Evaluation of Staining Methods and Rapid Urease Test for the Detection of *Helicobacter pylori* in Gastric Biopsy Specimen among Patients with Dyspeptic Symptoms at a Tertiary Care Centre in Jammu

P. Sharma[1], R. Tomar[2*] and S. Sudan[1]

[1]Government Medical College, Jammu, India
[2]SMC, Ghaziabad, India

*Corresponding author

Abstract

In a developing country like India, *Helicobacter pylori* infection is a prevalent entity. It is an important human pathogen involved in the causation of pathogenesis of a number of diseases including Peptic Ulcer Disease (PUD) and Gastric Carcinoma. Diagnosis of *Helicobacter pylori* infection may be established by a number of Invasive and Non Invasive tests. In the present study we have evaluated two staining methods viz. Gram staining, Loeffler's Methylene Blue Staining and Rapid Urease Test (RUT) for the detection of *Helicobacter pylori* in biopsy samples. *Method:* A total of 30 patients with gastric disorders (chronic gastritis and peptic ulcer disease) underwent upper gastrointestinal (GI) endoscopy with biopsy. *Helicobacter pylori* infection in gastric biopsies was identified after histopathological examination by microscopy and rapid urease test. *H. pylori* was detected in 14 (46.6%) out of 30 samples by histopathological method. 9 biopsy samples (30%) were positive by Gram Staining and 13 samples (43.3%) were positive by Loeffler's Methylene Blue (LMB) staining. Of the total 30 samples, 11 samples (36.6%) were positive for Rapid Urease Test (RUT). 8 samples of the 9 samples positive in Gram Staining were also positive by RUT and all the 13 samples positive by LMB were also positive by RUT. *Conclusion*: LMB Staining method to stain and detect *H. pylori* is a better method than Gram Staining.

Introduction

Helicobacter pylori is a Spiral Shaped/Curved Gram Negative Rod (Seagull-Shaped Morphology), microaerophilic bacterium measuring approximately 2 to 4 μm in length and 0.5 to 1 μm in width that is a colonizer of human gastric mucosa of nearly one-half of the world's population and the infection may last for decades. *Helicobacter pylori* prevalence has shown discrepancy among different population as well as in different countries, transmission of the infection known to be influenced by the socioeconomic conditions. Approximately 90% prevalence have been reported in developing nations in comparison to that of 50% in developed countries[1,2]. *H. pylori* is an important human pathogen involved in the causation of pathogenesis of a number of diseases such as Acute gastritis, Peptic ulcer disease (PUD), Chronic atrophic gastritis, Autoimmune

gastritis, Adenocarcinoma of stomach, Non-Hodgkin's gastric lymphoma, Idiopathic thrombocytopenic purpura (ITP), Iron deficiency anemia and Vitamin B12 Deficiency (Pernicious Anemia). WHO (World Health Organization) has listed *Helicobacter pylori* in the list of known carcinogens[3]. Diagnosis of *Helicobacter pylori* infection may be established by a variety of Invasive and Non Invasive tests. These tests vary in their sensitivity and specificity, and the choice of test will depend on the situation, for example, whether the test is to detect infection or the success of eradication treatment. Non - Invasive methods include Urea Breath test, Stool Antigen Assay and Antibody Detection by ELISA. Invasive methods includes Histopathology with Warthin Starry Silver Staining and Microbiological Methods like Gram staining, Culture Media and Biochemical Testing[4]. Culture is probably the most difficult approach for the diagnosis of *Helicobacter pylori*. The advantages are that it is highly specific and the antibiotic sensitivity can be detected. The presence of IgG antibodies to *H.pylori* can be detected by immunoassays. Serology is sensitive for primary diagnosis but is not useful in assessing post treatment *H.pylori* status[5]. The urea breath test relies on the urease activity of *H.pylori* to detect the presence of infection. Sensitivity is excellent because the whole stomach is sampled. Unlike serology it is useful for determining the success of the eradication therapy. Urea Breath Test (UBT) sometimes show false-negative results frequently (up to 40%) due to decreased bacterial loads in the stomach mucosa, and include the following clinical conditions: Use of Proton pump inhibitors (PPI) medication; Antibiotic use; Bleeding peptic ulcer; Atrophic gastritis (with or without intestinal metaplasia); Gastric carcinoma; MALT lymphoma, and Partial gastrectomy. Since the late 1990's, it has been well established that UBT also gives false positive results in cases where urease-producing bacterial species are colonizing an achlorhydric stomach due to atrophic gastritis or a long term use of proton pump inhibitors (PPIs). With the advent of Polymerase chain reaction (PCR), many possibilities have emerged for diagnosing *H. pylori* infection. PCR allows identification of the organism in samples with few bacteria and it has been successfully used to detect *H. pylori* CagA and VacA virulence genes in gastric biopsy samples[6]. The potential advantage of PCR includes high specificity, quick results and the ability to identify different strains of bacteria for pathogenic and epidemiologic studies. However the limitations of PCR methods include the propensity for false-positive results in part due to the detection of cDNA from non-*H. pylori* organisms. False-negative results may also occur due to a low number of organisms

or to the presence of inhibitors in the sample. Another rapid test is smear evaluation, smears being stained by Giemsa or Gram stain.

In the present study we have evaluated Loeffler's methylene blue stain and Gram Staining to stain *H.pylori* per se and Rapid urease test for detection of *H. pylori* in endoscopy guided biopsy samples.

Materials and Methods

This was a prospective study conducted in the Department of Microbiology, Government Medical College (GMC), Jammu, India. The study was performed on samples received from Surgery Department, Government Medical College (GMC), Jammu, India.

Inclusion Criteria: Patients of age group 16-65 years and both sexes were included in the study during a period from December 2018 to April 2019.

Exclusion Criteria: Patients with age less than 16 years, Patients who had a history of Proton pump inhibitor (PPI), H2 receptor antagonist, Warfarin, Fluoxetin, or Steroid use within 1 week before endoscopy or, antibiotic use within 4 weeks before endoscopy as well as those with severe medical illness, active Gastrointestinal (GI) bleeding, and history of gastric surgery and *H. pylori* eradication, lesions suggestive of malignancy on endoscopy were excluded from the study.

Sample Collection: Before taking the sample, patient was informed about the procedure and the consent for the same was taken. A standard protocol was followed for obtaining the sample by endoscopic guided biopsy. Approximately 5 mm of biopsy sample from the infected site or normal gastric antral mucosa was collected.

Sample Transport: The tissue biopsy sample was cut with a sterile scalpel blade in a sterile Petri dish into 3 pieces. 1 piece was sent to the Pathology Department for HPE and 2 pieces were sent to Microbiology Department within 1 hour of collection in 2 seperate 12 x 100 mm clean and sterile test tubes, one test tube filled with 1.5 ml of Sterile Normal Saline and the other test tube filled with 1.5 ml of a Christensen's Urea Broth solution (HiMedia) prepared freshly and checked with a known urease positive control *Proteus mirabilis* strain and a known negative control strain of *Escherichia coli* strain. Both the test tubes were well marked with the media it contains, Patient's Name, Age, Sex and the Sample Number.

Methods

HPE: Biopsy sample were sent to Pathology Department where it was fixed in Formalin (10%) processed overnight and embedded in paraffin wax. The sections of few micron thickness were stained with Haematoxylin and Eosin (H and E) Stain so that morphological changes (if any) can be appreciated. If spirally coiled shaped bacteria were seen in oil emulsion, they were stained with Giemsa Stain and the findings were noted.

Gram Staining: The biopsy specimen was picked from the tube containing normal saline and a crushed smear was made over a couple of clean sterile microscopic glass slides in the centre making a smear of approximately 1-1.5 cm in size and oval in shape. Both the slides (one for Gram Staining and the other for LMB Staining) were well labelled. After air-drying, in one of the slides, the smear was fixed with uppermost flame of the Bunsen burner and a direct modified Grams staining was performed after heat fixing the smear. The method used was modified Gram-stain, where the counter stain safranin was replaced with carbol fuchsin. Carbol fuchsin used was freshly prepared on the day of use. Stained slide was washed with water and air dried. The smears were examined under oil immersion (100x) objective examining at least 50 fields for the presence of spiral or comma shaped bacilli. Presence of such bacilli was assumed to be positive for *Helicobacter pylori*.

Loeffler's Methylene Blue Staining: The other slide with the crushed smear was stained with LMB stain for 30 seconds and examined for the presence of deep blue colored bacilli.

Rapid Urease Test: The urease broth samples were incubated at a temperature of 37°C along with uninoculated urea broth as control for 24 hours observing the broth after every one hour till the close of the working hours and the following morning i.e. approximately after 18 hours. Broth showing a change of color from orange yellow to pink indicated alkalinisation and urea hydrolysis and was considered as positive for urease test.

Results and Discussions

Of the 30 patients with Dyspeptic symptoms who underwent upper gastrointestinal (GI) endoscopy with biopsy, 23 (77%) were Male and 7 (23%) were Female (Figure 1). A maximum of 18 (60%) patients with dyspetic symptoms were in the age group of 31-40 years followed by 6 (20%) patients in the age group of 21-30 years (Table 1). 2 (6.6%) out of 30 patients who underwent the procedure were endoscopically diagnosed as Gastric Ulcer, 7 (23.3%) as Gastroduodenitis and a maximum of 21 patients (70%) as Duodenal Ulcer (Figure 2). *H. pylori* was detected in 14 (46.6%) out of 30 samples by histopathological method (Figure 3). 9 biopsy samples (30%) were positive by Gram Staining and 13 samples (43.3%) were positive by Loeffler's Methylene Blue (LMB) staining. Of the total 30 samples, 11 samples (36.6%) were positive for Rapid Urease Test (RUT). 8 samples of the 9 samples positive in Gram Staining were also positive by RUT and all the 13 samples positive by LMB were also positive by RUT (Table 2).

Table.1 Age Wise Distribution of Patients with Dyspeptic Symptoms of Chronic Gastritis and Peptic Ulcer Disease

S.No.	Age Group (in years)	No. of Cases (n=30)
1	16-20 years	1
2	21-30 years	6
3	**31-40 years**	**18**
4	41-50 years	2
5	51-65 years	3

Table.2 Results of various test methods (Gram Staining, LMB Staining and RUT)

S.No.	Positive Finding	Total (n = 30)	% Positivity
1	Gram Staining	09	30 %
2	LMB Staining	13	43.3 %
3	RUT	11	36.6 %
4	Gram Staining + RUT	08	26.6 %
5	LMB Staining + RUT	13	43.3 %

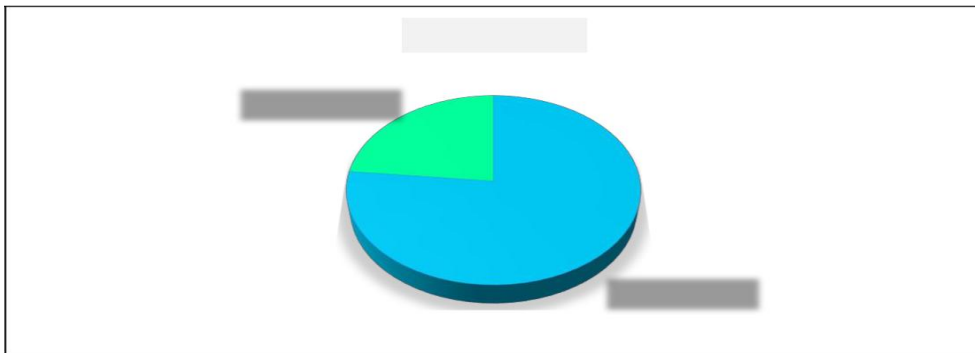**Figure.1** Gender wise distribution of patients with dyspeptic symptoms of chronic gastritis and peptic ulcer disease (n=30)

Figure.2 Differentiation of patients with dyspeptic symptoms based on endoscopic diagnosis (n=30)
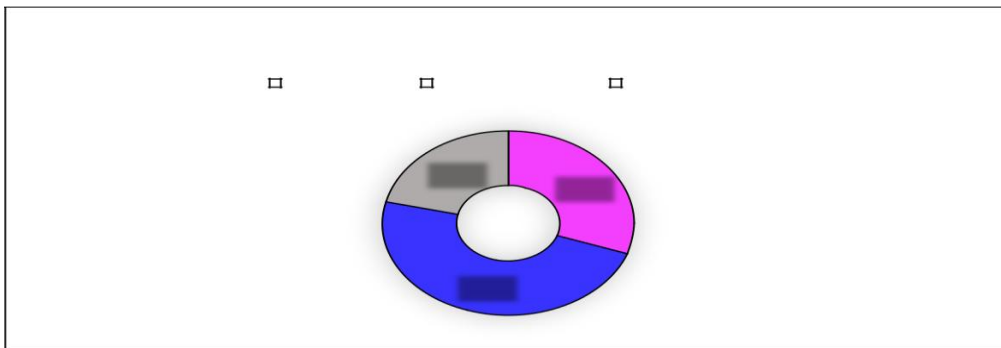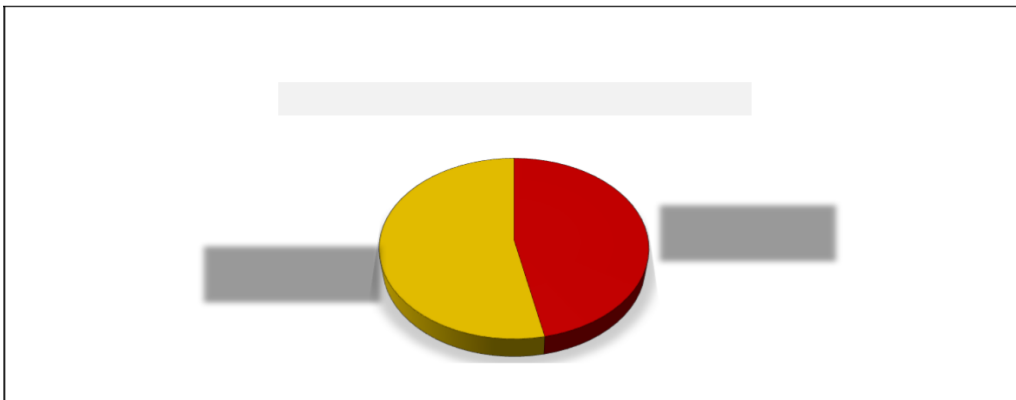

Figure.3 Result of histopathological findings

Diagnosis of *Helicobacter pylori* infection is very essential as it leads to life threatening complications ranging from chronic gastritis and PUD to malignancy. In the staining methods, each stain has its own advantages and disadvantages in terms of cost-effectiveness, time-consumption, technical level, accuracy and availability. Although *H. pylori* is readily visualized on routine Hematoxylin and Eosin (H & E) Staining, it is better identified by special stains. Different stains have been proposed such as Warthin Starry, Giemsa, Steiner Silver, Genta, Toluidine blue, Carbol fuchsin and Immunohistochemical stain. Some stains can demonstrate both the organism as well as highlighting the metaplastic morphology. Warthin-Starry stain, for example, can detect *H. pylori* easily but has a higher cost, is difficult to prepare and is time-consuming. Giemsa stain is easier to perform but has a higher cost. Immunohistochemical studies are reliable but need sophisticated preparation and are costly. Similarly molecular method like Polymerase Chain Reaction (PCR) has the disadvantage of being expensive and high false positives due to risk of contamination of the sample. As yet reported elsewhere, none of the diagnostic assay is stand alone and universal for disease diagnosis because of several extrinsic and intrinsic limitations. In resource poor nation like India with lack of adequate and modern diagnostic facilities, it is more practical to use stains with lower cost, which are easier to perform and readily available. Since LMB stain has all those features and is a simple stain for microbiologic studies, we used it for staining *H.pylori* from biopsied samples. Rapid urease test (RUT) on the other hand has the advantage of being simple, inexpensive and rapid.

Of the 30 patients with Dyspeptic symptoms of Chronic Gastritis and Peptic Ulcer Disease (PUD) who underwent upper gastrointestinal (GI) endoscopy with biopsy, 23 (77%) were Male and 7 (23%) were Female which shows a male to female ratio of 3.2:1, male clearly outnumbering female. Similarly, a male to female ratio of 1.07:1 was observed in a study by Mujawar *et al.,*[7]. *H. pylori* in our study was detected in 14 (46.6%) out of 30 samples by histopathological method. Positivity ranging from ~ (45 to 67%) was observed in various studies by different authors[8,9,10,11,12,13]. The disadvantage of this technique is the need for invasive endoscopy to obtain the tissue sample, however, histopathological examination allows for the definitive diagnosis of *H. pylori* infection. Thus HPE can be considered as gold standard for the demonstration of *H. pylori* in the biopsy samples. Crushed smears stained with Gram staining showed a percentage positivity of 30%. On LMB staining, *H. pylori* took up deep blue color and mucus took up faint blue colour and stood highly appreciable against the background. Smears stained with LMB showed higher percentage positivity of 43.3% as compared to Gram Staining which showed a percentage positivity of 30%. In a study by Vijaya *et al.,*[14] LMB was concluded to be sufficient for the detection of *Helicobacter pylori* in an ordinary set up. Other similar studies by Misra *et al.,*[15] and Ahluwalla *et al.,*[16] also showed LMB Stain to be a good method with promising results.

Conclusion

Although HPE being considered as the gold standard test, LMB staining is technically simple, rapid (few minutes of staining time), reliable, inexpensive and showed a promising result in our study, hence found to be excellent stain for the detection of *H. pylori* as it can pick up a very light load of infection, compared to technically demanding, expensive and slow histopathology. The present study infers Loeffler's Methylene Blue (LMB) staining to be a better staining method for the detection of *Helicobacter pylori* in a resource poor setting. We also advocate for the combination of both LMB and RUT to increase the strength of diagnostic accuracy.

References

1. H. Cheng, F. Hu, L. Zhang *et al.,* "Prevalence of Helicobacter pylori infection and identification of risk factors in rural and urban Beijing, China," Helicobacter, vol. 14, no. 2, pp. 128–133, 2009.
2. B. Salih, "*Helicobacter pylori* infection in developing countries: the burden for how long?," Saudi Journal of Gastroenterology, vol. 15, no. 3, pp. 201–207, 2009.
3. Prasad KN, Singhal S, Ayyagari A, Sharma S, Dhole TN. Association of Helicobacter pylori in patients of Non ulcer dyspepsia. Indian J Microbiol. 1991; 31: 415-8.
4. Barthal JS, Everett ED. Diagnosis of Campylobacter pylori infections: The gold standard and the alteration. Rev Inf Dis. 1990; 12(1): 107-14.
5. Lerang, F., *et al.,* 1998. Accuracy of IgG serology and other tests in confirming Helicobacter pylori eradication. Scand. J. Gastroenterol. 33: 710-5.
6. Rudi, J., *et al.,* 1999. Direct determination of *H.pylori VacA* genotypes and *CagA* gene in

gastric biopsies and relation to gastrointestinal disease. Am. J. Gastroentrol. 94: 1525-1531.

7. Mujawar P, Nikumbh DB, Suryawanshi KH, Pagare PS, Surana A. *Helicobacter pylori* Associated Gastritis in Northern Maharashtra, India: A Histopathological Study of Gastric Mucosal Biopsies. Journal of Clinical and Diagnostic Research. 2015 Jun, Vol-9(6): EC04-EC06.

8. Yakoob MY, Hussainy AS. Chronic gastritis and Helicobacter pylori: A histopathological study of gastric mucosal biopsies. Journal of the college of Physicians and Surgeons Pakistan. 2010; 20(11): 773-75.

9. Dandin AS, Pawale J, Athanikar S. Helicobacter pylori associated gastritis. J Clin Diagn Res. 2012; 6(2): 211-14.

10. Satoskar A, Vora IM. Incidence of Helicobacter pylori associated gastritis in the urban population from India. Trop Geogr Med. 1994; 46: 167-68.

11. Maitra TN, Ghosh S. Gatritis and *Helicobacter* (*Camylobacter*) *pylori*- Merely one more piece in the jigsaw puzzle or the final answer? Indian J Pathol Microbiol. 1991; 34(1): 67-79.

12. Akanda MR, Rahman AN. Comparative study of different methods for detection of Helicobacter pylori in gastric biopsies. Dinajpur Med Col J. 2011; 4(1): 1-6.

13. Abu-Ahmad NM, Odeh A, Sallal AKJ. Prevalence of Helicobacter pylori gastritis at the north of Jordan. Jordan Jr of Biological Sciences. 2011; 4(2): 71-76.

14. Vijaya D, Chandrashekar N, Nagarantnamma T, Shivarudrappa AS. Simple stain for Helicobacter pylori. Journal of Clinical and Diagnostic Research. 2012 May (Suppl-2), Vol-6(4): 664-666.

15. Misra V, Misra SP, DWivedi M, Gupta SC. The Loeffler's methylene blue stain. An inexpensive & rapid method of detection of Helicobacter pylori infection. J Gastroenterol Hepatol 1994; 9: 512-13.

16. Ahluwalla C, Jain M, Mehta G, Kumar N. Comparison of endoscopic brush cytology with biopsy for detection of Helicobacter pylori in patients with gastroduodenal diseases. Indian J Pathol Microbiol 2001; 44(3): 283-88.