

Clinicopathological Study of Helicobacter Pylori Infection in Patients with Acid Peptic Disorder Attending Tripura Medical College and Dr B.R.A.M Teaching Hospital

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Abstract:

Introduction: Acid peptic disorder (APD) is a global issue caused by imbalances in gastric secretions, leading to conditions like gastritis, ulcers, and duodenal ulcers. *H. pylori*, a Gram-negative bacterium, are a common human pathogen, colonizing 60% of the world's population. It causes chronic inflammation in human gastric mucosa, leading to progressive damage to the stomach lining and various stomach diseases without treatment. This study aims to determine the prevalence of *H. pylori* infection in adult patients with APD symptoms, compare the sensitivity and specificity of Rapid Urease Test and Histopathological Analysis, and study its staining patterns.

Material& Methods: Adult patients with acid reflux disease participated in the study, which was carried out at Dr. B.R.A.M Teaching Hospital and Tripura Medical College. There were 110 samples in all, and a number of instruments were employed, such as an endoscope, rotary microtome, automatic tissue processor, microscope, and HPE staining. The urease enzyme was found using the Rapid Urease Test (RUT), which was utilised to diagnose *H. pylori*. Patients who met the inclusion criteria had their written, informed consent obtained before any data were collected.

Results: There were 110 patients in the research. Of them, 98 patients (89.1%) had an *H. pylori* infection. These patients data Analysed Based on Age, Gender, Location, Occupation and Socioeconomic status.

Discussion: The World Health Organisation classifies *H. pylori* infection as a "definite biological carcinogen" and notes that it affects half of the world's population and is a primary cause of gastrointestinal illnesses. According to a research, 86.4% of men and 92.2% of women who were mostly from rural areas and aged 31 to 40 tested positive. Abdominal pain and a burning feeling are common symptoms, and the most common endoscopic diagnosis are Gastritis (77.3%) followed by Gastric ulcer (20%) and Gastric carcinoma (2.7%).

Conclusion: *H. pylori* infection, affecting 75% of the global population, increases gastritis risk and gastric carcinoma risk. Early diagnosis is crucial, and histopathology, including Warthin Starry silver stain, can improve diagnostic yield in gastric biopsies, enhancing the detection process.

Keywords: APD, *H. pylori*, alcohol, NSAIDS.

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Introduction

Acid peptic disorder (APD) is a global issue caused by an imbalance in gastric secretions, resulting in conditions like gastritis, gastric ulcers, and duodenal ulcers. Gastritis, inflammation of gastric mucosa, can be caused by alcohol, NSAIDS, or other factors.[1] Rathbone and Heatley (1992) explored the historical links between bacteria and acid reflux disease.[2] Since its presentation to the medical world by Warren and Marshall in 1983, *H.*

pylori has been the subject of fundamental biochemical and clinical controversy.[3] In 1997, *H. pylori* was identified as a common human pathogen, colonizing 60% of the world's population. Marshall and Warren won the Nobel Prize for Medicine in 2005 for their discovery of *H. pylori* in gastric biopsy samples.[4] *Helicobacter pylori*, a Gram-negative bacterium, can cause chronic inflammation in human gastric mucosa,

leading to progressive damage to stomach lining and various stomach diseases without treatment.[5]

H. pylori infection is a prevalent global bacterial infection, affecting 75% of populations, especially in developing countries. Rates vary, with higher prevalence in developing countries. Most colonized individuals develop chronic inflammation. Long-term *H. pylori* carriage increases the risk of site-specific diseases, peptic ulcers, stomach cancer, and mucosa-associated lymphoid tissue lymphoma.[6] *H. pylori* infects half of the world's population, with India's highest burden in developing countries. Infections start as young as 10 years due to overcrowding and poor hygiene.[4]

This study analysed *H. pylori* infection prevalence in Acid Peptic Disorder patients at Tripura Medical College and Dr B R A M Teaching Hospital, comparing Rapid Urease Test sensitivity and specificity to gold standard histopathological analysis.

Aim and Objectives

To find out association of *Helicobacter pylori* with Acid Peptic Disorder among adult patients. The study aims to determine the prevalence of *Helicobacter pylori* in gastric mucosa in adult patients with Acid Peptic Disorder symptoms, compare the sensitivity and specificity of Rapid Urease Test and Histopathological Analysis, and study its staining patterns.

Materials and Methods

Study Design: Hospital based Cross sectional study.

Study Site: Department of Pathology in collaboration with Department of Medicine, Tripura Medical College and Dr B.R.A.M Teaching Hospital.

Study Duration: March 2021 to August 2022 (1year 6 months).

Study Population: All adult patients with history suggestive of Acid Peptic Disorder attending Out Patient Department of Tripura Medical College and Dr B.R.A.M Teaching Hospital fulfilling the inclusion criteria were included in the study.

Sample Size: Using a "single population proportion sample size calculation" technique, the sample size was determined taking into account the following assumptions: P [prevalence or proportion] = 41%, d = 10% margin of error with a 95% confidence interval. To optimise the study's power, the sample size was doubled and came out to be 93. Even though 103 was the estimated final sample size, 10% non-response rate was taken into account.

Inclusion Criteria: Adult patients with history suggestive of Acid Peptic Disorder attending Out Patient Department of Tripura Medical College and Dr B.R.A.M Teaching Hospital.

Exclusion Criteria:

- Patients on Proton pump inhibitor for last 2 weeks.
- Patients on H2 Blocker for last 1 week.
- Patients on Antibiotics for last 2 weeks.
- Patients not fit or have other contraindication for Upper Gastrointestinal Endoscopy.
- Patients not willing to participate in the study.
- Sampling Method: Until the sample size is reached, use a universal sampling method.

Study Tools:

1. Microscope
2. Automatic tissue processor
3. Rotary Microtome
4. Endoscope: Olympus CV70 (Processor)/Olympus GIFTTYPE V70(Scope) or Olympus CV170(Processor) / Olympus gift type Q150 (Scope)
5. Olympus standard fenestrated forcep with needle
6. Rapid Urease Test (RUT) using H-P Test Kit manufactured by Lenus Medicare & Research Private Limited (Kolkata, India).
7. HPE Stains (Hematoxylin and Eosin, Warthin Starry Silver Stain)
8. 10% Formaldehyde solution

Specimen collection, transport and storage:

Patients were informed about the procedure and consented to Upper Gastrointestinal Endoscopy at Tripura Medical College and Dr B.R.A.M Teaching Hospital. Endoscopic biopsy was obtained from the stomach using Olympus standard forceps. RUT was performed on one portion of the biopsy specimen, while other portions were collected and sent to the Department of Pathology for histopathological examination.[7]

Procedure of HPE:

The following steps were followed to prepare histological slides:

Fixation: after obtaining the tissue from department of medicine it was fixed for overnight with 10% neutral buffer formalin at a ratio of 1:10[8,10]

Dehydration: Dehydration replaces residual fixative and cellular water in tissue processing, removing free water and leaving bound water intact to prevent over processing artifacts. Graded alcohols of increasing concentration are used.[12]

Clearing: The clearing agent, xylene, was utilized to displace dehydrating solutions and make tissue

components more receptive to the infiltrating medium.

Infiltration: Infiltration is crucial to prevent softening and crumbling of the tissue, allowing for smooth sectioning with microtomy. After clearing, tissue sections are infiltrated with paraffin wax for support

Embedding (Blocking): Embedding involves transferring tissue samples into molten paraffin wax in L metallic block, creating a tissue block suitable for sectioning.

Section cutting: Sectioning involves cutting tissue specimens into sections at a thickness of 3-4 micrometers using a microtome, for staining and examination.[97]

Staining: Hematoxylin and eosin stain and Warthin-Starry Silver stain used for staining the slides.

Hematoxylin and eosin (H&E) stain: H&E is a widely used histological stain, while Hematoxylin is a nuclear stain with a mordant for penetrating particles. Eosin is a cytoplasmic stain, easy to use, and demonstrates different tissue structures. Hematoxylin stains acidic structures and cell nuclei blue-black.[97]

Requirements for Hematoxylin & Eosin Staining: The list includes various tools such as Coplin Jars, Dropping bottles, Coverslips, specimen slides, Slide washing tray, DPX mounting media, microscope, reagents, water bath, and Microtome.

Staining procedure of Hematoxylin & Eosin: Deparaffinization of the sections by dipping in xylene for 2 times, 10 minutes each. Re-hydration by dipping in graded alcohols as follows; 2 changes of absolute alcohol for 5 minutes each followed by 95% alcohol for 2 minutes and 70% alcohol for 2 minutes. Washed in distilled water. Stained with Harris hematoxylin solution for 8 minutes. Washed in running tap water for 5 minutes. Differentiation done with 1% acid alcohol for 30 seconds. Washed in running tap water for 1 minute. Bluing done in 0.2% ammonia water solution for 30 seconds to 1 minute. Washed in running tap water for 5 minutes. Rinsed in 95% alcohol. Counterstained with eosin Y solution for 30 seconds to 1 minute. Dehydration done with graded alcohols as follows: 50% alcohol> 70% alcohol> 95% alcohol> absolute alcohol for 5 minutes in each grade. Cleared by dipping in 2 changes of xylene, 5 minutes each. Mounted with DPX.

Warthin Starry silver stain: The Warthin Starry silver stain is used to detect *H. pylori* in histological specimens by binding silver ions from a solution. The process involves impregnating silver atoms on the organism, treating it with a

reducing solution, and then counterstaining with Tartrazine. This stain can be used to visualize *H. pylori* on the foveolar epithelium and deep inside the gastric pits.

Reagents of Warthin Starry silver stain

- Acidulated Silver Nitrate (0.25%) (Reagent A)
- Acidulated Silver Nitrate (2%) (Reagent B)
- Acidulated Gelatin (Reagent C)
- Acidulated Hydroquinone (Reagent D)
- Tartrazine (Reagent E)

Following reagents were combined for preparation of Developer solution:

- 6 ml Acidulated Gelatin (Reagent C)
- 2ml Acidulated Silver Nitrate Solution (2%) (Reagent B)
- 2ml Acidulated Hydroquinone Solution (Reagent D)

Staining Procedure of Warthin Starry silver stain

The slides were deparaffinized and hydrated with distilled water. Placed in 30ml of Reagent A in a plastic coplin jar. The solution was pre-warmed to 60° C in water bath. The slides were then allowed to stand for 5-7 minutes for the silver impregnation. Removed from Reagent A and treated with the freshly prepared warm Developer solution. Allowed to remain in the developer solution until the sections appeared gray-brown. Washed thoroughly under warm tap water. Rinsed in two changes of distilled water. Counterstain was done with Reagent E for 15 seconds. Then again rinsed in two changes of distilled water. Dehydrated in graded alcohols. Cleared in three changes of xylene. Mounted with DPX. Then the slides were examined under microscope.

Rapid Urease Test (RUT) kit : *H. pylori* can be quickly diagnosed with RUT, a rapid diagnostic test. The test's foundation is *H. pylori*'s capacity to secrete the urease enzyme, which catalyses the conversion of urea into carbon dioxide and ammonia. After a day, the RUT kit will be read to determine the final results. When the test well's colour changes from yellow to either pink or red, it is deemed positive.

Data Collection: Data were collected in a predesigned and structured proforma from patients who fulfilled the inclusion criteria after obtaining informed and written consent.

Data Analysis: Data were analysed using the SPSS version 26. Data were expressed as Bivariate analysis presented in tables.

Results and Observations

Distribution of patients: There were 110 patients in the research. Of them, 98 patients (89.1%) had

an H. pylori infection. The percentage of cases that tested positive for H. pylori infection is displayed

in Figure 1.

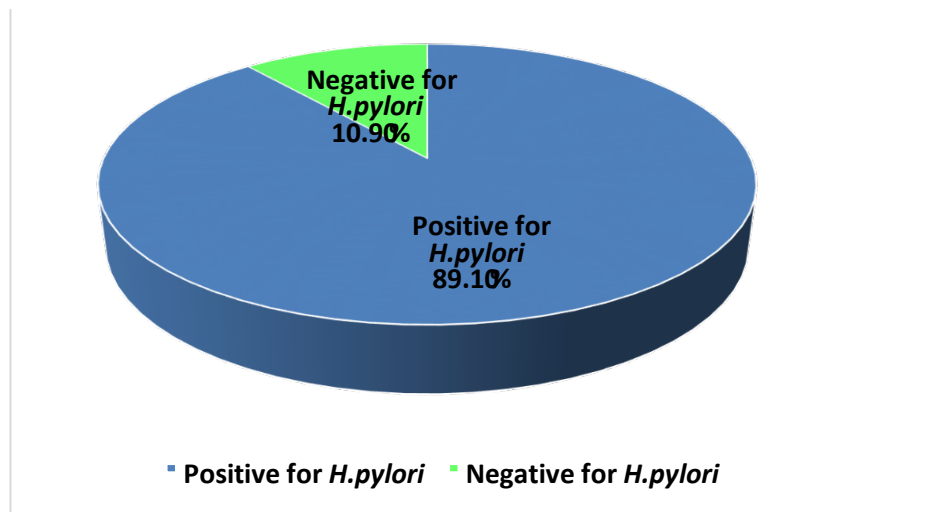


Figure no1: Percentage of cases which were positive for H.pylori infection.

Gender wise distribution of patients: Out of 110 patients, 59 (53.6%) were males and 51 (46.4%) were females. Out of 59 males, 51 (86.4%) were positive for H.pylori and out of 51 females, 48 (92.2%) were positive for H.pylori. Table number 1 shows Gender wise distribution of patients of APD and Table number 2 shows Gender wise distribution of H.pylori positive patients.

Table 1: Gender wise distribution of patients:

Gender	Frequency	Percentage (%)
Male	59	53.6%
Female	51	46.4%
Total	110	100%

Table no 2: Gender wise distribution of H.pylori infection:

Gender	H.	pylori	Total
	Positive	Negative	
Male	51 (86.4%)	8 (13.6%)	59
Female	47 (92.2%)	4 (7.8%)	51
Total	98	12	110

Age wise Distribution of patients: Of the 110 patients, the majority were between the ages of 31 and 40 (25.5%) and 41 and 50 (22.7%). 3.6% of them were in the age group of under 20. The distribution of age groups is shown in Table 3.

Table 3: Age group distribution:

Age group	Frequency	Percentage (%)
<20 years	4	3.6%
21 – 30 years	17	15.5%
31 – 40 years	28	25.5%
41 – 50 years	25	22.7%
51 – 60 years	15	13.6%
>60 years	21	19.1%
Total	110	100%

Age wise distribution of percentage of H.pylori positive cases: Majority of the H.pylori positive cases were from the age group 31-40 years followed by 41-50 years and >60 years. Figure number 24 shows age wise distribution of H.pylori positive cases in the study.

Distribution of patients according to their location: Out of 110 APD patients, 68 (61.8%) were from rural areas and 42 (38.2%) were urban. Of these, 60 (88.2%) had H. pylori infection in rural areas, while 38 (90.5%) had it in urban areas. The distribution of patients is depicted in Table 4.

Table 4: Distribution of H. pylori infected individuals in rural and urban areas:

Location	H.	pylori
	Positive	Negative
Rural	60	8
Urban	38	4
Total	98	12

Occupation: The study found that 28.2% of patients were day laborers, as depicted in Table 5.

Table 5: Occupation wise distribution of patients:

Occupation	Frequency	Percentage (%)
Student	11	10%
Government employee	10	9.1%
Retired personal	5	4.5%
Day labourer	31	28.2%
Housewife	16	14.5%
Farmer	14	12.7%
Businessman	15	13.6%
Unemployed	8	7.3%
Total	110	100%

Socioeconomic status: The majority of patients (53.6%) were from the lower middle class, followed by the middle class (20%), as per the Modified BG Prasad socioeconomic status scale. Table 6 display the distribution of patients and H.pylori positive cases.

Table 6: Distribution of patients according to socioeconomic status:

Socioeconomic status	Frequency	Percentage (%)
Upper class	10	9.1%
Upper middle class	14	12.7%
Middle class	22	20%
Lower middle class	59	53.6%
Lower class	5	4.5%
Total	110	100%

Risk Factors: The majority of patients (30.90%) had a history of tobacco use, followed by alcohol intake (25.40%) and smoking combined.

Drug history: 60.9% of the patients had no notable history of drug use. A history of NSAID and steroid use was reported by 25.50% and 6.4% of patients respectively.

Chief complaints of patients: Out of 110 patients, 83 (75.5%) had burning sensation with abdominal pain, 7 (6.4%) had presented with burning sensation, abdominal pain and vomiting, 6 (5.5%) had only vomiting, 4 (3.6%) each had hematemesis and malaena, 3 (2.7%) had presented with both hematemesis and malaena and dysphagia with regurgitation. Table number 7 shows the distribution of chief complaints.

Table7: Distribution of Chief complaints of patients:

Chief complaints	Frequency	Percentage (%)
• Burning sensation with abdominal pain	83	75.5%
• Vomiting	6	5.5%
• Dysphagia with regurgitation	3	2.7%
• Hematemesis	4	3.6%
• Melena	4	3.6%
• Hematemesis + Melena	3	2.7%
• Burning sensation with abdominal pain and vomiting	7	6.4%
Total	110	100%

Clinical/Endoscopic diagnosis: Out of 110 cases, most of the cases were diagnosed Gastritis (77.3%) followed by Gastric ulcer (20%) and Gastric carcinoma (2.7%). Table number 8 shows the percentage of various cases diagnosed clinically/endoscopically.

Table 8: Clinical/Endoscopic diagnosis:

Clinical/Endoscopic diagnosis	Frequency	Percentage (%)
Gastritis	85	77.3%
Gastric ulcer	22	20%
Gastric carcinoma	3	2.7%
Total	110	100%

Presence of H.pylori in Clinical/Endoscopic Diagnosis: Of the 85 instances of gastritis, 80 (94.1%) had an H. pylori infection, while 5 did not. 16 (72.7%) of the 22 instances of gastric ulcers were positive for H. pylori infection. Two (66.7%) of the three instances of gastric cancer were positive for H. pylori infection.

Table 9: Number of H.pylori positive cases in various Clinical/Endoscopic diagnoses

Clinical/Endoscopic Diagnosis	H.pylori		Total
	Positive	Negative	
• Gastritis	80	5	85
• Gastric ulcer	16	6	22
• Gastric carcinoma	2	1	3
Total	98	12	110

H. pylori in H&E Stain: Out of 110 cases, 78.2% (86) were detected using H&E stain, as shown in Table 10.

Table 10: H. pylori positive in H&E Stain:

H. pylori in H&E Stain	Frequency	Percentage (%)
• Positive	86	78.2%
• Negative	24	21.8%
Total	110	100%

H. pylori in Warthin Starry Silver Stain: The Warthin Starry Silver stain identified 98 (89.1%) cases of H. pylori infection out of 110 cases, as shown in Table 11.

Table 11: H. pylori positive in Warthin Starry Silver Stain

H. pylori in Warthin Starry Silver Stain	Frequency	Percentage (%)
• Positive	98	89.1%
• Negative	12	10.9%
Total	110	100%

H. pylori in RUT: RUT identified 74 (67.3%) H. pylori infections out of 110 cases, as shown in Table 12.

Table 12: H. pylori positive cases in RUT

H. pylori in RUT	Frequency	Percentage (%)
• Positive	74	67.3%
• Negative	36	32.7%
Total	110	100%

Comparison between H&E and RUT: RUT identified 74 instances of H. pylori infection out of 110 cases. In the H&E stain, 12 instances that were negative for H. pylori in the RUT test turned out to be positive. H. pylori were not detected in 24 instances in either RUT or H&E. When compared to RUT, H&E's sensitivity and specificity were found to be 86% and 100%, respectively.

Comparison between RUT and Warthin Starry Silver stain: Out of 110 cases, RUT detected 74 H.pylori infections,. 24 cases which were found to be negative in RUT was found positive in Wathin Starry Silver stain. 12 cases were negative in both RUT and Warthin Starry Silver stain.

The sensitivity and specificity of Warthin Starry Silver Stain were 100% and 75.5%, respectively, as shown in Table 13.

Table 13: Comparison between RUT and Warthin Starry Silver stain:

RUT	Warthin Starry Silver Stain	
	Positive	Negative
Positive	74	0
Negative	24	12

Comparison between H&E and Warthin Starry Silver stain: Out of 110 cases, H&E detected 86 H.pylori infections, 12 cases which were found to be negative in H&E found to be positive in Warthin Starry Silver stain. 12 cases were negative in both H&E and Warthin Starry silver stain. The Warthin Starry Silver stain had a sensitivity and specificity of 87.7% and 100%, respectively, indicating a significant difference between the tests. Table 14 shows comparison between H&E and Warthin Starry Silver stain.

Table 14: Comparison between H&E and Warthin Starry Silver stain:

H&E	Warthin Starry Silver Stain		Significance (P value)
	Positive	Negative	
Positive	86	0	<0.001
Negative	12	12	

Histological Diagnosis: The majority of cases were diagnosed histologically as H. pylori associated gastritis (80%), followed by H. pylori associated gastritis with intestinal metaplasia (5.5%). 3.6% were diagnosed with gastric carcinoma, and 0.9% were gastric carcinoma without H. pylori.

Staining pattern of H.pylori in Histopathological stains: H. pylori can be detected in gastric biopsies using H&E and Warthin Starry Silver stains. The pink coloration in Hematoxylin-eosin stain and dark brown coloration in Warthin Starry Silver stain enhances detection sensitivity.

Discussions

H. pylori infection affects half of the global population and is a major cause of gastric diseases like chronic gastritis, ulcers, and gastric carcinoma. In 1994, the World Health Organization designated H. pylori a "definite biological carcinogen." Various methods for accurate detection, including noninvasive and invasive methods, have been developed, with histological examination being a useful diagnostic test.[13,14,15]

The study investigates the association of H. pylori with APD and its prevalence in gastric mucosa using Histopathological Analysis and Rapid Urease Test(RUT). It compares RUT's sensitivity and specificity in gastric mucosa detection and studies H. pylori staining patterns using Warthin Starry Silver and H&E stain.

The study involved 110 adult patients with APD, with 53.6% males and 46.4% females. Among them, 86.4% males and 92.2% females tested positive for H.pylori infection. This finding aligns with previous studies indicating a female predominance, although male predominance has also been reported in India.[16,17] The study found that the majority of patients were aged 31-40 (25.5%), followed by those aged 41-50years(20.4%). This is consistent with previous studies indicating a higher prevalence of H. pylori infection in the age group of 41-50 years, 36-45 years, and children, as well as in a 2012 study.[18]

The study found that the majority of patients (61.8%) were from rural areas, similar to previous research by Contreras M and colleagues in 2015 and Hoang TT et al in 2005. Agumon BD et al in 2005 found a higher prevalence of 72.3% in rural areas.[19,20]

The study revealed that day labourers, housewives, and businessmen were the most common occupations infected with H.pylori, with unemployed individuals and farmers having a higher prevalence. Previous studies have also found skilled workers and farmers to be more susceptible to the infection.[21,22]

The study found that the majority of patients (53.6%) belonged to the lower middle class, followed by the middle class (20%), with the least number of patients from the upper class (9.1%). This is consistent with previous studies indicating a higher prevalence of H. pylori infection in lower socioeconomic classes due to factors like overcrowding, poor hygiene, and sanitation.[23]

The study found that the majority of patients had a history of tobacco consumption (30.9%), followed by a combination of tobacco, smoking, and alcohol (24.5%). A 2019 study linked smoking to increased H. pylori infection persistence, while a 2013 study found no association.[24]

The study revealed that a significant percentage of patients (60.90%) had no significant drug history, while 25.50% had NSAIDs, 6.4% steroids, and 7.3% had NSAIDs and steroids, a finding consistent with previous research. [25] The study found that burning sensation and abdominal pain were the most common symptoms (75.5%), consistent with previous research by Javed M, Somro S, and Kadam PN et al.[26]

The study revealed that gastritis (77.3%) was the most common endoscopic diagnosis among 110 patients, followed by gastric ulcer (20%) and gastric carcinoma (2.7%). This finding aligns with previous studies by Ogutu EO, Poudel A et al[28]., and KC RS et al[29]., which found gastric ulcer as the most common endoscopic diagnosis.

The study found that H. pylori positivity in gastric biopsies was 78.2% in H&E stain, 89.1% in Warthin Starry Silver stain, and 67.30% in RUT. Warthin Starry Silver had better sensitivity and specificity for H. pylori detection compared to H&E and RUT, similar to a 2020 study that found 100% and 82.8% sensitivity and specificity for H&E stain and 92.3% for Warthin Starry Silver.[27]

The study found that H. pylori associated gastritis was the most common diagnosis, followed by intestinal metaplasia, carcinoma, and gastric ulcer, which is consistent with previous research.[26]

Summary

The study conducted at Tripura Medical College aimed to determine the prevalence of H. pylori in gastric mucosa and its association with Acid peptic disorder (APD) patients. 110 patients were included, with 59 (53.6%) males and 51 (46.4%) females. H.pylori infection was detected in 98 patients (89.1%). The majority of patients were from rural areas (61.8%), with day labourers, (28.5%), housewives (14.5%), and businessmen (13.6%). The most common symptoms were burning sensation with abdominal pain (75.5%) and abdominal pain and vomiting (6.4%). The most common endoscopic findings were gastritis in 85

patients (77.3%), gastric ulcer in 22 patients (20%), and gastric carcinoma in 3 patients (2.7%). The Warthin Starry Silver stain had better sensitivity and specificity for *H. pylori* detection in gastric biopsies compared to H&E stain and RUT. The most common diagnosis was *H. pylori* associated gastritis in 88 patients (80%), followed by *H. pylori* associated gastritis with intestinal metaplasia in 6 patients (5.5%), *H. pylori* associated gastritis with carcinoma in 4 patients (3.6%), and gastric ulcer in 4 patients (3.6%).

Conclusion

H. pylori infection is a prevalent bacterial infection globally, affecting 75% of the population, particularly in developing countries. It causes gastritis and increases the risk of gastroduodenal ulcers and gastric carcinoma. Early diagnosis is crucial for preventing complications, especially carcinogenesis.

Histopathology is essential for detecting *H. pylori* infection, providing information about gastric mucosa, inflammation, and pathology. H&E stain is commonly used, but special stains like Warthin Starry silver stain can improve diagnostic yield. This study recommends using Warthin Starry silver stain as an additional special stain alongside H&E stain for *H. pylori* detection in gastric biopsies.

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