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Studies on the Association Between *Helicobacter pylori* Genotypes and Histopathological Findings on Biopsies Obtained from Endoscopy Referral Patients of Benue State University Teaching Hospital Makurdi, Benue State Nigeria

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Abstract

Background: *Helicobacter pylori is one of the most common chronic bacterial infections worldwide. It causes gastritis, peptic ulcer, gastric cancers and gastric malt lymphoma. The outcome of the infection may be associated with virulence-associated genotypes. The aim of the study was to determine the association between H. pylori genotypes and histopathological findings on biopsies obtained from endoscopy referral patients of Benue State University Teaching Hospital Makurdi, Benue State Nigeria.*

Methods: *Eighty (80) patients referred for endoscopy were enrolled and two biopsy samples were collected from each of them. One was placed in Brain Heart Infusion (BHI) broth and was used for PCR while the second sample was placed in 10% formal saline and was used for histopathological examination following standard techniques.*

Results: *Histopathological examination showed that 30 (N = 80; 37.5%) of the patients had chronic gastritis, while 50 (N = 80; 62.5%) had normal mucosa. While there was no significant difference between the histopathological findings and H. pylori genotypes using 2-factor ANOVA (p = 0.22), there was however significant difference in the occurrence of the different vacA genotypes (p = 0.005).*

Conclusion: *In this study, H. pylori genotypes was not associated with histopathological findings.*

Keywords: Gastric biopsies, *Helicobacter pylori*, Histology, Immunohistochemistry, Genotypes.

Introduction

Helicobacter pylori is a Gram-negative bacterium which colonizes the human stomach and causes gastritis, peptic ulceration, gastric cancers and gastric Mucosa Associated Lymphoid Tissue (MALT) Lymphoma (Ahmed et al., 2007). The prevalence of *H. pylori* related diseases is not directly proportional to the prevalence of H. pylori colonization. This may be due to several factors such as genetic diversity among humans (Azuma et al., 1998), environmental factors such as diet (Corea, 1995; Corea, 1992; Varis et al., 1998) etc. There is evidence for the existence of distinct genetic lineages (Achtman et al., 1999) that may play a role in pathogenicity. The importance of several specific *H. pylori* genes has been studied in the past. vacA encodes a vacuolating cytotoxin which is excreted by *H. pylori* and damages epitherial cells (Cover, 1996). In recent years, the relationships between histological parameters of gastritis, *H. pylori* infection and virulence genes of this microbe have started to be investigated. H. pylori infection is acquired in early childhood and the current view claims that prolonged infection with *H. pylori* is the major determinant for development of intestinal metaplasia (IM) in the stomach; aging and inflammation of the antrum burns out the microbes (Haruma et al., 2000; Asaka et al., 2001; Lijima et al., 2004; Faller & Kirchner, 2005). The bacterium has been rated as a "class one" carcinogen to the gastrointestinal tract by the World Health Organization (Aguemon et al., 2005).

Transmission pathways of *H. pylori* are not clear. However, risk factors of transmission include precarious hygiene standards, over-crowding, contaminated environments and water sources amongst others (Ndip et al., 2003). It has been found that *H.*

pylori can survive in milk and water in its infectious bacillary form and in river water for several months in a non-culturable but viable form (Bragonca et al., 2007). Previous serological studies have related a high prevalence of antibodies against *H. pylori* among some professions (Abattoir workers, shepherds and veterinary workers) who are in direct contact with *H. pylori* infected animals (Papiez et al., 2003). *H. pylori* has also been isolated from the intestinal tract of cats, dogs, and sheep (Dore et al., 2001). Lack of proper sanitation, safe drinking water, and basic hygiene as well as poor diets and overcrowding all play a vital role in determining the overall prevalence infection (Hunt et al., 2010). Prevalence of 82% has been reported in children 5-9 years, 95% in adults of middle age and 70 – 90% in older adults (Hunt et al., 2010). It has been estimated that more than 80% of Africans are infected with *H. pylori* (Campbell et al., 2010).

Disease outcome may be the result of many factors including host factors, environmental factors and differences in the expression of bacterial elements (Bami-Hani, 2002). Among several pathogenic determinants, expression of the cytotoxin-associated gene A (cagA) and the vacuolating cytotoxin gene A (vacA) products enhance the pathogenicity of this bacterium, Individuals harbouring cagA genes are at increased risk of gastroduodenal ulcerations (Peek et al., 1997).

Since the discovery of *H. pylori* as an important agent in gastritis, and peptic ulcer disease, investigation for this bacterium during endoscopy has become a standard clinical practice to establish active *H. pylori* infection (Ndububa et al., 2001). In Nigeria, there is poor management of *H. pylori* infection, no national consensus guidelines and very few documented pathological roles of the bacterium (Adesanya et al., 2003).

Aim of the Study

The main aim of the study is determining the Association between *Helicobacter pylori* Genotypes and Histopathological Findings on Biopsies Obtained from Endoscopy Referral Patients of Benue State University Teaching Hospital Makurdi, Benue State Nigeria Objectives of the Study

1. To detect *H. pylori* from biopsy specimens using PCR and Histology.
2. To determine the different strains of *H. pylori* by genotyping.
3. To investigate the relationship between genotypes and histopathological findings.

Ethical Approval

Ethical approval was obtained from the Health Research Ethics Committee of the Benue State University Teaching Hospital, Makurdi and volunteer participants were informed of the details of the study and their consent sought.

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 endoscopy on the informed-consenting participants. Tiny biopsy tissue samples were collected from the antrum of patients into two different bottles, sterile McCartney bottles containing Brain Heart infusion broth with 1.5% glycerol and stored in the freezer at -20 ^{o}C within 2 hours of collection until transported in ice packs to Safety Molecular Pathology Laboratory, Enugu for analysis, and in plastic universal bottles containing 10% formal saline which were stored at room temperature and transported to Enugu in sterile plastic bags.

Histology Tests

Haematoxylene and Eosin Staining (H&E)

This test was carried out specifically to group the patients into two: those with normal mucosa and those with abnormal mucosa (gastritis).

Sections on slides were placed on a staining rack, dipped into Haematoxylene stain for 5 minutes, rinsed in water, placed in 1% acid alcohol for a few seconds, blued in warm water to intensify the blue colour of the Haematoxylin and washed in tap water. They were then placed in Eosin stain for 5 minutes and washed in tap water. The sections were dehydrated by putting them in ascending order of ethanol concentration (70 %, 95% 100%) for 10 minutes each and mounted in Dextrene plasticizer xylene (DPX) to increase refractive index for better resolution.

Haematoxylene and Eosin stains help to demonstrate general tissue structure where the nucleus is stained blue – black with Haematoxylene and the cytoplasm and other tissues stain with varying shades of pink e.g., muscle fibre - deep pinky red, fibrin deep pink, red blood cells – orange/red which is the colour of the Eosin.

Immunohistochemical (IHC) Staining for Cellular Antigens using Enzyme Digestion

The sections were deparaffinized/hydrated by placing slides on a hot plate (slides warmer) for 10 minutes, after which the sections were incubated in two washes of xylene for 5 minutes each and incubated in two washes of absolute ethanol (100%) for ten minutes each. They were also incubated in two washes at 95% ethanol for 10 minutes each. Then washed twice in distilled water for three minutes each. Then the slides were brought to a boil in citrate buffer pH 6.0 in a microwave oven for 15 minutes to unmask the antigenic sides which were masked during fixing of the tissue.

The slides were cooled under running tap water and the Abcam kit (kit code ab64264; from Fitzgerald USA) was used. The slides for staining were labelled clearly and arranged in a staining rack including two slides for positive and negative controls. The negative control was a known gastric biopsy without *H. pylori* while the positive control was a section known to have *H. pylori*. The immedge hydrophobic pen was used to carefully mark the area of slide staining.

Eighty microliters (80 µl) (two drops) of Hydrogen peroxide block was added to cover the sections and incubated at room temperature in a humid chamber for 10 minutes to avoid drying, then washed two times in buffer, for three minutes each and 80 µl of protein block was applied to cover sections and incubated at room temperature in a humid chamber for 5 minutes and then washed once in buffer for three minutes. The sections were then covered with 80µl of primary antibody (specific to the cellular antigen), unconjugated *H. pylori* antibody was applied and incubated at room temperature in the humid chamber for 40 minutes and washed four times in buffer for 3 min eac. Eighty microliters (80 µl) of Biotinylated goat from the ABCAM kit was applied and incubated at room temperature for 10 minutes in a humid chamber and washed four times in buffer for three minutes each.

Eighty microliters of streptavidin peroxidase was applied and incubated at room temperature for ten minutes in the humid chamber and the slides rinsed four times in buffer. One hundred microliters (100 µl) of DAB solution was added to the slides. The slides were incubated at room temperature for 10 minutes and rinsed four times in buffer. One hundred microlitres of filtered

Haematoxylin (counterstain) was added and incubated at room temperature for one minute. This was rinsed 7 times in tap water, dehydrated, cleaned in three rounds of xylene and mounted, each slide with DPX mountant, avoiding air bubbles, and it was ready for viewing under the microscope.

Giemsa Stain for Tissue Sections

Sections on slides were put on a staining rack and dipped into Haematoxylene stain and allowed to stay for 5 minutes. The sections were rinsed in water and placed in 1% acid alcohol for a few seconds. The sections were blued in warm water to intensify the blue colour of the Haematoxylin and were washed with tap water, placed in Giemsa stain for 5 minutes and washed in tap water. The sections were dehydrated by placing in ascending order of ethanol concentration (70%, 95 %, and 100 %) for 10 minutes each and mounted in Dextrene plasticizer xylene (DPX) to increase refractive index for better resolution.

Giemsa stain helps to demonstrate the presence of the organism.

Genomic DNA (gDNA) Extraction

The Genomic DNA was extracted using ReliaPrep gDNA miniprep kit (Promega, Southampton, UK).

The 2 ml tubes to be used were selected and labeled, and 200 µl of specimen were placed in the tubes. Proteinase K (25 µl) was dispensed into the tubes and the content mixed by vortexing for one minute to destroy other proteins and release the bacteria. Lysis buffer (200 µl) was added and mixed by vortexing for 10 seconds and incubated at 56 0C for 10 minutes in a water bath to help the buffer work maximally. Binding buffer (250 µl) was added to each tube and mixed for 10 seconds. Spin columns were selected, labelled and placed into collection tubes. The lysates were transferred into the corresponding spin columns which had silica membrane and the binding buffer to help release DNA and to adsorb to the silica membrane. The columns were centrifuged (14000 revolutions per minute (rpm)) and the flow through discarded and new collection tubes inserted. Column wash buffer (500 µl) was used to wash the columns three times and contrifuged for 3 minutes at 14000 rpm. The flow through discarded at each step. Spin columns were placed in clean collection tubes and centrifuged (14000 rpm, 1 min) to remove residual wash buffer and were placed in clean 1.5ml recovery tubes. Sterile nuclease free water (200 µl) was added into each tube and incubated at room temperature for one minute and then centrifuged (13000 rpm, 1 min). The DNA quality was checked at 260/280nm using Eppendorf Bio photometer Plus (Eppendorf, Germany). Nuclease free water was used as blank. Figure 1.2 and above were taken to be pure. The genomic DNA was labelled and used for further tests immediately and the remaining one stored in the fridge.

Genotyping for *H. pylori* Virulence Genes and Strains

Singleplex PCR for Detection of cagA Region Gene of *H. pylori*

A single multiplex PCR system was used to amplify the two regions of the cagA gene: 394 bp and 717 bp respectively.

The primer sequences used were:

CAGA394F	GATAACAGGCAAGCTTTTGAGGGA
CAGA394R	CCATGAATTTTTGATCCGTTC
CAGA717F	ATGGGGAGTCATGATGGCATAGAACC
CAGA717R (Broutet et al., 2001)	ATTAGGCAAATTAAAGACAGCCACC

Water was used as no template control (NTC), *E. coli* DNA as Negative Control (NC) and *H. pylori* samples as positive control (PC).

Twelve point five microliters (12.5 µl) of 10 x PCR master mix (or multiplex mix), 7.5 µl of the CAG mix and 5.0 µl of genomic DNA was pipetted into each sample well making a total reaction volume of 25 µl.

The thermal profile set in the Eppendorf PCR machine as cag M mix was as follows: 95°C for 3 min, 94°C for 60 sec, 58°C for 60 sec, and 72°C for 60 sec, 72°C for 5 min for 40 cycles.

Electrophoresis was run in 2.0 % agarose gel with 20 µl ethidium bromide placed in 0.5 x TBE buffer at 100 V for 45 minutes and viewed in ultra violet (UV) light. Product sizes 394 bp or 717 bp were considered cagA gene positive.

Multiplex PCR for Detection of vacA Mid Region (m) Gene of *H. pylori*

A single multiplex PCR system is used to amplify the mid region of the vacA gene, to identify the m1, m2, m1/m2 or m2/m1 alleles (strains).

The primer sequences used were:

VA7-F	GTAATGGTGGTTTCAACACC
VA7-R	TAATGAGATCTTGAGCGCT
VA4-F	GGAGCCCCAGGAAACATTG
VA4-R	CATAACTAGCGCCTTGCAC (Atherton *et al.,* 1999)

Water was used as no template control (NTC), *E. coli* DNA as Negative Control (NC) and *H. pylori* samples as positive control (PC).

Twelve point five microliters (12.5 µl) of 10 x PCR master mix (or multiplex mix), 7.5 µl of the vac m mix and 5.0 µl of genomic DNA was pipetted making a total reaction volume of 25 µl and put in each sample well.

The thermal profile was set in the Eppendorf machine as vac m mix as follows: 95°C for 3 min, 95°C for 30 sec, 56°C for 60 sec, 72°C for 90 sec, and 72°C for 5 min for 35 cycles.

Electrophoresis was run in 2.0 % agarose gel with 20 µl ethidium bromide placed in 0.5 x TBE buffer at 100 V for 45 minutes and viewed under UV light. The following product sizes were considered positive: 630 bp for m1 alleles, 352 bp for m2 alleles, 705 bp for m1/m2 alleles and 277 bp for m2/m1 alleles.

Multiplex PCR for Detection of vacA Signal Region(s) Gene of *H. pylori*

Two different multiplex PCR mixes are used to type the signal coding region 's' region into s1 and s2 (s1 is sub-typed to s1a, s1b and s1c). Product sizes include 190 bp for s1a, 187 bp for s1b, 199 bp for s2; 286 bp for s2 and 259 bp for s1 respectively.

The primer sequences used were as suggested by Atherton et al. (1999) and are:

VA1-F	ATGGAAATACAACAAACACAC
VA1-R	CTGCTTGAATGCGCCAAAC
VA1-s2-F	ATGGAAATACAACAAACACAC
VA1-s2-R	CTGCTTGAATGCGCCAAAC
SS1-F	GTCAGCATCACACCGCAAC
SS3-F	AGCGCCATACCGCAAGAG
SS2-F	GCTAACACGCCAAATGATCC

Water was used as no template control (NTC), *E. coli* DNA as Negative

Control (NC) and *H. pylori* strain from ATCC number 43526 as Positive Control (PC). Twelve point five microliters (12.5 µl) of 10x PCR master mix (or multiplex mix), 7.5 µl of the primer mix and 5.0 µl of genomic DNA was pipetted making a total reaction volume of 25 µl and put in each sample well.

The Thermal Profile was set in the Eppendorf Machine as '*H. pylori* mix' as follows: 95°C for 3 min, 95°C for 15 sec, 52°C for 60 sec, 72oC for 60 sec, 72oC for 5 min, for 35 cycles.

Electrophoresis was run in 2.0 % agarose gel (with 20µl ethidium bromide placed in 0.5 x TBE buffer) at 100 V for 30 minutes and the bands viewed in UV light.

The Platinium Multiplex PCR master mix (Invitrogen, UK) was used in all multiplex reactions while all the primers were HPLC grade, synthesized by Eurofins, Germany.

Statistical Analysis

Data obtained from the study were analysed using Statistical Package for Social Sciences (SPSS) version 20, IBM Inc. Chi square was carried out to measure association between variables. Two – factor ANOVA was used to determine group differences and Duncan Multiple Range test was used to separate means. Alpha level of significance was set at 0.05.

Results

Of the eighty (80) biopsy specimens collected, 50(62.5 %) had normal mucosa and 30(37.5 %) had abnormal mucosa (gastritis) as detected by Haematoxylene and Eosin stain. Seventeen (21.3 %) of the patients had *H. pylori* infection as detected by Giemsa stain, 19 (23.8 %) by Immunohistochemical stain (Table 1).

Test	Frequency (%)
Haematoxylene and Eosin Stain	
Gastritis	30 (37.5)
Normal mucosa	50 (62.5)
Giemsa stain	
Positive	17(21.3)
Negative	63(78.8)
Immunohistochemical stain	
Positive	19 (23.8)
Negative	61(76.3)

Table 1: Distribution of *Helicobacter pylori* by Histologic Methods (n=80)

The distribution of *H. pylori* genotypes detected in biopsies are shown in Table 2 where s1+m2 had the highest frequency of 22 (92 %) followed by s1c+m2 19 (79 %). The least were s2+m1, s1c+m1+m2 and s1c+s2+m2 1 (4 %) each. Seven (29%) of the positive cases were cagA positive, 71% of the *cagA* positive were s1c+m2.

There was no significant difference in the three histopathological test findings and the vacA genotypes using two-factor ANOVA ($p = 0.220$). However, there was a significant difference between the *vacA* genotypes ($p = 0.005$). The means of the genotypes which were separated using Duncan Multiple Range Test showed that there was no significant difference in the genotypes except s1c+m2 which was different (Table 3).

In the distribution of *H. pylori cagA* genotypes in relation to Histopathological findings. There was no association between the *cagA* genotypes and the histopathological findings (Chi-square=0.586; $p = 0.746$) (Table 4).

Genotypes	Number	%
vacA		
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+s2+m2	1	4
s1c+m1+m2	1	4
cagA		
cagA Positive	7	29
cagA++ s1c+m2	5	71

Table 2: Frequency of Helicobacter pylori Genotypes Detected in Biopsies

VacA genotypes	Haemato-xylene and Eosin stain Number (%)	Giemsa stain Number (%)	Immunohis-tochemical stain Number (%)	Mean Value
s1b+m1	2(6.7)	1(5.9)	1(5.3)	1.3[a]
s1c+m2	18(60)	14(82.3)	16(84.1)	16[b]
s1c+m1 +m2	1(3.3)	1(5.9)	1(5.3)	1.0[a]
s1+s2+m1	1(3.3)	1(5.9)	1(5.3)	1.0[a]
Untypeable	8(26.7)	-	-	2.7[a]
Total	30(100)	17(100)	19(100)	
Mean Value	6.0[a]	3.4[a]	3.8[a]	

Table 3: Distribution of *Helicobacter pylori vacA* Genotypes in Relation to Histopathological Findings

$F_{4,8} = 9.034$, $p = 0.005$

$F_{2,8} = 1.84$, $P = 0.220$

cagA genotypes	Haematoxylene and Eosin stain Number (%)	Giemsa stain Number (%)	Immunohisto-chemical stain Number (%)
cagA genotype			
cagA positive	6(20)	5(29.4)	5(26.3)
cagA negative	24(80)	12(70.6)	14(73.7)
Total	30(100)	17(100)	19(100)

Table 4: Distribution of *Helicobacter pylori cagA* Genotypes in Relation to Histopathological Findings

$\chi 2 = 0.586$ df = 2 $p = 0.746$

Discussion

The main purpose of this study was to determine the association between *Helicobacter pylori* Genotypes and Histopathological Findings on Biopsies Obtained from Endoscopy Referral Patients of Benue State University Teaching Hospital Makurdi, Benue State Nigeria.

Chronic gastritis as detected by Haematoxylene and Eosin stain was associated with *H. pylori* infection. Similar results based on seroprevalence were reported among patients with gastritis in Kaduna State (Nwodo et al., 2009), and in Enugu State (Neri et al., 2009). Likewise, previous studies based on histology of biopsies for *H. pylori* infection in Ibadan, Western Nigeria (Jemilohon et al., 2010) and Olokoba et al. (2013) in Maiduguri, Northern Nigeria detected *H. pylori* from histology of gastric biopsies among gastritis patients.

Infection with *H. pylori* causes gastritis which is associated with peptic ulcer disease, gastric carcinoma etc. (Graham, 2000). Although the infection causes gastritis in most patients, not all develop severe gastric pathology. This could be as a result of host and environmental factors (Azuma et al., 1998) but there is also evidence for the role of specific *H. pylori* virulence genotypes. The less virulent genotypes are more likely to cause mild gastritis in patients while the more virulent genotypes cause more serious gastric disorders (Athertyon et al., 1995). Several studies have shown that *vacA* genotype is associated with disease in patients from many countries (Kidd et al., 1999). However, in this study, there was no significant association between *vacA* and histopathological findings which agrees with the findings of Yamaoka et al. (1999).

The *cagA* genotype encode proteins that enhance virulence by increasing production of interleukin-8 by gastric epithelial cells (Shimoyama & Crabtree, 1997) resulting in severe inflammation of the gastric epithelium. However, in the present study, there was no significant association between *cagA* genotype and histopathological findings. Several other studies did not confirm these relationship (Maeda et al., 1998). These discrepancies could be as a result of geographic differences.

Conclusion

This study showed no significant association between *vacA* and *cagA* genotypes of *H. pylori* and Histopathological findings. However, there was a significant difference between the *vacA* genotypes.

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