 (52%) and males 61 (48%) females. The average age of the patients was 44.87 years.

The representation per age group was 4 (3.15%) patients under 20yrs; 52 (40.94%) between 20 and 40 years; and 71 (55.91%) patients were above 40 years.

Endoscopic findings

Gastritis was the single highest clinical findings at 63.8% of all the enrolled patients followed by GERD 13.4%, NUD 11.8%, Peptic Ulcer 9.4% and Gastric Cancer 7.9%. Gastric Polyps, Gastric nodules, gastrophathy and pre-pyloric mass were rare at 0.01%.

***Helicobacter pylori* infection.**

A total of 84 (66.14%) of 127 patients were positive by histology and 80 (62.99%) by molecular diagnosis (**criteria:** presence of at least three genotypes/alleles). For all associations with clinical outcome only those positive by molecular diagnosis was used.

Prevalence of *H. pylori* by age and sex in the enrolled patients.

Patients aged 40 and above years had highest ***H. pylori*** prevalence (62.5%); followed by those aged 20-39 years (64%); and lowest those under 20 years (50%). Male patients had a lower prevalence at 59.01% against female patients at 67.21%. However, neither was there any significant association between *H. pylori* infection and age ($p=0.083$) nor sex ($p< 0.34$).

Prevalence of *H. pylori* in different disease conditions.

We established that all the patients diagnosed with duodenal ulcer were positive for *H. pylori* infection while it was positive in 74.4% patients with gastric ulcer. Though the association not significant ($P < 0.0870$) 69.1% of patients with gastritis were *H. pylori* positive implying a significant risk to the development of these pathologies. Significantly, ($P< 0.0015$) GERD was associated with *H. pylori* infection while 60% of patients diagnosed with gastric cancer *H. pylori* positive.

Distribution of vac and cagA genotypes.

All the *H. pylori* positive samples were vacA gene positive as compared to 48.75% which had cagA gene. The most occurring alleles were vacA m2, vacA i2 and vacA s2 at 65%, 52% and 49%, respectively. This study established that the vacA m1, vacA i1 and vacA s1were lowly distributed in the Kenyan *H. pylori* strains as shown in Table 2. The most occurring pairs of alleles were vacA i2/m2 (50%) and s2/m2 (45%). The vac s1/m1 and vac s/1i1were distributed at 30% and 28% among all the positive samples. The vac s2/m1 combination was completely absent. Considering the multiple combinations, s2/m2/i2 was the most common multiple combination at 41.25% while s1/m1/i1 was distributed at 23.75% in all the *H. pylori* positive samples.

Associations of pathologies with various genotypes

We further established a significant association between vacA m1, vacA i1, and vacA s1 with peptic ulcer, intestinal metaplasia and gastric cancer (all at $P < 0.05$) as shown in Table 3. While vac s2 was significantly associated with gastritis, vac i2 and vac m2 were not significantly associated with any of the above pathologies. However, s2, m2 and i2 were significantly associated with gastritis. In this study, cagA gene had no significant association with any of the pathologies despite being established in 54.4% and 60% of patients diagnosed with peptic ulcer and gastric cancer.

Table 2 - Distribution of vacA genotypes and cagA gene among the positive samples

Genotype	Number (out of 80)	%	Genotype	Number (out of 80)	%	Genotype	Number (out of 80)	%
***vac* s1**	**36**	**45**	***vac* s1/m1**	**22**	**28**	***vac* s1/m1/i1**	**19**	**23.75**
***vac* s2**	**39**	**48.8**	***vac* s1/i1**	**24**	**30**	***vac* s1/m/1i2**	**3**	**3.75**
***vac* m1**	**22**	**27.5**	***vac* s1/m2**	**15**	**19**	***vac* s1/m2/i1**	**6**	**7.5**
***vac* m2**	**52**	**65**	**vac s1/i2**	**9**	**11**	***vac* s1/m2/i2**	**7**	**8.75**
***vac* i1**	**30**	**37.5**	**vac s2/m1**	**0**	**0**	***vac* s2/m1/i1**	**0**	**0**
***vac* i2**	**42**	**52.5**	***vac* s2/m2**	**36**	**45**	***vac* s2/m1/i2**	**0**	**0**
***cag*A**	**39**	**48.8**	***vac* i1/m2**	**9**	**11**	***vac* s2/m2/i1**	**3**	**3.75**
			***vac* i2/m2**	**40**	**50**	***vac* s2/m2/i2**	**33**	**41.25**

Table 3 - Association between specific *H. pylori* genotypes and gastric pathologies

Gastritis (55)					**Duodenal Ulcer (5)**				
	P value	OR	95% C.I OR			P value	OR	95% C.I OR	
vac s1 (18)	0.002	0.189	0.067	0.535	vac s1 (5)	0.011	All vacA s1 Positive		
vac s2 (33)	0.004	4.750	1.638	13.777	vac s2 (0)	-	All vac s2 Negative		
vac m1 (22)	0.000	0.134	0.045	0.397	vac m1 (4)	0.027	12.667	1.329	120.716
vac m2(41)	0.010	3.727	1.377	10.090	vac m2(1)	0.062	0.118	0.012	1.110
vac i1(15)	0.006	0.250	0.092	0.677	vac i1(5)	0.003			
vac i2(33)	0.50	2.667	1.002	7.096	vac i2(0)	-			
cag A(26)	0.695	0.828	0.321	2.132	cag A(3)	0.606	1.625	0.257	10.290
Gastric Ulcer (6)					**Peptic Ulcer (11)**				
	P value	OR	95% C.I OR			P value	OR	95% C.I OR	
vac s1 (5)	0.084	6.935	0.771	62.351	vac s1 (10)	0.009	16.538	2.000	136.756
vac s2 (0)	0.013	0.473	0.372	0.602	vac s2 (0)	-	-		
vac m1 (2)	0.740	1.350	0.229	7.951	vac m1 (6)	0.039	3.975	1.070	14.760
vac m2(3)	0.430	0.510	0.096	2.714	vac m2(4)	0.041	0.250	0.066	0.940
vac i1(4)	0.146	3.692	0.633	21.530	vac i1(9)	0.005	10.286	2.044	51.752
vac i2(1)	0.103	0.161	0.018	1.446	vac i2(1)	0.013	0.068	0.008	0.564
cag A(3)	0.949	1.056	0.200	5.574	cag A(6)	0.679	1.309	0.365	4.696
Intestinal Metaplasia						**Gastric Cancer**			
	P value	OR	95% C.I OR			P value	OR	95% C.I OR	
vac s1 (17)	0.002	5.667	1.922	16.710	vac s1 (9)	0.014	14.333	1.718	119.568
vac s2 (6)	0.013	0.257	0.088	0.748	vac s2 (0)	0.001	0.403	0.341	0.576
vac m1 (11)	0.012	3.833	1.342	10.952	vac m1 (6)	0.022	5.062	1.270	20.177
vac m2(10)	0.012	0.275	0.100	0.757	vac m2(3)	0.022	0.184	0.043	0.780
vac i1(13)	0.029	3.059	1.124	8.322	vac i1(7)	0.034	4.768	1.28	20.156
vac i2(8)	0.047	0.361	0.132	0.989	vac i2(2)	0.43	0.188	0.037	0.948
cag A(11)	0.916	0.949	0.360	2.502	cag A(6)	0.450	1.682	0.436	6.483

Correlation between the cagA and the vacA genotypes with severity of various pathologies.

We further assessed whether severity of various pathological outcomes were associated with various genotypes as shown in Table 4. The degree of inflammatory activity according to the density of neutrophils in gastric mucosa was not significantly correlated with any genotype while intensity of the superficial epithelial damage significantly correlated with vacA s1 ($p= 0.002$), m1 ($p= 0.023$), and i1 ($p= 0.008$). The vacA s1 was the only genotype that significantly correlated with degree of chronic inflammatory infiltrate in the gastric mucosa (Lymphocytes, plasma cells) ($p= 0.016$). the intensity of intestinal metaplasia

was graded according to the amount of glandular tissue replaced by intestinal – type epithelium and in this case vac s1,m1 and i1 was significantly associated with its severity at, p=0.001, p= 0.010 and p=0.023, respectively. The cagA gene was not significantly correlated with severity of any of these disease conditions.

Table 4 - Correlation between the density of colonization, the cagA and the vacA genotypes with severity of various pathologies

	Severity of Gastritis *P value*	Degree of Inflammatory activity *P value*	Intensity of Epithelial Damage *P value*	Degree of Mucosa Infiltration *P value*	Level of Intestinal Metaplasia *P value*
vac s1	.019	.064	.023	.016	.001
vac s2	(-) .032	(-).146	(-).035	(-).036	(-).006
vac m1	.106	.059	.026	.108	.010
vac m2	(-).297	(-).095	(-).052	(-).194	(-).009
vac i1	.073	.053	.008	.138	.023
vac i2	(-).201	.146	(-).070	(-).068	(-).033
cag A	(-).700	.858	.560	(-).917	(-).932

(-) Negative correlation

DISCUSSION

Gastritis, like in many other studies in Kenya was a more frequent gastroduodenal disease while gastric cancer remained rare, [17,18] despite the high prevalence of *H. pylori* infection. The prevalence of *H. pylori* was found to be 62.99% among the dyspeptic patients slightly lower than the by findings Kimanga *et al*., (2013), [19] who documented 67.5% in all age groups in dyspeptic patients. Other studies [20,21] in other African countries found *H. pylori* prevalence levels of >90%. Larger proportions of those aged 40 years and above had more violent pathologies such as peptic ulcer disease, intestinal metaplasia, and gastric cancer than those aged less than 40 years. Similarly, Ogutu, *et al*., (1998) [22] reported the presence of gastric cancer in patients aged 50 years and above accounting for 17.4% of dyspeptic symptoms in this age group. All cases of duodenal ulcers were reported in patients aged below 40 years, similar to an earlier study. [22] There was no association of *H. pylori* infection with age or sex. Therefore, other underlying factors contribute to disease risk. There was a significant association between age and gastritis, p<.0001, with 75.76% of patients aged below 40 years having gastritis.

In this study the prevalence of cagA gene was 48.75% lower than other reports in African countries. [19,23] probably giving a reason as why there are rare virulent pathologies in Kenya. Further, cagA gene had no significant association with any of the pathologies despite being established in 54.4% and 60% of patients diagnosed with peptic ulcer and gastric cancer in concordance with western countries [8,16,24,25] but in agreement with another study [16] in Kenya. Studies have shown that cagA is polymorphic [10] and the distribution of EPIYA motif patterns and combination differs geographically. Possibly, Kenya's cagA EPIYA motif pattern induce less pathological changes.

On analysis of vacA gene polymorphisms, the most occurring alleles were vacA m2, vacA i2 and vacA s2 at 65%, 52% and 49%, respectively. These polymorphisms have been found to be less virulent. [7,10,26,27] This study established that the more virulent isoforms, vacA m1, vacA i1 and vacA s1 [7,10,26,27] to be lowly distributed in the Kenyan *H. pylori* strains, possibly giving an explanation of low occurrence of serious pathologies in Kenya. Next we assessed the distribution of the vacA pairs of alleles. The most occurring pairs of alleles were vacA i2m2 (50%) and

s2m2 (45%). The vac s1m1 and vac s1i1 which are highly toxigenic [7,26,28] were distributed at 30% and 28% among all the positive samples this explains the lower incidence of dangerous pathologies in Kenya. The vac s2m1 combination was completely absent as was documented elsewhere in Africa. [29]

Significant association between vacA m1, vacA i1, and vacA s1 with peptic ulcer, intestinal metaplasia, atrophy and gastric cancer (all at $P < 0.05$) was established as documented elsewhere. [8] While vac s2 was significantly associated with gastritis, vac i2 and vac m2 associated with any of the above pathologies. The less virulent polymorphisms m2 and i2 [7,10,26,27] were significantly associated with gastritis.

In this study, cagA gene was not significantly correlated with the severity of mucosa infiltration which is in concordance with a study by Basso et al., 2008, [8] who associated cagA gene with more severe antral and corpus activity but not with the degree of inflammatory activity.vac s1, m1, and i1 were correlated with the severity of intestinal metaplasia and intensity of epithelial damage. To the best of our knowledge this is the first study in Kenya to correlate the various genotypes with the severity of gastroduodenal diseases. In this study, it was only the Vac s1 genotype that was significantly associated with the intensity of mucosa infiltration. This was in agreement with another study, [8] which also in addition to vacA s1, the vacA m1 and i1 significantly correlated with intensity of mucosa infiltration.

CONCLUSION

The findings of this study have provided information on the genotypes of the Kenyan *H. pylori* strains. Despite the high prevalence of *H. pylori* infection, the less toxigenic vacA isoforms s2, m2 and i2 are highly distributed in Kenya. This study has further confirmed the potential of vac s, m1 and i1 to induce gastric cancer and peptic ulcer. Surprisingly, we can conclude that it's not the cagA gene per se that lead to severe gastroduodenal diseases but other factors within it. We cannot rule out the possibility of the presence of unidentified strain or certain host or environmental factors that trigger *H. pylori*-induced cancer and peptic ulceration. More studies will help increase our understanding of bacterium-host interactions in colonization and disease and offer insight into the epidemiology of the disease hence increased disease surveillance and control benefit.

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