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Detection of *Helicobacter pylori* by Histopathological Examination of Gastric Biopsies at A Tertiary Hospital in Makurdi, Benue State, Nigeria

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Abstract

Helicobacter pylori are bacteria implicated in numerous common chronic bacterial infections worldwide, and cause gastritis, peptic ulcer, gastric cancers and gastric malt lymphoma. The aim of the study was to detect *H. pylori* among patients referred for endoscopy at Benue State University Teaching Hospital, Makurdi by histopathological methods. Eighty (80) patients referred for endoscopy were enrolled into the study. Biopsy samples were collected from each patient, placed in 10% formal saline and used for histopathological examination by standard techniques. Histopathological examination showed that 30 (N = 80; 37.5 %) of the patients had chronic gastritis, while 50 (N = 80; 62.5 %) had normal mucosa. In this study, there was a high prevalence of *Helicobacter pylori* in dyspeptic patients in Makurdi, Benue State of Nigeria. *H. pylori* infection was however found not to be associated with gender and age using Haematoxylene and Eosin stain ($x^2 = 1.516; p = 0.218$). However, there was significant association between the infection and gender using Giemsa and Immunohistochemical staining methods ($^2 = 4.9001; p = 0.027$ and $^2 = 7.591; p = 0.006$).

Key words: Gastric biopsies, Giemsa stain, Haematoxylene stain, Histology, *Helicobacter pylori*, Immuno-histochemical stain

Introduction

Helicobacter pylori are Gram-negative bacteria. They infect more than half the world's human population, with prevalence ranging from 25% in developed countries, to more than 90% in developing countries (Bardan, 1997). The bacteria colonize

the gastric epithelium and causes chronic gastritis, peptic ulceration, gastric cancers and gastric Mucosa Associated Lymphoid Tissue (MALT) Lymphoma (Ahmed *et al*., 2007). *Helicobacter pylori* have been rated a "class one" carcinogen to the gastrointestinal tract (Aguemeon *et al*., 2005).

Risk factors of transmission include precarious hygiene standards, over-crowding, contaminated environments and water sources amongst others (Ndip *et al*., 2003).

H. pylori are believed to be transmitted primarily by faecal oral or oral-oral routes, with water and food as possible vehicles of infection. However, exact modes of transmission are not easily determined because *H. pylori* are difficult to culture from environmental samples. There is some evidence for iatrogenic transmission through inadequately sterilized endoscopes. *H. pylori* has been detected in vomitus, indicating the potential for gastro-oral transmission (Tanih *et al*., 2010).

According to Hunt *et el*. (2010) inadequate sanitation, safe drinking water, basic hygiene as well as poor diets and overcrowding all play a role in determining the overall prevalence *H. pylori* infection. 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). There is an estimate of more than 80% of Africans infected with *H. pylori* (Campbell *et al*., 2010). A study on seroprevalence of *H. pylori* infected patients with peptic ulcer in Kaduna State of Nigeria revealed that out of the 225 patients tested, 181 (80.4%) were positive for *H. pylori* and 44 (19.6%) were negative (Nwodo *et al*., 2009). A similar study was carried out in Enugu State, Nigeria, where out of 103 patients, 63 (62%) were positive (Neri *et al*., 2009). *H. pylori* still continues to present itself as a serious health concern (Aguemeon *et al*., 2005). However, literature is scarce on its prevalence in Makurdi, Benue State, Nigeria.

To help bridge the information gap, this study sought to determine the prevalence of *H. pylori* in Makurdi, Benue State, Nigeria, using histopathological method.

MATERIALS and METHODS

Ethical Approval

Ethical approval 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. Volunteer participants were informed of the details of the study and consented.

Sample Size Determination:

Sample size was calculated using Raosoft Sample Size Calculator (www.raosoft.com). 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.

Patient Recruitment

Patients were recruited from the Gastroenterology unit of the Benue State University Teaching Hospital (BSUTH), Makurdi. Subjects were patients who had various *H pylori*-associated dyspeptic symptoms including epigastric pain, fullness, vomiting, nausea and flatulence. A Consultant Gastroenterologist performed the endoscopies.

Sample Collection

Gastric biopsy samples were taken from the antrum of the patients. Tiny pieces of tissue samples were collected in plastic universal bottles containing 10 % formal saline which were stored at room temperature and transported to the Laboratory in sterile plastic bags.

HISTOLOGY TESTS

Tissue Processing

The biopsy tissues in 10% formal saline were processed for histopathological tests using an automated tissue processor (ATP) comprising of the following stages:

Fixation: The tissue was placed in 10% formal saline for two hours to prevent

putrefaction and deterioration.

Dehydration: In this stage, the tissue was dehydrated using absolute alcohol starting from 70 % (1½ h) 90 % (1½ h) and absolute (1 h) (ascending order).

Clearing: This was done by placing the tissue in xylene three times for one hour each to ensure proper clearing. Clearing increased the refractive index of the tissue so that it could be clearer during viewing. It also removed tissue associated fat.

Infiltration: The tissue was impregnated with molten paraffin wax by placing it in paraffin wax baths 1 and 2 at 65 °C for 1½ hours each. Infiltration helped to fill the pores/holes created by the dehydrating and clearing agents.

Embedding station/centre: The tissue was first put in the pre-warming chamber, to maintain the molten state of the paraffin wax. A metallic mold was put under the opened for paraffin wax to flow into. Forceps was used to put the tissue in the mold and then taken to the cooling chamber to solidify into Formalin Fixed Paraffin Embedded (FFPE) Tissue Sections. Embedment was necessary to stabilize the tissue for easy cutting.

Sectioning: This involved getting thin slices of tissue about 4 – 5 µm thick and was done using a microtome (Rotary microtome blades).

The formalin fixed paraffin embedded tissue was placed in ice for 10 minutes, the slides labeled, trimmed to 20µm, placed on a slide containing 20% alcohol for 1 minute and dropped in a water bath for 45 minutes to spread and unfold any folding. The water bath was kept at a temperature below 65°C to prevent the wax from melting. After 45 minutes, slides were coated with positively charged poly-L-lysine by placing a drop on each slide and squash spread. The tissues were picked from the water bath onto slides warmer to dewax for 15 minutes at 70°C and kept for staining.

Haematoxylene and Eosin (H&E) Staining

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 Haematoxylene and washed in tap water. They were then placed in Eosin stain for 5 minutes and again washed in tap water. The sections were dehydrated in increasing ethanol concentration (70%, 95% and 100%) for 10 minutes each and mounted in Dextrene plasticizer xylene (DPX) to increase refractive index for better resolution.

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

Immunohistochemical (IHC) Staining for Cellular Antigens using Enzyme Digestion

The sections were deparaffinized/hydrated by placing slides on a slide warmer for 10 minutes, then incubated in two washes of xylene for 5 minutes each, and two washes of absolute ethanol (100%) for ten minutes each. They were further washed in 95% ethanol for 10 minutes each, then washed twice in distilled water for three minutes each. The slides were brought to a boil in citrate buffer (pH 6.0) in a microwave oven for 15 minutes to unmask the antigens, then cooled and stained using the Abcam kit (kit code ab64264; Fitzgerald, USA). The slides were labelled and arranged, including one slide as positive and the other as negative control. The negative control was a known gastric biopsy without *H. pylori*, while the positive control was a section positive for *H. pylori*.

Eighty microliters (80 µl) of hydrogen peroxide was added to cover the tissue sections and incubated at room temperature in a humid chamber for 10, washed in buffer.

Subsequently 80 µl of protein block was applied to cover the sections, incubated at room temperature in a humid chamber for 5 minutes, and washed in buffer for three minutes. The sections were covered with 80µl of primary antibody (specific to the cellular antigen), unconjugated *H. pylori* antibody was applied, incubated at room temperature in the humid chamber for 40 minutes, and washed four times in buffer for 3 minutes each. 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 (80 µl) of streptavidin peroxidase were 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 Haematoxylene (counterstain) was added and incubated at room temperature for one minute. This was rinsed under tap water, dehydrated, cleaned in xylene and mounted, with DPX mountant, and viewed under the microscope (x100).

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 under tap water, placed in Giemsa stain for 5 minutes, and again washed under 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.

Results

Out 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 (H&E) staining. Seventeen (21.3 %) of the patients had *H. pylori* infection as detected by Giemsa stain (GS), and 19 (23.8 %) by Immuno-histochemical (IHC) stain (Table 1).

Table 1: *Helicobacter pylori* Isolated using Different Stains *(n=80)*

Test	Frequency (%)
H & E Stain	
Gastritis	30 (37.5)
Normal mucosa	50 (62.5)
Giemsa Stain	
Positive	17(21.3)
Negative	63(78.8)
IHC Stain	
Positive	19 (23.8)
Negative	61(76.3)

Key: H & E = Haematoxylene and Eosin; IHC = Immunohistochemical Stain

Plate 1 shows the photomicrograph of normal gastric mucosa, negative for Giemsa stain showing the normal epithelial cells (arrowed)

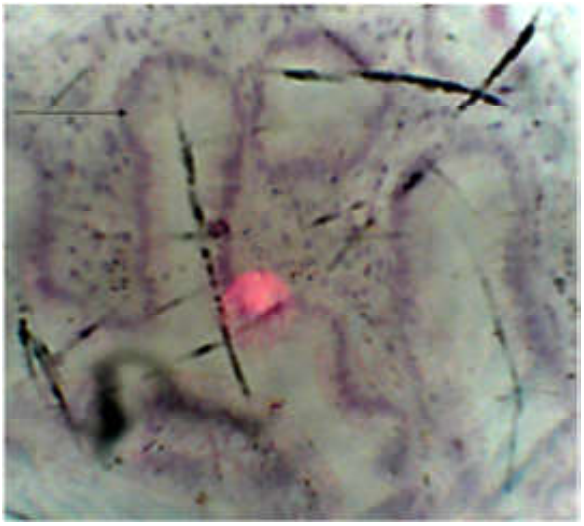

Plate 1: Photomicrograph of normal Gastric mucosa: Negative for Giemsa stain showing normal epithelial cells (arrowed)

Photomicrograph of chronic gastritis showing the infiltration of white blood cells (arrowed) is shown in Plate 2.

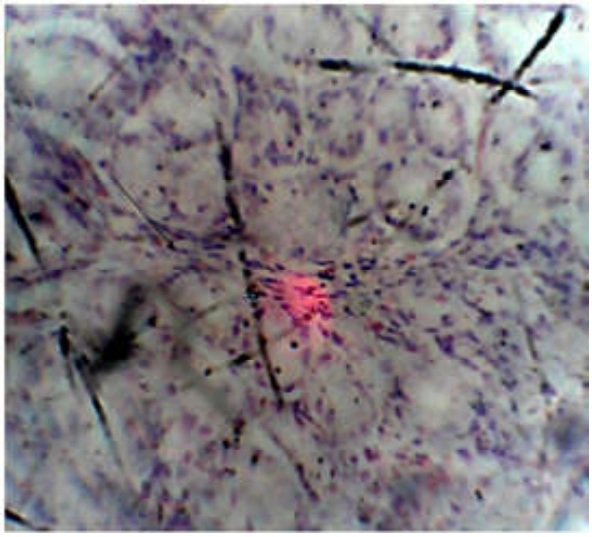

Plate 2: Photomicrograph of Chronic Gastritis showing infiltration of white blood cells (arrowed)

Plate 3 presents the photomicrograph of chronic gastritis positive for Giemsa showing the infiltration of white blood cells and the presence of *H. pylori* (arrowed).

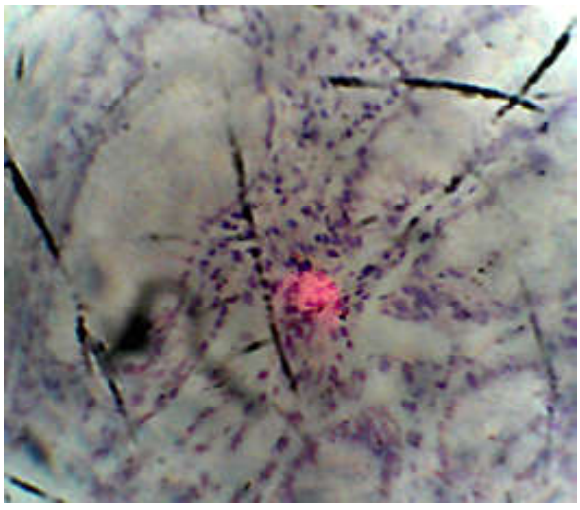

Plate 3: Photomicrograph of chronic gastritis with positive Giemsa stain showing *H. pylori* (arrowed)

Plate 4 is the photomicrograph of chronic gastritis with *H. pylori* positive in immunohistochemistry. It shows the infiltration of white blood cells and the dark brown colouration around the epithelial cells (arrowed).

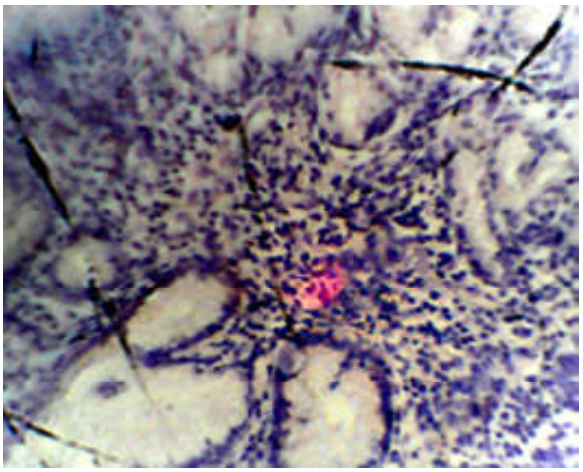

Plate 4: chronic gastritis with *H. pylori* positive by IHC stain showing dark brown coloration (arrowed)

The photomicrograph of normal mucosa negative for *H. pylori* in immunohistochemistry showing the normal epithelial cells without the dark brown colouration (Plate 5)

Plate 5: Photomicrograph of normal mucosa: Negative for *H. pylori* by IHC staining showing normal white blood cell without the dark brown coloration (arrowed)

Table 2 shows that out of the eighty patients examined out of which 33 (41.25%) were males and 47 (58.75%) were females, gastritis was not significantly associated with sex according to Haematoxylene and Eosin staining which indicated that the outcome of infection was not dependent on sex (^{2} *=1.516; p = 0.218).*

Table 2: Distribution of *Helicobacter pylori* by Sex in gastritis patients using Haematoxylene and Eosin Stain

Sex	Gastritis No. (%)	Normal mucosa No. (%)	Total
Female	15(31.9)	30(68.1)	47(100)
Male	15(45.5)	20(54.5)	33(100)
Total	30(37.5)	50(62.5)	80(100)

^{2} *= 1.516, df =1, P = 0.218*

The Distribution of *Helicobacter pylori* by Sex using Giemsa and Immunohistochemical Staining Methods showed that there was a significant association between *H. pylori* infection and sex *(^{2} = 4.9001; p = 0.027, ÷^{2} = 7.591; p = 0.006 respectively)* (Table 3).

Table 3: Distribution of *Helicobacter pylori* by Gender using Giemsa and Immunohistochemical Staining Methods

Test/Sex	Positive (%)	Negative (%)	Total (%)	Chi-Square	P-Value
Giemsa Stain					
Female	6(12.8)	41(87.2)	47(100)		
Male	11(33.3)	22(66.7)	33(100)	4.901	0.027
Total	17(21.13)	63(7.88)	80(100)		
Immuno-histochemical stain					
Female	6(12.8)	41(87.2)	47(100)		
Male	13(39.4)	20(60.6)	33(100)	7.591	0.006
Total	19(2.38)	61(76.3)	80(100)		

The distribution of *H. pylori* infection in relation to age is shown in Table 4. There was however no significant association between age and the infection according to Haematoxylene and Eosin staining.

Table 4: Distribution of *Helicobacter pylori* by Age in Gastritis patients using Haemataxylene and Eosin stain

Age group (years)	Gastritis No. (%)	Normal mucosa No. (%)	Total (%)
< 30	9(69.2)	4(30.8)	13(100)
31 – 43	9(33.3)	18(66.7)	27(100)
44 – 56	11(32.4)	23(67.6)	34(100)
> 57	1(16.7)	5(83.3)	6(100)
Total	30(37.5)	50(62.5)	8(100)

$\chi^2 = 7.280$, df =3, P = 0.063

Discussion

Haematoxylene and Eosin, Giemsa and Immunohistochemistry staining techniques have helped in identification of organisms in gastric biopsies. Several methods have been used but histological detection remains the most common (Patnayak *et al.,* 2015).

Chronic gastritis as detected by Haematoxylene and Eosin stain was significantly associated with *H. pylori* infection. Similar observations were reported by Toulaymat *et al.* (1999) and Wang *et al.* (2010). Results based on seroprevalence were also reported among patients with gastritis in Kaduna State, and in Enugu, all in Nigeria (Nwodo *et al*., 2009; Neri *et al*., 2009). Likewise, previous studies based on histology of biopsies for *H. pylori* infection in Ibadan, Western Nigeria and in Maiduguri, Northern Nigeria detected *H. pylori* from histology of gastric biopsies among gastritis patients (Jemilohon *et al*., 2010; Olokoba *et al*., 2013). The finding of the study that males had a higher prevalence than females as detected by Eosin and Immunohistochemical stains is in accordance with the findings of Dandrin *et al.* (2012) and Abu-Ahmad *et al.* (2011) and may be attributed to the fact that several studies have suggested that oestrogen protects women from this kind of inflammation (Ibrahim *et al*., 2017). While no evaluation was undertaken from females enrolled into the study, it seems reasonable to assume that they had higher levels of the hormone oestrogen than would occur in males. Similarly, women with delayed menopause and increased fertility are reported to generally have a lower risk of gastric infections and cancer (Ibrahim *et al*., 2017).

Conclusion

This study undertaken to detect *H. pylori* among patients referred for endoscopy at Benue State University Teaching Hospital, Makurdi, using histopathological methods, showed a high prevalence of *Helicobacter pylori* in dyspeptic patients. *H. pylori* infection in the study was found not to be associated with gender and age using Haematoxylene and Eosin stains. However, there was a significant association between the infection and gender using Giemsa and Immunohistochemical staining methods.

This study, therefore, implies that detection of *H. pylori* can be readily detected and diagnosed using staining methods. Health practitioners and Doctors treating and managing suspected *H. pylori* infections in patients could rely on histopathological staining for effective diagnosis.

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