Original Article

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Microbiology Section

Comparative Evaluation of the Diagnostic Tests for *Helicobacter pylori* and Dietary Influence for Its Acquisition in Dyspeptic Patients: A Rural Hospital Based Study in Central India

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ABSTRACT

Introduction: Dyspepsia is a very common group of symptoms referring upper gastrointestinal tract for which patients consult the physician, accounting for about 4% to 14% of their consultations. Annual Incidence of Helicobacter pylori is 6%-14% in developing countries with faeco-oral route of transmission. In this context this study has been undertaken to look for the association of Helicobacter pylori in patients of dyspepsia with comparative evaluation of diagnostic test for detection of helicobacter pylori and also look for effects of smoking and alcohol consumption over symptomatic colonization in dyspeptic patients attending the NKP Salve Institute of Medical Sciences and Research Centre, Digdoh hills, Nagpur, a tertiary care hospital catering to rural population of central India.

Materials & Methods: It was a cross sectional prospective study involving 92 patients. After obtaining history of their dietary and personal habits, 3 mucosal biopsy specimens were collected from pyloric end of the stomach along with blood sample. Specimens were processed immediately for culture, direct gram staining, rapid urease test, histology and serology along with Urea breath test by HeliprobeTM.

All the data was managed in Microsoft Excel and statistical analysis was done using Epi-info. Pearson's chi square test was used for comparative evaluation between two groups.

Results: 55% patients were considered as positive by the three test criteria and taken as Reference Standard as being infected by *H. pylori*. Diagnostic tests used as well as dietary habits were evaluated against the reference standard.

Conclusion: Culture and in absence of culture; RUT, Direct Gram Stain, and Serology in combination, followed by RUT, Direct Gram Staining with Histology are the best for increasing the specificity for diagnosis of *Helicobacter pylori* infection in patients of dyspepsia. Alcohol consumption is not significantly associated but smoking and tobacco chewing or smoking along with alcohol consumption is highly significant for symptomatic colonization of *H. pylor*i in dyspeptic patients.Key-words: Helicobacter pylori, Dyspepsia, Reference Standard, Evaluation of diagnostic tests, Dietary influence, Smoking, Alcohol.

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INTRODUCTION

Dyspepsia is a very common human experience for which there are numerous causes. Dyspepsia is the group of symptoms which refers to the upper gastrointestinal tract for which patients consult the physician, accounting for about 4% to 14% of their consultations. However, the prevalence of dyspepsia in the general population is much higher, with 20%-30% patients experiencing dyspeptic symptoms and with many of them self medicating themselves [1,2].

Until the discovery of *Helicobacter pylori,* the physicians and microbiologists believed that the stomach was likely to be sterile because of the presence of its acid 'milieu'. The successful isolation of spiral gram negative bacilli by them has changed the focus from a non-infectious to an infectious aetiology i.e. from pH to Hp [3].

H. pylori is common bacterium and approximately 50 % population of the world is said to be infected [4]. Humans are the only known reservoirs of *H. pylori* and the risk factors which have been described for acquiring the infection include residence in a developing country, poor socioeconomic conditions, overcrowding and an ethnic and genetic predisposition. Upto 80% of the children under the age of 10 years are infected in the developing countries. The prevalence of this infection in India is 22%, 56% and 87% in the 0-4 years, 5-9 years and in the 10-19 years age group respectively [5].

The annual incidence of the *Helicobacter pylori* infection is 0.3% to 0.7 % in the developed countries and it is 6-14 % in the developing countries [6].

The accepted routes of its transmission are the faeco-oral route in the developing countries and the gastro-oral route (through the vomitus and the saliva) in the developed countries [7].

Today, the role of *Helicobacter pylori* has been established in chronic antral gastritis, duodenal ulcers, chronic gastric ulcers, dyspepsia, gastric cancer and gastric lymphoma. The World Health Organization has added *H. pylori* to its list of known carcinogens [8].

There is a paucity of data on the incidence of *Helicobacter pylori* in patients of dyspepsia from the rural areas of central India. The

literature about the dietary influence of alcohol consumption, smoking and in particular, tobacco chewing, which is not common in the western world, is also lacking. These habits are commonly prevalent in the rural areas and it is necessary to study their actual role in symptomatic colonization.

In this context, this study was undertaken to look for the association of *Helicobacter pylori* in patients of dyspepsia, with a comparative evaluation of the diagnostic tests for the detection of *Helicobacter pylori* and also to look for the dietary influence of alcohol consumption, smoking and tobacco chewing over the symptomatic colonization in dyspeptic patients who attended the tertiary care hospital which catered to the rural population of central India.

MATERIALS AND METHODS

This cross sectional, prospective study was carried out in the Department of Microbiology from December 2006 to November 2008 after obtaining permission from the institutional ethical committee. Patients of either sex and of any age who had the symptoms of dyspepsia were selected for the study. After obtaining the informed written consent, the patient's details were noted down according to the case record form, including their dietary and personal habits. The chronic consumption of alcohol and tobacco along with their smoking habits were identified.

Collection of the Specimen

The diagnostic modalities were considered under:

(1) Invasive
   (a) Culture
   (b) Microscopic examination:
      (i) Direct Gram Staining
      (ii) Histopathology
   (c) Biopsy urease or Rapid Urease Test

(2) Non-invasive
   (a) Urea Breath Test
   (b) Serology

The patients were taken for upper G.I. Endoscopy after keeping them nil by mouth overnight. The patients who were on proton pump inhibitors were asked to discontinue the same for 10 days before being given an appointment for upper G.I. endoscopy, as it decreased the diagnostic yield.

Collection and Transport of the Biopsy specimen

Three mucosal biopsy specimens were collected from the pyloric (antral) end of the stomach.

Two specimens were taken in 0.2 ml of normal physiological saline which was just enough to keep them moist for the culture, direct gram staining and for the rapid urease test (RUT). The biopsies were transported within two hrs to the Microbiology Laboratory and in case of a delay, they were stored at 4^0C for 5-6 hours [9,10].

One specimen was taken in 10% formalin for histopathology.

Collection of blood for serum

The blood samples were collected by taking all the aseptic precautions and they were transferred to a collecting tube till the serum separated. The separated serum was divided into small aliquots and these were properly labeled and stored at -20° C to be processed collectively for IgG against H. pylori later.

Processing of the specimens

All the samples were processed immediately after (within 2 hours) their transportation to the Microbiology Laboratory.

Culture

One biopsy specimen was homogenized by using a sterile mortar and pestle, in little sterile broth. This was used for the inoculation of the plates and for preparing the smears for gram staining.

The Brucella agar base and the Columbia agar base were procured from Himedia laboratories. The media were prepared by the addition of 5%-7% defibrinated sheep blood and they were made selective by the addition of the Campylobacter supplement (Skirrow's) – FD-008 from Himedia.

All the selective and the non-selective media were inoculated with homogenized material and they were incubated at 37°C in a candle jar with a pad of cotton which was soaked in water, which was placed at the bottom, to provide a humidified microaerophilic environment. The plates would be examined on the 3rd, 5th and the 7th day of their incubation [9,11]. Characteristic, shiny translucent colonies were identified by gram staining and the urease, catalase and the oxidase tests [12].

Direct Gram Staining

The smears were prepared on clean, grease free slides from the tissue homogenate which was fixed with 95 % ethanol, by putting 2-3 drops of it on the slide and allowing them to evaporate.

The smears which were thus prepared were stained by using Jensen's modification of gram staining, but instead of using Saffranin, dilute Carbol fuschin (0.2 %) was used as a counterstain, which helped in the better visualization of gram negative, spiral or sea gull wing shaped bacteria, which were present mucosally and submucosally [10,11,13,14].

Histopathology

The biopsy specimens which were collected in 10 % formalin as a fixative were transported to the Pathology Laboratory after proper labeling and with the requisition form for the Haematoxalin and eosin staining. After this, they were observed and reported by the pathologists [15].

The Rapid Urease test (RUT)

The Rapid urease or the Biopsy urease test was performed for putting the second biopsy specimen into 0.5 ml of freshly prepared unbuffered 10 % urea solution in de ionized water, with 1% phenol red as an indicator, whose pH was adjusted from 6.8 to 7.2. An uninoculated tube was also kept as a negative control. The inoculated and the control tubes were incubated and the results were read at the end of 1 hour, 4 hours and 24 hours. The hydrolysis of the urea liberated ammonia, which increased the pH and changed the colour of the indicator medium from yellow to pink, thus indicating a positive test [16,17].

IgG against Helicobacter pylori

The EIAgen *Helicobacter pylori* IgG Kit from Adaltis Italia S.p.A., (Bologna) Italy-12, which was an ELIZA based kit, was used. All the serum samples which were stored at -20°C were thawed and all the test procedures were carried out according to the manufacturer's instructions. Titres above the cut off value of 15 AU/ml were noted as positive.

The Urea Breath test

"Heliprobe"™, which was marketed by Rad- Probe and developed by Kibion Diagnostic, Uppsala, Sweden, was used for the urea breath test.

The Heliprobe Procedure [18]

After a 6 hour fasting period, a 37- kBq^{14} C Urea capsule was given to the patients, with 50 ml of water. After 10 min, the breath samples are collected by making the patients blow into the Heliprobe Breath card till it got saturated, which was indicated by the change of colour of the indicator from orange to yellow. The change of colour indicated complete saturation of the CO_2 in the LiOH (Lithium hydroxide) pads. These Heliprobe Breath Cards were the put into the slot of the Heliprobe Breath Analyzer. The analysis was based on the number of beta particles that hit the two Geiger Muller Counters during the 250 second measurement cycle and the results were presented as counts per minute (cpm), together with the test results- negative, equivocal or positive and they were reported as:

Heliprobe O : Negative (<25 cpm)
Heliprobe 1 : Equivocal (25 to 50 cpm)
Heliprobe 2 : Positive (>50 cpm)

All the data was managed in Microsoft Excel and the statistical analysis was done by using Epi-info. The Pearson's Chi square test was used for the comparative evaluation between the two groups.

RESULTS

A total of ninety two antral (92) biopsy samples were obtained and 57 blood samples were collected for serum.

A male predominance of 58/92 (63.04%) was observed, as compared to that of 34/92 (36.96 %) in females. Most of the patients presented in the active age group of 20 to 40 years.

[Table/Fig-1] shows the number of samples which were processed by various diagnostic methods for the diagnosis of *Helicobacter pylori* and their overall sensitivities.

Tests performed	Samples processed	Positive	Percentage
Culture	92	8	8.69%
Rapid Urease Test	92	76	82.60%
Direct Gram staining	92	72	78.26 %
Histology	65	56	86.15 %
Serum IgG	57	42	73.68 %
Urea Breath test (Heliprobe)	10	6	60.00 %

[Table/Fig-1]: Sample positivity by different diagnostic methods

Tests	Number of Positive Patients & (%)
Culture Positive	8 (14.54%)
RUT* +Direct Gram Stain +Serology	17(30.90%)
RUT +Direct Gram Stain +Histology	16 (29.09%)
Direct Gram Stain +Serology + Histology	8(14.54%)
Direct Gram Stain + Serology +Heliprobe	3 (5.45%)
Serology + Heliprobe + Histology	3(5.45%)
Total	55

[Table/Fig-2]: Positivity for H. pylori with different combination of tests applying three test criteria (Reference standard)
* RUT= Rapid Urease Test

There is no failsafe method for the diagnosis of *Helicobacter pylori* with a sensitivity and specificity of 100 %. To increase the specificity, the three test criteria has been adopted for the diagnosis of the *Helicobacter pylori* infection. By this criteria, all the culture positive cases; and in the absence of culture positivity, patients with any three (3) diagnostic tests which are positive out of five (5) test that have been carried out were taken as being infected for *Helicobacter pylori*. A total of 55 patients were found to be positive either by culture or by any other three tests [Table/Fig-2].

These 55 patients were considered as positive (infected by *H.pylori*) by the three test criteria and they were taken as the Reference Standard.. The comparative evaluation of the different tests which were done to study the incidence of *Helicobacter pylori* in the patients of dyspepsia has been shown in [Table/Fig-3].

The effect of alcohol and tobacco chewing was also seen over the symptomatic colonization of *Helicobacter pylori* in dyspepsia. [Table/Fig-4] .

DISCUSSION

In our study, a clear male predominance of 58/92 (63.04%) was observed, as compared to that of 34/92 (36.96 %) in females who presented with symptoms. The biological differences, whatever they are, must stem ultimately from the greater quantity of genetic material which is possessed by the female. The presence of one X chromosome in the male infant thus confers less immunological protection as compared to that in the female counterparts [19].

Similar findings were found in a study which was done in India by Ahmed et al., who also observed the active age group of 20 to 40 yrs to be more vulnerable, as was observed in our study [20].

8/92 samples were positive by culture with a sensitivity of 8.69%. A sensitivity of over 90% was reported by various workers like Marshall et al., Jones et al., and Goodwin et al., [9,15,21]. In contrast, the Indian studies reported a sensitivity which ranged from 1.09% (as reported by A. Ayyagari) to 63% (as reported by Akbar and Eltahawy) [11, 22].

Our culture sensitivity was in accordance with these results. The low rate of isolation may be because of the fastidious nature of *H. pylori* and because of a number of other factors like the patchy distribution of the organism, inadequate mincing of the biopsy material, the presence of oropharyngeal flora, the loss of viability of the specimen during transportation, etc. These factors are difficult to control. All these factors together, result in low sensitivity and a low negative predictive value.

The rampant use of over the counter prescriptions of the antisecretory agents, as well as of Metronedazole for the protozoan infestations, which are quite common in the rural regions of India, decrease the positive outcome [23, 24].

Therefore, though it was considered as a gold standard, it is now used only in research settings [25].

76/92 (82.60%) samples in our study were positive by the Rapid urease test, which is in agreement with the findings of almost all the studies in which the sensitivity ranged from 72 % (as reported by U. arora et al) to 99 % (as reported by Pinit Kullavanijaya) [26].

With the use of un-buffered 10 % urea solution, our results were found to be in agreement with those of other workers like A.S.Arvind et al., U. arora et al., and Vaira D., et al [16,17,27].

A total of 65 samples were processed for the histo-pathoplogical examination by haematoxilin and eosin staining, out of which a

Test	Reference standard positive	Reference standard negative	Total	Chi square value	p value**	Interpretation
Culture	8	0	8	5.89	p<0.05	Significant
Rapid Urease test	41	35	76	6.18	P<0.05	Significant
Direct Gram staining	52	20	72	21.31	P<0.001	Highly Significant
Histopathology	35	21	56	0.43	p>o.05	Not Significant
Serology IgG	39	3	42	35.16	P<0.001	Highly Significant
Urea Breath test "Heliprobe"	6	4	10	0.002	p>o.05	Not Significant

[Table/Fig-3]: Comparative evaluation of different test with reference standard
**p-value- p<0.05=Significant association, p<0.001= Highly significant association.

Habits	No. of patients positive in Reference standard	No. of patients Negative in Reference standard	Total	Chi square value	p value	Interpretation
Alcohol consumption	16	14	30	0.77	p>o.05	Not Significant
Smokers/tobacco chewer	30	10	40	6.18	P<0.05	Significant
Chronic Alcoholic and Tobacco Chewer	14	1	15	8.39	P<0.05	Significant

[Table/Fig-4]: Evaluation of Effect of dietary habits over the Symptomatic colonization of Helicobacter pylori in dyspepsia

total of 56 (86.15%) showed the changes of antral gastritis. Like in our study, most studies which have been reported in the western literature, which include those by Warren et al,, Goodwin et al., and Morris et al., have found *H.pylori* to be associated with chronic active gastritis [21,28,29].

A total of 92 biopsy specimens were processed by using modified gram staining in which the slides were counterstained by using dilute Carbol Fuschin. 72 out of the 92 samples (78.26%) were positive for *Helicobacter pylori* [Table/Fig-1].

Ayyagari reported the staining of the crushed tissue smears by Gram's method by using dilute Carbol Fuschin as counter stain as the simplest and reliable technique with 80.8 % sensitivity. K.N. Prasad et al., reported a sensitivity of 34 % for the same method [11,30].

D'Costa F Grace et al., found gram staining to be 74.2% sensitive. With 78.26% positivity, our findings were in accordance with those of other workers [31].

40/57 samples were positive (70.17%) for IgG against *Helicobacter pylori* by this ELISA based test. Pinit Kullavanijaya et al reported 120/191 patients to be positive i.e. 62.8 %, with a sensitivity of 96.8% and a specificity of 96.8 %, while Abida Malik et al reported a seropositivity of 58.3 % [26,23]. Daad H Akbar et al reported 212/289 (73%) patients to be positive for IgG [22]. Our positivity rate of 70.17 % was comparable to that which was reported by all the authors.

For the Urea Breath test, the HELIPROBE system was procured late in the course of the study. Though it was subsidized, the higher cost was a constraint for the patients, as a rural population mainly attended our tertiary care unit. Thus, only 10 patients could be included for Heliprobe. 6/10 (60%) patients were found to be positive for the Urea Breath test.

The low rate of positivity in our study may be attributed to the low number of samples, over the counter prescription of the proton pump inhibitors and also to the use of Metronedazole for protozoan infestations, which were very common in the rural settings in India, which could increase the false negative cases [23, 24].

In the absence of a failsafe method, a battery of tests are required for increasing the specificity of the diagnosis [32]. Many workers, in the past, have considered culture as a gold standard and in the absence of culture, a positive outcome of more than one test (invasive or non invasive) was taken as a reference standard for the diagnosis of the *Helicobacter pylori* infection. This kind of reference standard has been used by various workers [11,22,26,29,33,34,35].

A total of 55 patients were positive by the reference standard which has been described above. 40/58 (68.96%) males and 15/34 (44.11%) females in our study were found to be infected with *Helicobacter pylori* by using the three test criteria [Table/Fig-2].

There were 8 patients which were positive by culture. All the eight patients were also positive by the Rapid Urease Test (RUT), histology, direct gram staining and serology [22,26].

55 out of 92 patients i.e. 58.78 % were diagnosed by the three test criteria (Reference standard). This rate was similar to that which was reported by other investigators like Bulk et al., who reported a rate of 69% and M.A. El Barrawy who reported a rate of 61% [35].

RUT and direct gram staining with the non invasive test of serology was more effective in combination in diagnosing 17/55 patients (30.90%), followed by RUT and direct gram staining with histology 16/55 (29.09%), as was reported also by Pinit Kullavanijaya et al., and Daad H. Akbar et al, followed by a combination of direct gram staining, serology and histology [8/55 (14.54%)] and culture [8/55 (14.54%)] [26,22].

[Table/Fig-3] shows that culture and the Rapid Urease Test were significantly associated and that direct gram staining and IgG detection were highly significant for the diagnosis of H.pylori. [22,26,33,34,35].

Histology alone was not significant in the diagnosis of the *Helicobacter pylori* infection, but along with the combination of RUT and direct gram staining, histopathology was quite useful for its diagnosis [Table/Fig-2]. The lower sensitivity and the lack of a significant association may be due to the small no of samples which were processed for histopathology as well as drug induced gastritis, because of the rampant use of NSAIDs which are easily available as over the counter prescriptions in rural India.

Good sensitivity and specificity were reported by the workers for Heliprobe. This is a new system and the time interval after which

the patient exhales into the Heliprobe card also has an effect over the test positivity. Ideally, it should be 10 min and a delay may cause the ^{14}C labeled urea from getting hydrolyzed by other urease producing organisms like Campylobacter jejuni. The protease which is present in the intestine can give false positive results [18,36,37].

The effect of alcohol, smoking and tobacco chewing in patients as a possible risk factor, was also evaluated for the symptomatic colonization of *Helicobacter pylori* in patients of dyspepsia by using the patients who were diagnosed by using a reference standard [Table/Fig-4].

With 16/30 patients being diagnosed by using the reference standard, the alcoholic patients were not significantly associated with the symptomatic colonization, while with 30/40 patients who were diagnosed as positive for the *Helicobacter pylori* infection had smoking or tobacco chewing habits. They were significantly associated with the symptomatic colonization, as was also reported by Cardenas VM et al., and el-Gueid A et al., [38,39].

14/15 patients with chronic alcoholism along with tobacco chewing were found to be significantly associated with the symptomatic colonization. The increased C-X-C chemokine mRNA expression which was seen in smokers and tobacco chewers could play a role in inducing an enhanced inflammatory activity in gastritis and in the consequent severe disease which was associated with the *Helicobacter pylori* infection. Shimoyana T et al., and MA El-Barrawy reported similar findings [35,40].

To conclude: In culture and in the absence of culture RUT, direct gram staining, and serology in combination was the best [17/55 patients (30.90%)], followed by RUT and direct gram staining with histology [16/55 (29.09%)] for increasing the specificity for the diagnosis of the *Helicobacter pylori* infection in patients of dyspepsia.

Alcohol consumption was not significantly associated with the symptomatic colonization of *H.pylori* and it was found to have a protective effect against the colonization.

Smoking and tobacco chewing or smoking along with alcohol consumption was highly significant for the symptomatic colonization of H. pylori in dyspeptic patients.

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