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Comparative Study of Some Staining Methods for the Demonstration of *Helicobacter pylori* in Gastric Biopsies in Gombe State, Nigeria

A. Umar [a*], G. K. Owolagba [a], S. M. Aliyu [b], M. D. Hassan [c], U. Abubakar [a], M. O. Mohammed [a] and M. S. Sani [a]

[a] Department of Histopathology, School of Medical Laboratory Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria.
[b] Department of Histopathology, Federal Teaching Hospital, Gombe, Nigeria.
[c] Department of Medical Microbiology, Federal Teaching Hospital, Gombe, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Authors AU and GKO designed the study, wrote the protocol and the first draft of the manuscript. Authors SMA and MDH managed the literature searches and analyses of the study. Authors UA, MOM and MSS took care of logistics and data interpretation. All authors read and approved the final manuscript.

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ABSTRACT

Background: Currently there are different staining methods available for the demonstration of *Helicobacter pylori* in gastric biopsies sections. Most of these methods are technically demanding, slow and expensive with varying sensitivity and specificity.
Aims: This study was to compare some of these techniques for the detection of *H. pylori* in routine gastric biopsy tissue sections in order to ascertain their reliability and effectiveness.

**Corresponding author: Email: abdu.umar@udusok.edu.ng, aupalladan@gmail.com;*

Methodology: Sixty-five positive and seven negative cases of *H. pylori* were selected based on the results of rapid urease test, serology test and histopathological examination of the tissue sections. Histopathological examinations of sections stained by Hematoxylin and Eosin staining method (H&E), Giemsa staining method, modified Giemsa staining method, modified McMullen staining method and immunohistochemical staining were performed.
Results: The sections were evaluated by two independent observers. The sensitivity and specificity for the techniques were as follow: immunohistochemistry, 100% and 100%; modified Giemsa stain, 96.9% and 85.7%; Giemsa stain, 92.3% and 81.4%; modified McMullen stain, 89.2% and 52.9%; H&E, 90.8% and 77.1%. The interobserver agreement was analyzed by Kappa (k) statistics using SPSS version 23 software. The agreement between the two observers was k: 0.859 (98.4%) for immunohistochemistry; k: 0.777 (96.9%) for modified Giemsa stain; k: 0.533 (95.2%) for Giemsa stain; k: 0.267 (92.1%) for modified McMullen stain; and k: 0.421 (93.7%) for H&E stain.
Conclusion: The best results were obtained by the immunohistochemical staining and the Giemsa stainings. Although the immunohistochemistry staining method gives the best result and is very reliable, but it is fairly time consuming and expensive. Therefore, considering the cost, applicability and the reliability of the modified Giemsa or Giemsa stain, we recommend both for the detection of *H. pylori* in gastric biopsies sections especially in resource constrained laboratories.

Keywords: Biopsies; giemsa; Helicobacter pylori; immunostaining; peptic ulcer; staining.

1. INTRODUCTION

In their important work published in 1984, Marshall and Warren revealed the connection between gastritis and peptic ulcer disease caused by *Helicobacter pylori*, formerly known as *Campylobacter pylori* infection. This discovery had such a significant impact on research that they were awarded the Noble Prize in Medicine in 2005 [1]. The bacteria can cause acute gastritis and chronic gastritis in the gastric mucosa [2]. "*H. pylori* gastritis has major complications including gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma. Therefore, its eradication is a preventative measure for gastric cancer" [3]. "This is the reason why the International Agency for Research on Cancer (IARC) reported that *H. pylori* is a carcinogen in humans" [4].

About 50% of the world population are infected with *H. pylori* [5]. Infection is linked to geographical region, ethnicity, age, and socioeconomic circumstances, and its prevalence varies greatly; it is higher in poor countries and lower in the developed world [5,6]. However, there has been a global trend in recent years towards a decline in the prevalence of *H. pylori* [2]. In many developing nations, the frequency among middle-aged adults is over 80%, as opposed to 20–50% in industrialized nations [7]. According to Hunt et al, [8] the prevalence rate in Nigeria was 82% among children and 70-91% among adults. These Gram-negative bacilli can be detected using a variety of techniques, which include invasive techniques such as histological investigation, and non-invasive methods such as C13 urea breath tests, stool culture, serological method and stool antigen test [9]. A single test alone cannot provide a correct diagnosis; culture must also be considered in some cases [10].

H. pylori is identified in histological sections as short, curved or spiral bacilli that are found on the stomach mucosa's epithelial surface or in its mucus layer. They are also found in the deep gastric pits [11]. Following therapy, *H. pylori* density decreases or even disappears, and the bacteria may become a round (coccoid form) or vibrio shape. Haematoxylin and Eosin (H&E) stain and modified Giemsa stains can be used to distinguish these changed forms [12]. *H. pylori* is typically detectable in an excellent H&E stained paraffin section when examined closely at a high magnification. However, this method has a low sensitivity, especially if the paraffin section contains scanty organisms [13]. "Most laboratories use an additional histochemical staining method in conjunction with the H&E method for the identification of the organisms, as timely and accurate diagnosis is crucial given the well-documented consequences of *H. pylori* infection" [14]. For the histological evaluation of *H. pylori* infection, numerous special or histochemical staining techniques have been developed, and some earlier methods have been improved upon; however, all of them have some defects in terms of sensitivity, practicality, stability, visibility of the organisms, or cost [15].

The diagnosis of *H. pylori* infection using histopathological techniques is becoming a common practice. There are various staining techniques that are used, these include *H. pylori* antibody immunohistochemical stains, Giemsa, modified Giemsa, Warthin-Starry, modified McMullen method, and Genta stains. Immunohistochemical staining is currently regarded as the "gold standard" for the histopathological diagnosis of *H. pylori* due to its excellent sensitivity and specificity, [16]. “Apart from Genta staining method, which is complex and time consuming, the other staining techniques require an additional routine H&E stained slides to assess the pathology associated with the infection. Therefore, the sensitivity and specificity of the various staining techniques have been a subject of investigations” [17,18].

Despite the fact that each method has its advantages and disadvantages, none with the exception of the immunohistochemistry method has been proven to be more effective in terms of cost, ease of use, and sensitivity [14]. “In contrast to earlier approaches, two recently published methods; a modified McMullen's and the *H. pylori* silver stain were both asserted to match the aforementioned standards. While the latter is a silver staining approach distinct from the Warthin-Starry method, the former is a variation of the Gimenez stain” [19].

Most of our histopathology laboratories rely on conventional Giemsa staining method for the demonstration of *H. pylori* in routine gastric biopsy paraffin sections. It has been discovered that Giemsa technique stains only the inner portion of the organisms, thereby making it appear thinner and more difficult to distinguish from the other tissue structures [20]. We may therefore ask ourselves whether we are missing some positive biopsies by regarding them as negative. This is very possible where the organisms are scanty. So, the question now is whether we can still rely exclusively on Giemsa method for routine demonstration of *H. pylori* in our histopathology laboratories.

The study was designed to compare some of the available histological staining techniques for the demonstration of *Helicobacter pylori* in routine gastric biopsy paraffin sections in order to ascertain their reliability and effectiveness and come up with a simple, reliable and cost-effective method for the detection of *H. pylori* in our laboratories.

2. METHODOLOGY

2.1 Study Location

The study was carried out in the Laboratory of the Department of Histopathology, Federal Teaching Hospital, Gombe; a referral tertiary hospital, to compare some staining methods for the demonstration of *Helicobacter pylori* in gastric biopsies in Gombe state, Nigeria.

2.2 Study Design

This was a retrospective study that analyzed sixty-five (65) endoscopic biopsies of patients reported as having gastritis or gastric ulcers, and seven (7) negative gastric tissue samples as control. Formalin fixed and paraffin embedded gastric biopsies tissue blocks randomly selected from the archive of the Department of Histopathology, Federal Teaching Hospital, Gombe, were retrieved. The patients were previously investigated for dyspepsia and their *H. pylori* status had been determined by urease test and serology in addition to histology. Standard histological sections were cut at 3µm using a rotary microtome. The sections were picked on clean glass slides and stained by the following histological staining methods for the detection of *H. pylori* organisms on tissue sections. The slides were interpreted by two independent pathologists (observers) who were unaware of the results before evaluation.

1. Haematoxylin and Eosin (H&E) staining method.
2. Giemsa staining method.
3. Modified Giemsa (Sheehan’s May-Grunwald Giemsa) staining method.
4. Modified McMullen staining method.
5. Anti-*H pylori* immunohistochemical (IHC) staining method.

Emphasis was placed on the issues that affect the techniques; time, availability, preparation of solutions, staining variations, ease of performance of the techniques, and the reproducibility of the methodologies described.

2.3 Haematoxylin and Eosin Staining Method

Sections were dewaxed in two changes of xylene for 10 minutes in each, and then hydrated through descending grades of alcohol to water. Sections were then stained in Mayer’s haematoxylin for 10 minutes and then were washed in water. Sections were then briefly differentiated in 1% acid alcohol and washed in

water. Sections were then blued in Scott's tap water and then washed in water. Sections were then counterstained with 1% Eosin Y solution briefly and washed in water. Sections were then dehydrated through ascending grades of alcohol, cleared in xylene and mounted in DPX [21].

2.4 Giemsa Staining Method

"Sections were dewaxed in two changes of xylene and hydrated through descending grades of alcohol to distilled water. Then rinsed in pH 6.8 buffered distilled water. Sections were then stained in working Giemsa solution for 25 minutes and then rinsed in distilled water. They were then differentiated in 0.5% aqueous acetic acid until sections became pinkish. Sections were then washed in tap water and blotted until almost dry. Sections were then dehydrated rapidly through ascending grades of alcohol, cleared in xylene and mounted in DPX" [22].

2.5 Modified Giemsa (Sheehan's May-Grunwald Giemsa) Method

Sections were deparaffinized in two changes of xylene, brought to alcohol and treated with methanol, three changes. Slides were then placed on staining rack, covered with Wright stain for 5 minutes. Equal amount of distilled water was added to the stain until a metallic sheen appears and allowed to react for 5 minutes. The slides were directly transferred into working Giemsa solution for 45 minutes at room temperature. Sections were then differentiated, dehydrated and cleared by 3 dips in 1% acetic acid, 2 dips in distilled water, 3 dips in 95% alcohol, 3 dips in absolute alcohol and finally 3 dips in xylene respectively. The sections were then mounted in DPX [23].

2.6 Modified McMullen Staining Method

"Sections were dewaxed in two changes of xylene and hydrated through descending grades of alcohol to distilled water. They were then stained in working carbol-fuchsin solution for 2 minutes and washed well in tap water. Sections were then stained in malachite green for 15-20 seconds and washed thoroughly in distilled water until sections turned blue green to the naked eye. Sections were then blotted dry and drying completed in air, cleared in xylene and mounted in DPX" [19].

2.7 Anti-*H. Pylori* Immunohistochemical (IHC) staining Method

"Sections were deparaffinized in two changes of xylene and hydrated through descending grades of alcohol to distilled water. Antigen retrieval was performed with the sections placed in the target retrieval solution (0.1M Citrate buffer, pH 6.0) for 60 minutes at temperature of $95^{O}C$ using water bath method followed by cooling at room temperature for 20 minutes. Sections were then rinsed with phosphate buffer (PBS). Sections were treated with peroxidase blocking solution for 10 minutes, washed in the buffer solution and treated with protein blocking solution at room temperature for 10 minutes" [24]. "Sections were then incubated in humidified chamber for 60 minutes with the primary antibody; Rabbit polyclonal anti-*H. pylori* antibody (1:100 dilution, from Abcam Plc, Cambridge UK). After washing thoroughly with PBS, the sections were treated with the biotinylated secondary antibody for 15 minutes and then washed in PBS solution. The sections were then treated with streptavidin-HRP conjugate for 10 minutes and washed in two changes of PBS solution. A drop of diamino benzidene (DAB) + Substrate mixture (1 drop of DAB to 1ml of DAB substrate mixture) was then spread over the section and incubated for seven minutes in humidified chamber for colour development, it was then rinsed in PBS solution. The sections were then counter-stained with Harris haematoxylin for 5-10 seconds before rinsing with running water for three minutes and dehydrated in ascending grades of alcohol, then mounted in DPX" [24].

After staining, the slides were independently evaluated by two independent observers for the presence of *H. pylori*. The sensitivity and specificity for the different staining methods were evaluated. Each staining method was evaluated by two independent *blinded* observers (pathologists) without the knowledge of the results of the other set of staining methods. The data generated was analyzed using Statistical Package for Social Sciences software (SPSS ver. 23). The agreement between the observers regarding the interpretation of *H. pylori* staining was calculated for each staining method using Cohen's kappa (k) statistics based on the guidelines from Altman [25], and adapted from Landis and Koch [26].

3. RESULTS

The mean age of patients was 53.1 ± 10.5 years, with 43 males and 29 females. Among the 72 cases studied, 65 cases were positive for at least one of the staining methods, while all the 7 control cases were negative for all the staining methods. Out of the sixty five (65) positive cases;

Table 1. Number of positive cases with different staining methods for *H. pylori*

	H&E	Giemsa	Modified Giemsa	Modified McMullen	Immunostaining
Positive cases	59	60	63	58	65
%	90.8	92.3	96.9	89.2	100

Table 2. Comparison of the statistical values, cost and staining times for the different staining methods

	Interobserver agreement(%)	k	Interpretation	Cost	Staining time	Sensitivity %	Specificity %
H&E	80.7	0.421	Moderate	Cheap	40 mins	90.8	77.1
Giemsa	95.2	0.533	Moderate	Cheap	35 mins	92.3	81.4
Modified Giemsa	96.9	0.777	Good	Cheap	01 hr	96.9	85.7
McMullen	52.1	0.267	Fair	Cheap	30 mins	89.2	52.9
IHC	98.4	0.859	Excellent	Expensive	03 hrs	100	100

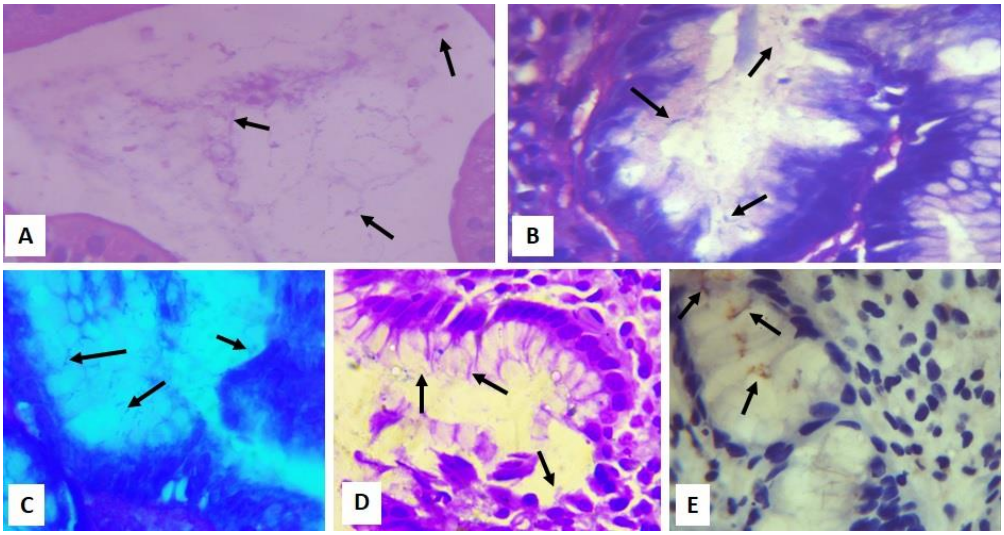

Fig. 1. Arrows indicate *Helicobacter pylori* microorganisms present (x1000) in H&E stain (A), Giemsa stain (B), Modified Giemsa stain (C), McMullen stain (D) and Immunohistochemistry stain (E)

59 (90.8%) cases were positive with Haematoxylin and Eosin (H&E) staining method, 60 (92.3%) cases were positive with Giemsa, 63 (96.9%) cases were positive with modified Giemsa, 58 (89.2%) cases were positive with modified McMullen and 65 (100%) cases were positive with Immunohistochemical staining method (Table 1). Interobserver agreement was excellent, good and moderate for immunohistochemical staining, modified Giemsa and Giemsa staining methods respectively, while for H&E staining and McMullen staining methods interobserver agreement was moderate and fair respectively. The sensitivity and specificity for the staining methods were as follows: Immunohistochemistry, 100% and 100%; modified Giemsa, 96.9% and 85.7%; Giemsa staining, 92.3% and 81.4%; H&E, 90% and 77.1%; and modified McMullen staining, 89.2% and 52.9% (Table 2).

The Haematoxylin and Eosin staining method (Fig. 1A) is very simple, straight forward, inexpensive, and takes about 40 minutes to perform. The staining method is easily reproducible and has the advantage of demonstrating the general tissue structures. The major disadvantage is that it takes an experienced observer to detect the organisms.

The Giemsa staining method (Fig. 1B) is very simple, straight forward, inexpensive, and takes about thirty-five minutes to perform and rarely

requires repeat staining (none was required in the study). The staining method is easily reproducible.

The modified Giemsa staining method (Fig. 1C) is also very straightforward, inexpensive, and takes about an hour to perform and rarely requires repeat staining. This staining method is easily reproducible. The major disadvantage is that contrast between the organisms and the tissue is not very good.

The modified McMullen's method (Fig. 1D) is also simple to perform and inexpensive. It takes about 30 minutes of technical time and gives a very good contrast when performed well, making identification of the organism easy. Concentration of the stains and the timings of staining are crucial, which cause variation in staining, both within the batch and from batch to batch. Hence, several repeats were required, which increased the cost and duration of producing a satisfactory slide for reporting.

The immunohistochemistry (IHC) staining method (Fig. 1E) gives an excellent result and the method is very reliable, hence regarded as the gold-standard technique. No variations were noted and therefore no repeats were required. The technical time required is approximately 3 hours, and the technique apart from been time-consuming, is fairly expensive and not applicable in resource constrained laboratories.

4. DISCUSSION

H. pylori is the most common gastric pathogen that colonizes the mucous lining of the stomach, and the only bacterium known to colonize the harsh acidic condition of the human stomach [3,27]. The bacterium produces urease enzyme which converts urea into bicarbonate and ammonia, thereby elevating the stomach pH [28]. There has been an increased interest in the correct identification of *H. pylori* in the tissue sections of gastric biopsies following studies by different researchers revealing the important role played by *H. pylori* in the pathogenesis of gastric carcinomas and lymphomas [29]. Currently, there are several tests available for the diagnosis of *H. pylori* infection such as; rapid urease test, breath test, cultures, serological tests and histopathological methods [13]. Some of these tests such as culture are very tedious and time-consuming process, therefore, have been abandoned in most of diagnostic laboratories [14]. Other methods such as Urease test and Urease breath test are relatively expensive and give false negative results especially when the bacterial load is low. Serological test has the disadvantage of preserving the level of antibody titers even after the eradication of the bacteria by antibacterial therapy. PCR methods have also been used for the detection of bacilli, but the method is expensive and requires technical support [30]. "Histological demonstration of *H. pylori* remains the most reliable method for demonstration of the bacteria in paraffin tissue sections. Although in most cases, *H. pylori* can be identified in a good H&E stained sections, the sensitivity of the method is low, especially when there are scanty bacteria in the sections. Therefore, most laboratories use an additional special staining method in the identification of the organism" [14].

In this study, 59 cases were found to be positive for *H. pylori* using H&E staining method, with sensitivity and specificity of 90.8% and 77.1% respectively, with an inter-observer agreement value of 80.7% and kappa (*k*) value of 0.421. This finding is in agreement with a finding in a similar study, where sensitivity and specificity of 97% and 80% respectively, and *k* value of 0.477 using H&E in demonstrating *H. pylori* in tissue sections were reported [30]. A sensitivity and specificity of 98.5% and 100% in the demonstration of *H. pylori* using H&E staining method on tissue sections was also reported in a related study [31].

Giemsa staining method as one of the commonest staining method routinely use in our laboratory for the demonstration of *H. pylori* performed moderately well in this study, despite the poor contrast between tissues and the microorganisms which makes it difficult to recognized the pathogens. In this study, sensitivity and specificity of the staining method were 92.3% and 81.4% respectively, while the inter-observer agreement was 95.2% and *k* value of 0.533. This is in line with the finding in a similar study which came up with sensitivity and specificity values of 98.5% and 97.8% respectively [18]. In another study it was concluded that Giemsa staining for *H. pylori* is preferred to other methods because of its good sensitivity, excellent specificity and low cost [32]. Modified Giemsa staining method gave comparatively high sensitivity and specificity values (96.9% and 85.7% respectively) second to immunohistochemistry which is the gold standard. It also has high inter-observer agreement of 96.9% and kappa (*k*) value of

0.777. This finding is in agreement with that of a similar study which reported sensitivity and specificity of 97% and 90% respectively and *k* value of 0.752 [30]. It is also in agreement with the findings of a study that reported a *k* value of 0.733 for modified Giemsa technique [14]. It differs with the findings in a study where a sensitivity and specificity of 77.6% and 57.4% respectively were reported [33]. The modified Giemsa stain is very straightforward, inexpensive, and takes few minutes to and the method is easily reproducible. The major disadvantage is that there is little contrast between the organisms and the tissue.

The modified McMullen's method has also been reported as simple to carryout and inexpensive, taking only few minutes and gives a very good contrast between the organisms and tissue, making identification of the organism easy [14]. However, in this study, there was variation in staining, both within the batch and from batch to batch. Hence, several repeats were made, which increased the time and cost of producing a satisfactory slide for reporting. For this reason, the interobserver agreement and *k* value were low; 52.1% and 0.267 respectively.

Immunohistochemistry (IHC) has been reported as a reliable technique for detection of *H. pylori* [34]. "The coccoid forms of the organisms, which may not be detected with other staining methods, were seen easily on immunohistochemical stained sections. Also, *H. pylori* antigen in the lamina propria and beneath the surface epithelial is detectable by IHC, while it can be hardly detectable by histochemical stains" [34]. For this reason, the method is regarded as the "gold standard" for the detection of *H. pylori* in tissue sections. In this study, both the sensitivity and specificity of the method were 100% with an inter-observer agreement of 98.4% and *k* value of 0.859 which shows an excellent inter-observer agreement. Our finding is in line with the findings of similar studies [14,30,34]). "The method is fairly expensive and a positive control slide needs to be used with every test. Therefore, not feasible in resource constrained laboratories. However, in cases of chronic active gastritis with negative histological result, IHC stain should be performed for the detection of *H. pylori"* [35].

5. CONCLUSIONS AND RECOMMENDATIONS

According to the results obtained in our study, immunohistochemical stain has high diagnostic accuracy than all the other staining techniques used in the demonstration of *H. pylori* in gastric biopsies. It has the highest sensitivity, specificity and inter-observer value compared to other staining techniques.

Although, immunohistochemistry staining method had the highest sensitivity and specificity values with high inter-observer agreement. However, due to its high cost and the hands-on time required, we therefore recommend that modified Giemsa or conventional Giemsa stains should be method of choice for the detection of *H. pylori* in our laboratories due to its low cost, availability, short time required for staining and very high sensitivity and specificity combined with a high inter-observer agreement. Furthermore, we recommend that researchers should look at the possibility of improving the poor contrast observed with Giemsa stains, as this will ultimately increase the sensitivity and specificity of the staining method.

6. LIMITATIONS OF THE STUDY

There are two major limitations in this study that could be addressed in future research; the study design limitations and the possible source of result bias. As with majority of studies, the design of the current study is subject to limitations of sample selection and sample size. A larger sample size ensures that the sample is a representative of a population and that the statistical result can be generalized to a larger population. The result bias might be due to the quality of the reagents used or flaws and human errors in the techniques or interpretations.

CONSENT

As per international standard or university standard, patient(s) written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

This study was approved by the Ethics and Research Committee of Federal Teaching Hospital, Gombe, with Reference Number: NHREC/10/09/2019.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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