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Helicobacter pylori Pathogenicity-Associated *cagA* and *vacA* Genotypes Among Nigerian Dyspeptic Patients

Yaji Mnena E[1]*, Nna Emmanuel[2] and Umeh EU[1]

[1]*Department of Biological Sciences, Federal University of Agriculture, Makurdi, Benue State of Nigeria, Nigeria*
[2]*Safety Molecular Pathology Laboratory, Enugu State, Nigeria*

Abstract

Objective: Although *Helicobacter pylori* infection is endemic in Nigeria, the specific genotypes that influence treatment responses are scarcely known. We aimed to determine the specific genotypes of *H. pylori* virulence genes, *vacA* and *cagA* in dyspeptic patients.

Subjects and methods: Gastric biopsy samples were obtained from 80 dyspeptic patients referred for endoscopy. Genomic DNA was extracted from biopsies using the ReliaPrep DNA kit. *H. pylori* DNA was detected by a singleplex PCR based on the genus specific 16s rRNA gene. The *vacA* subtypes for the s1 and s2 regions and the m1 and m2 alleles were detected by allele specific multiplex PCR. The *cagA* gene was amplified by a singleplex PCR.

Results: Of the 80 samples, 30 (37.5%) had abnormal mucosa which were chronic gastritis. The rest (62.5%) had normal mucosa lining. Of the 30 chronic gastritis cases, 22 (73%) had *H. pylori* infection as detected by 16s rRNA PCR. Only 2 (4%) of the normal mucosa cases had *H. pylori* infection. For the *vacA* genotypes, 79% of the *H. pylori* infections were s1c/m2 genotype, followed by 8% s1b/m2 genotype. Three different genotypes: s1c/m1/m2, s1c/s2/m2 and s1c/m1 occurred at 4% each. The most virulent *vacA* genotype, s1/m1 was only 8% while the least virulent *vacA* genotype s2/m2 was 4%. The moderate virulent *vacA* genotype, s1/m2 was the most prevalent (83%) in the Nigerian patients. The most prevalent subtype was the s1c/m2. Only 7 cases (29%) were *cagA* positive.

Conclusion: The pathogenicity-associated virulence genes present in Nigerian dyspeptic patients were moderate types. The endemicity of the disease may not necessarily lead to high rate of fatal outcomes or treatment failures as reported in other parts of the world.

Keywords: *Helicobacter pylori*; Signal region; Mid-region; *vacA* gene, Vacuolating cytotoxin A; *cagA*

Introduction

Helicobacter pylori is a gram-negative miroaerophic spiral bacterium, which was discovered in 1983 [1]. It infects more than half of the world's population with prevalence ranging from 25% in developed countries to more than 90% in developing countries [2]. The risk of infection is higher among those living in the developing world [3]. Infection with the bacterium causes chronic gastritis, peptic ulceration, gastric cancers and gastric Mucosa Associated Lymphoid Tissue (MALT) Lymphoma [3]. *Helicobacter pylori* is rated as a "class one" carcinogen to the gastrointestinal tract by the World Health Organization [4]. It is in the same category as cigarette smoke is to lung cancer.

H. pylori produces a variety of virulence factors such as motility, urease, catalase, flagella, phospholipases and cytotoxins [5]. The vacuolating cytotoxin *vacA* protein encoded by the *vacA* gene is present in all strains but only expressed in about 50% of the strains [6]. It was found that this protein in *H. pylori* broth culture filtrates could cause formation of large vacuoles in the cytoplasm of cultured mammalian cells [7]. The vacuolating activity is increased by exposure to acidic pH [6]. The *vacA* gene shows an allelic variation in two regions [8]. The signal region S has two different alleles s1 and s2. S1 has three sub-types: s1a, s1b, or s1c. The mid-region M has two different alleles m1 and m2 [9]. Strains with the gene s1/m1 have the highest level of cytotoxic activity and are associated with peptic ulcers, atrophic gastritis and gastric cancer, while the s2/m2 strains have non-toxic activity [6]. The bacterial strains with s1/m1 genotype produce higher amounts of toxin as compared with s1/m2 [7].

The *cagA* gene is part of the pathogenicity island (PAI-cag), a 40 kb DNA region that encodes proteins that are only found in a proportion of the strains (approximately 50%). This explains why not all strains are associated with clinical symptoms [10]. Strains of *H. pylori* with *cagA* positive are more virulent than others. A person with *cagA* is more likely to develop atrophic gastritis, intestinal metaplasia and gastric cancer [11]. The prevalence is generally high in developing countries.

In Nigeria, sero-prevalence of 82% has been reported in children 5 years to 9 years, 95% in adults of middle age and 70% to 90% in older adults [12]. A study on sero-prevalence of *H. pylori* infected patients with peptic ulcer in Kaduna State, Northwest Nigeria reported a prevalence of 80.4% [13]. A similar study carried out in Enugu state, South-East Nigeria reported a prevalence of 62% [14]. The burden of *H. pylori* infection in Nigeria is exacerbated by myths that the disease is 'incurable' as infected individuals live the rest of their lives taking drugs, avoiding certain foods and drinks [15]. Data on H. *pylori* genotypes in Nigeria is very scanty, majority of publications are on sero-prevalence. However, outcome of *H. pylori* treatment is associated with the bacterial genotype. We aimed at genotyping *H. pylori* strains from gastric biopsy samples using a combination of allele specific

***Corresponding author:** Yaji ME, Department of Biological Sciences, Federal University of Agriculture, Makurdi, Benue State, Nigeria, Tel: +234 70306 95000; E-mail: yajimnena@gmail.com

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multiplex PCR detection of *vacA* and single plex amplification of *cagA* gene alleles. Products of both genes are virulent factors that define *H. pylori* response to treatment.

Subjects and Methods

Ethical approval

Favourable ethical opinion was obtained from the health research ethics committee of the Benue State University teaching hospital, Makurdi. All participants had medical referrals for gastric biopsy at the department of gastroenterology of the Benue State University teaching hospital, Makurdi. Volunteered participants were informed-consented with written consent. Subjects were patients who had various *H. pylori*-associated dyspeptic symptoms including epigastric pain, fullness, vomiting, nausea and flatulence.

Sample size determination

Sample size was calculated using Raosoft (2014) sample size calculator. At 0.05 alpha level of significance, 95% confidence level and a patient population size of 99 and previous prevalence 50%, a sample size of 80 was obtained.

Sample collection

A consultant gastroenterologist performed the endoscopy on informed-consented participants. Gastric biopsy samples were taken from the antrum of the patients. Tiny pieces of tissue samples were collected into sterile McCartney bottles containing Brain Heart infusion broth and stored in the freezer at -200°C within 2 hours of collection until transported to the laboratory for analysis.

Extraction of genomic DNA

Genomic DNA was extracted from the tissue samples in the Brain Heart Infusion broth using ReliaPrep genomic DNA miniprep kit (Promega, Southampton UK). The Reliaprep uses spin columns that contain silica membrane for DNA purification. Briefly, about 200 µl of the macerated tissue materials in broth were dispensed into 2 ml Eppendorf tube containing 25 µl of proteinase K. The sample was mixed by gentle vortex and incubated for 5 minutes at room temperature. Then 200 µl of cell lysis buffer was added and the sample vortexed for 10 seconds before incubation in a water bath set at 56°C for 10 minutes. Thereafter, 250 µl of binding buffer was added to the sample and mixed by repeated pipetting. The mixture was transferred to the spin column and centrifuged at 14000 rpm for one minute. The flow through in the collection tube was discarded. The column was washed by addition of 500 µl of column wash buffer and centrifuged for 3 minutes at 14000 rpm. The washing was repeated twice. Columns were then placed into new collection tubes and centrifuged at 14000 rpm for 1 minute to remove residual wash buffer. Then 100 µl of nuclease-free water was added into the columns, which were placed into 1.5 ml tubes, incubated for one minute at room temperature and centrifuged at 13000 rpm for one minute. DNA quality was checked by reading at 260/280 nm using Eppendorf Biophotometer Plus (Eppendorf, Germany). The DNA elute was labeled and stored in the fridge until required for testing.

Detection of *H. pylori* 16s rRNA gene

H. pylori DNA was detected by using a singleplex PCR that amplifies 294 bp fragment using a final primer concentration of 0.5 µM in a 25 µl reaction volume. Primer sequences are contained in Table 1. The thermal profile comprised of initial denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 70°C for 60 s and 72°C for 60 s and a final extension of 72°C for 5 min. All amplifications were carried out in Eppendorf Nexus Gradient Master Cycler (Eppendorf, Germany) using 2x PCR master mix from Promega (Southampton, UK). PCR products were electrophoresed at 100 V for 30 minutes using 1.5% agarose gel stained with ethidium bromide.

Multiplex PCR for *H. pylori* genotyping

Two sets of multiplex PCRs were used to detect the signal regions (s1 and s2 including s1 subtypes) of the *vacA* gene. Another set of multiplex PCRs was used to detect the mid region of the *vacA* gene (m1 and m2). Primer sequences are contained in Table 1 as previously described [6,16,17]. A singleplex PCR was used to detect the *cagA* gene. The primers for the *cagA* gene were used at a final concentration of 0.05 µM. All primers were HPLC grade, synthesized by Eurofins, Germany. The thermal profile for the *cagA* singleplex PCR was initial denaturation of 95°C for 3 minutes and 40 cycles of 94°C for 60 s, 58°C for 60 s, 72°C for 60 s and final extension of 72°C for 5 min. All PCR products from the singleplex PCR were electrophoresed at 100 V for 45 minutes using 2.0% agarose gel. Images were captured using GenoMini Electrophoresis Gel system (VWR, UK).

The primers for the signal and mid regions of the *vacA* gene were used at a final concentration of 0.5 µM except for the reverse primers of the signal region, which were used at 1.0 µM. The thermal profile for the *vacA* multiplex PCR was initial denaturation of 95°C for 3 minutes and 40 cycles of 94°C for 60 s, 52°C for 60 s, 72°C for 60 s and final extension of 72°C for 5 min. The final reaction volume in all reactions was 25 µl. The platinium multiplex PCR master mix (Invitrogen, UK) was used

Name of Primer	5'-3' sequence	Amplicon size	Reference
CAGA394F	GATAACAGGCAAGCTTTTGAGGGA	394 bp	[16]
CAGA394R	CCATGAATTTTTGATCCGTTC		
VA7-F	GTAATGGTGGTTTCAACACC	630 bp for m1 alleles 352 bp for m2 alleles 705 bp for m1/m2 alleles 277 m2/m1 alleles	[17]
VA7-R	TAATGAGATCTTGAGCGCT		
VA4-F	GGAGCCCCAGGAAACATTG		
VA4-R	CATAACTAGCGCCTTGCAC		
VA1-F	ATGGAAATACAACAAACACAC	259 bp for s1 allele 190 bp for s1a allele 187 bp for s1b allele 199 bp for s2 allele 286 bp for s2 allele	[9]
VA1-R	CTGCTTGAATGCGCCAAAC		
VA1-s2-F	ATGGAAATACAACAAACACAC		
VA1-s2-R	CTGCTTGAATGCGCCAAAC		
SS1-F	GTCAGCATCACACCGCAAC		
SS3-F	AGCGCCATACCGCAAGAG		
SS2-F	GCTAACACGCCAAATGATCC		
Note: R stands for reverse primer and F stands for forward primer.			

Table 1: Primer sequences and amplicon sizes used in PCR.

in all multiplex reactions. All PCR reactions were performed in the Eppendorf Master Cycler-Nexus gradient (Eppendorf, Hamburg) using thin-walled PCR plates (Eppendorf, UK). The positive control used for all testing was the *Helicobacter pylori* strain from ATCC number 43526. Water was used as no template (negative control) while ATCC No 43827 strain ML35 *Escherichia coli* DNA sample was used as template (DNA) negative control.

Statistical analysis

Data were analyzed in GraphPad Prism version 7.02 (www.graphpad.com) and NCSS version 11 (www.ncss.com). Categorical data were presented in tables and analyzed with Fischer's Exact test. Numerical data were analyzed with Mann Whitney U test. Alpha level of significance was set at 0.05. Hierarchical cluster analysis was performed on *H. pylori* genotypes to assess the relative similarity.

Results

Of the 80 subjects sampled, 45 (56%) were male and 35 (44%) were female. The mean age was 42 years (range:13 years to 70 years). The average A260/280 for purified genomic DNA was 1.85 (range 1.50–2.40).

The patients were grouped into those with normal mucosa (62.5%) and those with abnormal mucosa (37.5%). Of the 30 cases with abnormal mucosa, who also had chronic gastritis, 22 of them (73%) were positive for *H. pylori* DNA detection. Of the 50 cases with normal mucosa, only 2 (4%) had *H. pylori* infection. Of the 24 detected cases of *H. pylori* infection, 79% were s1c/m2 genotype, followed by s1b/m2 genotype (8%) and 4% each for s1c/m1, s1c/m1/m2 and s1c/s2/m2 respectively (Table 2). The only s2/m2 (4% prevalence) was found in mixed infection genotype. Mixed infection genotype was 8%. Only 7 cases (29%) of *H. pylori* infection was positive for *cagA virulence gene*. Of the *cagA* positive cases, 71% were s1c/m2 genotype. Gel pictures are shown in Figures 1A-1C.

Hierarchical cluster analysis of the *H. pylori* genotypes showed a lower dissimilarity or increased similarity in the genotypes. There was mainly one predominant genotype while other genotypes were not relatively distant (Figure 2). We did not find s2/m1 genotype in the study group.

Alleles	N	%
S alleles		
s1a	0	0
s1b	1	4
s1c	23	96
s1	24	100
s2	1	4
s1+s2	1	4
M alleles		
m1	2	8
m2	22	92
m1+m2	1	4
vacA s and m genotpes		
s1+m2	22	92
s1+m1	2	8
s2+m2	1	4
s2+m1	0	0
s1c+m2	19	79
s1c+m1	1	4
s1b+m2	2	8
s1c+m1+m2	1	4
s1c+s2+m2	1	4
CagA Positive	7	29
CagA++ s1c+m2	5	71

Table 2: Alleles of signal and mid region of *vacA* gene in *Helicobacter pylori*.

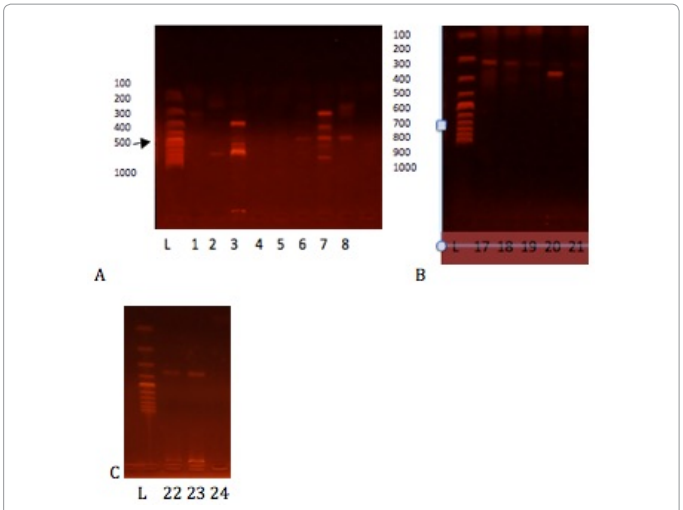

Figure 1: A. Gel bands for vac A genes, L is 100 bp DNA ladder, sample 1 is negative DNA control (*E. coli*), sample 2 IS 705 bp m1/m2 allele, sample 3 is 259 bp s1 (s1c), 630 bp m1 allele, sample 4 is negative, sample 5 is water control, sample 6 is 352 bp m2 allele, sample is 259 bp s1 allele and 352 bp for m2 allele and sample 7 is positive control. The intense band is in the DNA ladder is 500 bp.

B. Gel bands for vac A genes: L is 100 bp DNA ladder, sample 17 and 18 are same for 199 bp for s2 allele, sample 19 is negative and sample 20 is 259 bp for s1 allele.

C. Gel bands for vac A genes: L is 100 bp as annotated in A and B; sample 22 is 394 bp for cagA, sample 23 is postive control and 24 is positive DNA control.

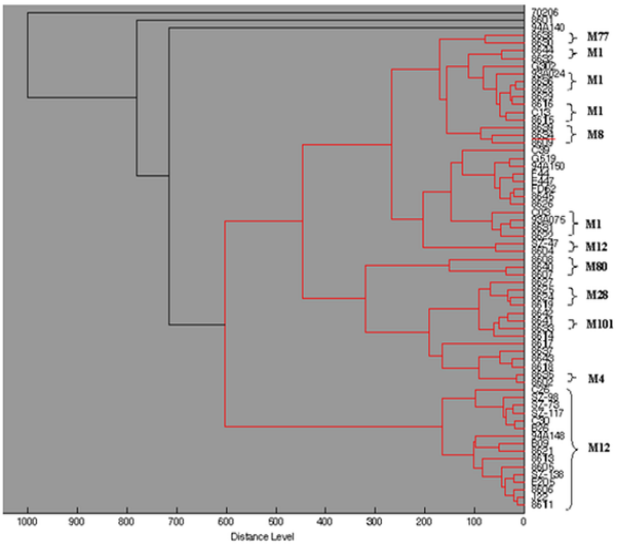

Figure 2: Dendrogram of *H. pylori* clusters in Makurdi.

Discussion

H. pylori infection is endemic in Nigeria but genotype specific information is scanty. Our study showed that 73% of chronic gastritis were associated with *H. pylori* infection, as detected by PCR method. Jemilohun et al. in 2010 reported 64% (based on histology of biopsy) for *H. pylori* infection in Ibadan, Western Nigeria [18]. Olokoba et al.

reported 80% prevalence from histology of gastric biopsies for *H. pylori* in Maiduguri, Northern Nigeria [19]. All the studies reported high prevalence of *H. pylori* infection.

The most prevalent *H. pylori* genotype in our area of study was the s1/m2. It had 83% prevalence. The most prevalent specific subtype was the s1c/m2, it had a prevalence of 79%. This genotype is known to have lesser virulence than the s1/m1 types. Smith et al. 2002 reported a prevalence of 73.2% for the s1/m2 in duodenal ulcer and non-ulcer dyspeptic patients in Nigeria. The prevalence agreed with our findings. However, the report from Smith et al. in 2002 did not include specific subtypes of the *vacA* genotypes. Our study, to the best of our knowledge, provides for the first time the specific subtypes of the *vacA* gene in Nigerian patients. Genotypes of *vacA* gene vary widely worldwide. In Pakistan, the most prevalent genotype is the s1b/m2 (54.5% prevalent) [20,21]. In Ethiopia and Afghanistan, amongst dyspeptic patients, the most prevalent genotype is the s1/m1 (48% and 53% respectively) [22,23]. In Thai, the most prevalent genotype is the s1/m1 (58%) [24]. In Kuwait patients, the *vacA* s1 type is prevalent among African Arabs while the s2 type is common in South Asian patients [25]. The Nigerian patients are infected mostly by the lesser toxic genotype. This may explain the lack of association between duodenal ulcers and *H. pylori* infection in Nigerian patients [20]. Although *H. pylori* infection is predominant in Nigeria and most developing countries in general, they do not share the same cytotoxicity genotypes. The low prevalence of very toxic genotypes (the s1/m1) in Nigerian dyspeptic patients, as reported in our study and other studies, may explain the low frequency of serious gastrointestinal disorders such as gastric lymphoma and duodenal ulcers often associated with *H pylori*. These findings may inform a better choice of eradication therapies to avoid using 'sledge hammers in killing mosquitoes'. This is further supported by the fact that the occurrence of *cagA virulence gene* was also low in the study group. However, the prevalence of *cagA* in chronic gastritis patients differed markedly from the prevalence of 92.7% reported by Smith et al. in duodenal ulcer and non-ulcer dyspeptic patients [20]. Hierarchical cluster analysis based on the alleles of s1, s2, m1 and m2 showed that the *H. pylori* strains in the study area were similar.

Conclusion

Although Nigeria has a high prevalence of *H. pylori* infection, the toxin producing genes found in the patients are less virulent. They produce less toxin and have not been associated with serious gastrointestinal problems. Since *H. pylori* genotypes influence outcomes of eradication therapies, it is therefore pertinent to consider the moderate nature of *H. pylori* strains in Nigeria which shares a high degree of similarity.

Significance of this Study

This study provides, for the first time, information on the specific subtypes of the *Helicobacter pylori vacA* genes in Nigerian dyspeptic patients. The information is vital to choose of eradication therapies for *H. pylori* associated gastrointestinal problems.

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