

REVISITING THE TRADITIONAL DIAGNOSTIC METHODS FOR
HELICOBACTER PYLORI

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ABSTRACT **Summary:** *Helicobacter pylori*, the common cause of Peptic Ulcer Disease (PUD), has a variety of traditional as well as modern diagnostic tests. This study aims to revisit these traditional diagnostic tests and know their efficacies in the current molecular diagnostics era. **Materials & Methods:** Antral biopsy samples were examined for Gram's stain and Rapid Urease Test (RUT). Serum samples were examined for IgG antibody detection by Enzyme Linked Immunosorbent Assay (ELISA). **Results:** The present study showed maximum efficacy with IgG antibody detection by ELISA. **Conclusion:** RUT combined with ELISA can be used as an alternative for PCR or Urease Breath Test for the diagnosis of *Helicobacter pylori* in peptic ulcer disease patients.

KEYWORDS : Peptic Ulcer Disease, Gram's stain, Rapid Urease Test, Enzyme Linked Immunosorbent Assay

INTRODUCTION

Helicobacter pylori (*H. pylori*) was discovered in the year 1982 by two Nobel laureates from Australia, John Robin Warren, and Barry James Marshall. They proved that there is an association which exists between *H. pylori* and peptic ulcer disease (PUD). In humans *H. pylori* causes one of the most common bacteriological infections of the body. *H. pylori* discovery had opened a new era of science which changed the understanding of PUD. Until the discovery of *H. pylori*, PUD was thought to be caused by gastric acid alone.¹ The prevalence of *H. pylori* varies according to age and according to the region of that particular area. In developing countries, children were more commonly involved. Today *H. pylori* regarded as one of the major pathogens affecting more than half of the world's population. It causes several diseases of the gastrointestinal system such as gastric ulcer, duodenal ulcer, mucous associated lymphatic tissue (MALT) lymphoma and gastric carcinoma.² In the past, before the discovery of *H. pylori*, factors leading to gastric acidity were thought to be the major factor causing PUD and treatment of PUD is mainly by antacids. Subsequently with the discovery of H_2 receptors which mediates gastric acid secretion, there is a drastic change in the understanding of the PUD and H_2 receptor antagonists were the safe and effective treatment for the PUD.¹ Nowadays PUD has become a severe problem in the community because of the recurrence of the disease. Warren and Marshall, in 1982, had proposed that there exists an association between PUD and some pathogen in the body. They isolated a spiral or curved urease producing organisms in the narrow interface between gastric epithelial cell surface and the overlying mucous gel and it was named as *Helicobacter pylori*. After clinical trials upon themselves (Barry Marshall and Arthur Morris), and on other volunteers, *H. pylori* was known to colonize the human stomach and causes gastric mucosal inflammation.¹ For diagnosing *H. pylori*, methods such as culture, histological stains and biopsy urease tests require an invasive test such as an endoscopic biopsy of gastric mucosa. Serology, Urea Breath Test and Stool Antigen Test are current non-invasive tests. Although accurate non-invasive methods are available, biopsy based invasive techniques are required to confirm the infection. Eradication of this organism leads to ulcer healing and markedly lowers the incidence and recurrence.² This study was conducted to compare the different diagnostic methods like Gram's stain, Rapid Urease Test (RUT) and IgG Enzyme Linked Immunosorbent Assay (ELISA) in the local population to select the most sensitive, specific, rapid and reliable method for the diagnosis of *H. pylori*.

Subjects and Methods

The present study is the collaboration between the Department of Microbiology and Department of Medical Gastroenterology, after obtaining permission from the Institutional Ethics Committee. Informed consent was obtained from the patients before their enrolment in the study.

Cases & Controls

Hundred patients of both sexes in age groups of ten to hundred years, attending gastroenterology department with complaints suggestive of antral gastritis, gastric ulcer, duodenal ulcer and gastric carcinoma were considered as cases. Ten healthy people were considered as controls.

Exclusion Criteria

Patients less than ten years of age, recent intake of H_2 blockers/proton pump inhibitors/antibiotics and who had gastric surgery were excluded from the study.

Sample Collection and Processing

Antral biopsy and blood samples were collected from all the cases. Blood samples were collected from controls.

Antral Biopsy

After preliminary investigation of the patient, under strict aseptic precautions and application of a local anaesthetic, two biopsies were taken from the gastric antrum. The biopsy samples were placed in a small screw-capped bottle containing 0.85% NaCl and processed within two hours.^{3,4} One sample was employed for Gram's stain (Jensen's modification with safranin as counter stain) (Fig. 1) and the other for RUT using freshly prepared RUT broth (HiMedia). RUT results were read within two hours (Fig 2).

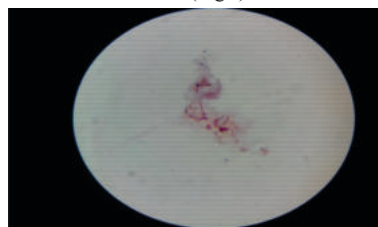


Fig 1: Gram's Staining of *H. pylori* (1000x Oil Immersion)



Fig 2: Rapid Urease Test

Blood

After aseptic precautions, two millilitres of blood was collected. Serum was separated after centrifuging the sample and stored in a sterile aliquot vials at -20°C until tested for qualitative detection of IgG *H. pylori* antibodies by ELISA (Microplate ELISA - XEMA). It's based on a two-site sandwich enzyme immunoassay principle, in which the microwells were coated with CagA protein of *H. pylori* (Fig. 3).

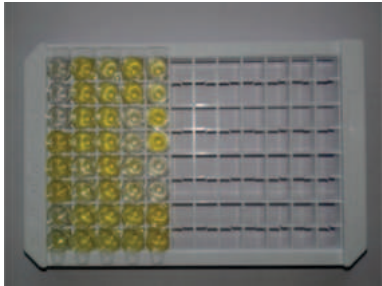


Fig 3: ELISA Microplate for *H. pylori* IgG Antibodies

Statistical Analysis

Efficacy for each test was evaluated. Odds Ratio (OR) and Fisher exact's p-value was calculated using Epi Info™ from Centers for Disease Control and Prevention (CDC).

RESULTS

The present study includes 100 cases of various ages from 17 to 83 years of age. In these 100 cases, 71 were males and 29 were females. 41-50 years age group has the highest frequency of all the cases. The frequency of cases was highest for gastritis (54), followed by duodenal ulcer (26) and gastric ulcer (20). (Table 1), (Chart 1)

Table 1: Predominance of Age Groups in Various Criteria

Age	Frequency	Predominant Age Group
Male	71	41-50
Female	29	61-70
Gastritis	54	41-50
Gastric Ulcer	20	51-60
Duodenal Ulcer	26	61-70
Gram's stain	17	21-30
RUT	34	21-30
ELISA	44	21-30
RUT+ELISA	30	21-30
All combinations	16	21-30

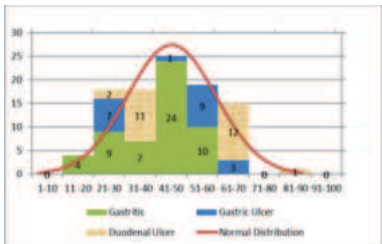


Chart 1: Normal Distribution of Cases Age & Distribution Histogram of Various Cases in Different Age Groups

Mean $\pm$ Standard Deviation for cases age was 44.01 $\pm$ 14.52 years. Q-Q plot for the age distribution curve shows that the data is normally

distributed. Most of the cases were within one standard deviation on either side of the mean of the distribution curve. (Chart 1) There were 49 cases tested positive for *H. pylori*. Among them, Gram's stain, RUT and ELISA showed positivity for 17, 34 and 44 cases respectively. Gram's stain, RUT and ELISA alone showed positivity in one, three and 14 cases respectively. There were no gastric carcinoma cases in the present study. (Table 2)

Table 2: Age Group Positivity in Different Criteria

Age Group	Gram's Stain	RUT	ELISA	RUT + ELISA	Other Combinations
1-10	-	-	-	-	-
11-20	-	1	1	1	-
21-30	10	18	18	18	10
31-40	7	11	15	11	6
41-50	-	-	-	-	-
51-60	-	-	5	-	-
61-70	-	4	5	-	-
71-100	-	-	-	-	-

Out of all gastritis, gastric ulcer and duodenal ulcer cases, maximum positivity was seen by ELISA in 14, 12 and 18 cases respectively. In RUT+ELISA combination and in other combinations of tests, maximum positivity was seen in duodenal ulcer cases in 13 and 6 cases respectively. (Table 3)

Table 3: Distribution of Cases According to Different Diagnostic Tests

S. No	Endoscopic findings	Total	Gram's Stain	RUT	ELISA	RUT + ELISA	Other Combinations
1	Gastritis	54	7	10	14	10 (18.5%)	6 (11.1%)
2	Gastric Ulcer	20	4	8	12	7 (35%)	4 (20%)
3	Duodenal Ulcer	26	6	16	18	13 (50%)	6 (23.1)
	Total	100	17	34	44	30	16

In RUT+ELISA combination, maximum cases (18) were seen in 21-30 age group (Males were 12 and females were 6). Highest percentage positivity for *H. pylori* was seen in duodenal ulcer (50%), followed by gastric ulcer (35%) and gastritis (18.5%). (Tables 1-3) Rest of the combinations showed maximum cases (10) in 21-30 age group. Highest percentage positivity for *H. pylori* was seen in duodenal ulcer (23.1%), followed by gastric ulcer (20%) and gastritis (11.1%). (Tables 1-3) Among 10 persons of control group, one was positive by ELISA.

DISCUSSION

Invasive (biopsy-based) tests vary in terms of sensitivity, whereas non-invasive tests, despite being more sensitive, have their own disadvantages.² Gram's staining is one of the rapid screening procedures for the diagnosis of *H. pylori*. Gram's stain positivity correlates with the study of Pilli and Kirani (18%).⁵ With carbol fuchsin instead of safranin as counter stain, highest and lowest percentage of smear positivity was shown by Kaore et al, (78.26%)⁶ and Pokhrel et al, (3.84%)⁶ respectively. There is a wide variation of results of Gram's stain from one study to another. This is due to sampling error.⁸ Another reason for variation in results is related to Hucker and Conn's counsel, which states that the significance of results can vary with the exact steps that followed in the staining procedure.⁹ RUT positivity in the present study correlates with Pilli and Kirani (36%)⁵ and Dewasi et al. (38%)¹⁰. RUT is a rapid and cheap test, which detects preformed urease in specimen and doesn't require the growth of the organism. False negativity can occur if there is less bacilli load and false positivity if other urea splitting organisms are present in the specimen.^{1,8} Marshall et al, observed that his biopsy urease test (*Campylobacter-like Organism* [CLO] test) had highest sensitivity (98%) when incubated at 30°C which is nearer to the optimum temperature for urease enzyme (45°C). This was confirmed by Morris et al, but contradicted by Vaira et al, (58% sensitivity). McNulty et al, reported that their two percent urea broth had good sensitivity because of increase in the concentration of phenol red.¹¹ ELISA positivity in the present study correlates with Pilli and Kirani (42%)⁵ and nearer to Sadik et al, (50.9%).¹² Highest positivity was shown by Selvi et al, (58%)¹³ and lowest positivity was shown by Dewasi et al, (34%)¹⁰. Serological assay results vary due to the reference method used to confirm *H. pylori* infection, antigen source and population under study. Added to this, ELISA can't differentiate between active and past infection.⁸ Serological assays should not be used to monitor treatment

due to their too slow drop in titre of *H. pylori*-specific antibodies.² Biopsy-based tests can be used for treatment monitoring. These tests shouldn't be performed within four weeks after intercurrent use of antibiotics or bismuth compounds or within two weeks after stopping proton pump inhibitors (PPI's). Even though non-invasive tests like ELISA can be used for treatment assessment, it must be confirmed by endoscopy to observe any healing from prior gastric ulceration or to exclude gastric carcinoma.³ Thus a positive IgG antibody test for *H. pylori* indicates a marker for infection rather than an indicator for active infection. RUT combined with ELISA is taken as a marker in this study because Gram's stain is less sensitive than RUT and ELISA, and ELISA was equal in accuracy to gold standard tests like Warthin-Starry staining.⁴ Combined positivity of RUT and ELISA in the present study correlates with the study of Selvi et al, (29%).¹³

Statistical Analysis

By taking RUT and ELISA as a marker for active *H. pylori* infection, efficacy of different diagnostic tests was evaluated (Table 4).

Table 4: Efficacy of Different Diagnostic Tests

Test	Sensitivity	Specificity	PPV	NPV	Accuracy
Gram's Stain	68.10%	98.50%	96.70%	83.30%	86.90%
RUT	100%	94.50%	88.20%	100%	96.10%
ELISA	100%	83.30%	68.10%	100%	87.70%

Of all the tests, Gram's stain has highest specificity and positive predictive value whereas RUT and ELISA has highest (100%) sensitivity and negative predictive value. RUT has highest (96.1%) accuracy. In the present study, IgG serology positivity in study and control groups is 44% and 10% respectively. This study correlates with the study of Pilli and Kirani, which shown 42% in study group and 30% in control group.⁵ Positivity of 10%-30% in healthy individuals has been reported by various authors which is in general agreement with our findings. The Odds Ratio (OR) and P-value for each test was calculated by comparing with RUT+ELISA combination with other combinations of tests (Table 5).

Table 5: Odds Ratio and 1-Tailed P-value for Different Diagnostic Tests Taking RUT+ELISA and Other Combinations as Control

Control			Gram's Stain	RUT	ELISA
RUT + ELISA	Odds Ratio	Estimate	0.4779	1.202	1.8333
		Lower	0.2434	0.6629	1.0243
		Upper	0.9383	2.1795	3.2813
	P-value		0.02233547	0.32473356	0.02828397
Other Combinations	Odds Ratio	Estimate	1.0753	2.7045	4.125
		Lower	0.5094	1.3756	2.1221
		Upper	2.2697	5.3173	8.0181
	P-value		0.5	0.00259594	0.00001221

RUT+ELISA combination was also compared with other combinations of tests and vice versa to find out which group is having more odds and probability for diagnosing *H. pylori* in a given sample. When compared with RUT+ELISA and other combinations of tests, ELISA has the highest odds and Gram's stain has the lowest odds for diagnosing *H. pylori* infection. When RUT+ELISA combination and other combinations of tests were compared against each other, RUT+ELISA combination had the highest odds for diagnosing *H. pylori* infection. (Table 6)

Table 6: Odds Ratio and 1-Tailed P-value for RUT+ELISA and Other Combinations Against Each Other as A Control

Test	Control			
RUT + ELISA	Other combinations	Odds Ratio	Estimate	2.25
			Lower	1.1346
			Upper	4.462
		P-value		0.01413239
Other combinations	RUT + ELISA	Odds Ratio	Estimate	0.4444
			Lower	0.2241
			Upper	0.8814
		P-value		0.01413239

When compared with RUT+ELISA combination, there is statistically significant result for Gram's stain and ELISA ($p < 0.05$, 2 degrees of freedom [df]), but not for RUT ($p > 0.05$, 2 df). When compared with other combinations of test, there is statistically significant result for both RUT and ELISA ($p < 0.05$, 2 df), but not for Gram's stain ($p > 0.05$, 2 df). (Table 5) When RUT+ELISA is compared with other combinations of tests and vice versa, there is a statistically significant

result for all the combinations of tests ($p < 0.05$, 2 df). (Table 6)

CONCLUSION

Among all the tests, RUT+ELISA showed highest sensitivity and specificity. So, it was the best combination of tests to confirm an active *H. pylori* infection in a patient. If conditions are not favourable for RUT+ELISA, in which RUT needs invasive procedure of endoscopy and biopsy, then ELISA alone showed 100% Sensitivity and 100% Negative predictive value. So, it can be used as a primary screening test.

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