Comparison of two transport media and three culture media for the primary isolation of *Helicobacter pylori* from perforated peptic ulcer biopsy specimens

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***Abstract*- Background**: Isolation of *H. pylori* on artificial culture media is hampered by lack of reliable confirmatory media. In this study, two transport media- Physiological saline and Stuart's transport medium and three culture media- Blood agar, Chocolate agar and Modified Thayer Martin agar were evaluated for primary isolation of *H. pylori*. The role of *Helicobacter pylori* in uncomplicated peptic ulcers has been definitively established. However its association in cases of perforated peptic ulcers is unclear. This study was also undertaken to identify *H. pylori* infection in the latter cases.

Materials and methods: Fifty intra-operative biopsies from cases of perforated peptic ulcers were aseptically collected, sent in transport media to the laboratory and processed by Gram staining and culture. Plates were incubated in a McIntosh Fildes' anaerobic jar under micro-aerophilic conditions for 7 days and any growth was identified using standard biochemical tests.

Results: Direct Gram staining showing curved, Gram negative bacilli suggestive of *Helicobacter pylori* was seen in 44% (22/50) specimens. *H. pylori* was recovered from biopsy specimens from 16% (8/50) patients. Comparison of all media combinations showed that the highest rate of isolation was 100% (8/8) with Stuart's transport medium & plating on Modified Thayer Martin medium followed by 75% (6/8) for Stuart's transport medium & plating on Blood agar.

Conclusion: Although *H. pylori* does not show very high rate of isolation from perforated ulcer biopsies, a combination of appropriate transport medium with culture media containing antibiotics can be used for isolation in suspected cases.

***Index Terms*-** *Helicobacter pylori*, perforated peptic ulcer, culture

I. INTRODUCTION

Helicobacter pylori is a micro-aerophilic, spiral or curved Gram negative bacillus which was discovered in 1984 by Warren and Marshall.[1] It has been found in the stomach of humans in all parts of the world. In developing countries, 70-90% of the population carries *H. pylori* with acquisition of infection before the age of 10 years. In developed countries, prevalence of infection is lower at 25-50%.[2] It is considered to play a major role in the pathogenesis of several gastro-duodenal diseases including chronic active gastritis, gastric and duodenal ulceration and gastric adenocarcinoma.[3] Although accurate non-invasive techniques like urea breath test, stool antigen detection test and serological diagnostic methods are available, biopsy based invasive techniques like rapid urease test and culture are required to confirm *H. pylori* as the causative agent of gastritis and peptic ulceration.[4] The rate of *H. pylori* isolation from biopsy specimens varies due to methodological factors such as bacterial density, transport media used, time from sampling to processing, culture media used and incubation conditions.[5] Reported isolation rates vary between 23.5% and 97% from different studies.[6,7]

Association between *H. pylori* and perforated peptic ulceration has not been proved conclusively. However, a close relationship between perforated ulcers and *H. pylori* density in gastric antrum has been reported.[8] Moreover, in contrast to uncomplicated peptic ulcer patients where association has been found in almost 90-100% cases, in perforations, a much lower rate of association (60-70%) has been reported.[9]

In this study, we aimed to evaluate the various combinations between two transport media and three culture media for the primary isolation of *Helicobacter pylori* from intra-operative biopsy specimens from cases of perforated peptic ulcers.

II. MATERIALS AND METHODS

Fifty consecutive intra-operative biopsy specimens from cases of perforated gastric or duodenal ulcers were processed for the isolation and identification of *H. pylori*.

Sample collection & transport: Two intra-operative, full thickness edge biopsies were taken from the site of ulcer perforation after thorough wash with sterile normal saline. Care was taken to include the underlying mucosa.

The two samples collected were put into two tubes containing sterile, physiological saline and Stuart's transport medium respectively. These were sent to the laboratory to be cultured for *Helicobacter pylori* within 30 minutes. Where delay was anticipated, the media with the specimens was refrigerated at 4˚C for not more than 24 hours. [10]

Isolation of *Helicobacter pylori*: The biopsy samples received in the laboratory were homogenized by grinding in a ground glass grinder and divided into 2 parts- one for microscopy and one for culture.

Microscopy: For Gram staining, the specimen was taken on a clean, grease-free glass slide over an area of around 2X2 cm^2 with a drop of normal saline to prevent drying of the sample.

An impression smear was also made by crushing the specimen onto another sterile glass slide. Freshly prepared Gram's reagents (Gram's Crystal violet & Safranin 0.5% w/v, Hi-Media Labs Pvt. Ltd., Mumbai, India) were used for staining the slides. Gram negative, pale staining, curved or 'S'-shaped, short bacilli were considered to be suggestive of *Helicobacter pylori*.[11]

Culture: The homogenized biopsy specimen was streaked on fresh, laboratory prepared Blood agar & Chocolate agar and Modified Thayer Martin agar (Thayer Martin Hi Veg medium Base, Hi-Media Labs Pvt. Ltd., Mumbai, India) augmented with 7% sterile, lysed blood and VCN supplement (Vancomycin, Colistin & Nystatin, Hi-Media Labs Pvt. Ltd., Mumbai, India) to inhibit commensal flora from the gastro-intestinal tract.[12,13] The plates were incubated in a McIntosh Fildes' anaerobic jar under micro-aerophilic conditions and incubated at 37˚C for 7 days. Growth was observed for at 3, 5 and 7 days. Growth generally appeared by 5 days. [14] If no growth was seen on Day 7, the plates were discarded and specimen labeled negative for *Helicobacter pylori*. Isolates were identified by typical colony morphology. Colonies on Modified Thayer Martin medium were small, 0.5-1mm, round, low convex, pale, grey-colored, translucent, smooth and easily emulsifiable.[10,14] Smaller colonies with similar morphology were seen on Blood agar and Chocolate agar in few cases.

Biochemical confirmation was done by a positive catalase, oxidase and urease test. The inability to reduce nitrates, hydrolyse hippurate and resistance to Nalidixic Acid was used to further confirm the isolate as *H. pylori.* [11, 14]

III. RESULTS

A total of 50 intra-operative biopsy specimens from perforated gastric and duodenal ulcers were processed for the primary isolation of *H. pylori*.

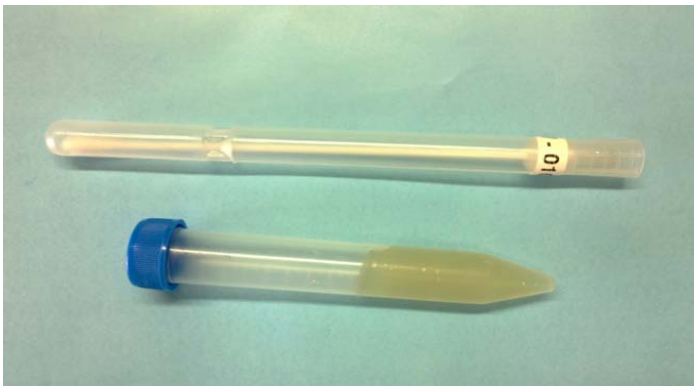

Figure 1: Stuart's Transport Medium with sterile swab

Total specimens (n)	Direct Gram smear positivity		Culture positivity	
	Number	%	Number	%
50	22	44	08	16

Table 1 shows direct Gram smear and culture positivity for *H. pylori* in cases of perforated peptic ulcers. Morphology suggestive of *Helicobacter pylori* on direct Gram staining was seen in 44% (22) specimens. Isolation of *H. pylori* on culture was achieved in 16% (08) of the specimens.

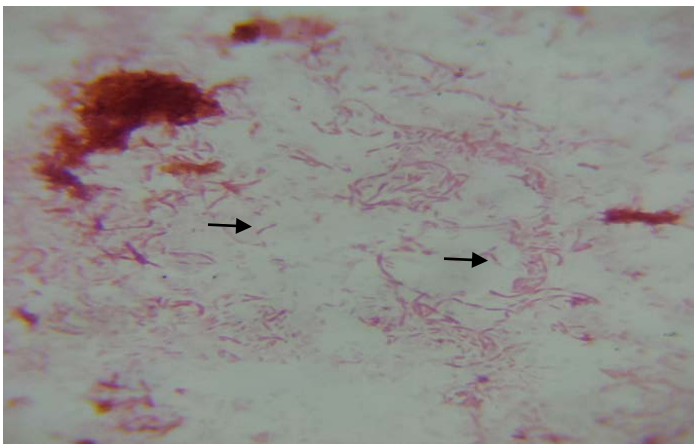

Figure 2: *Helicobacter pylori* in direct Gram Stain

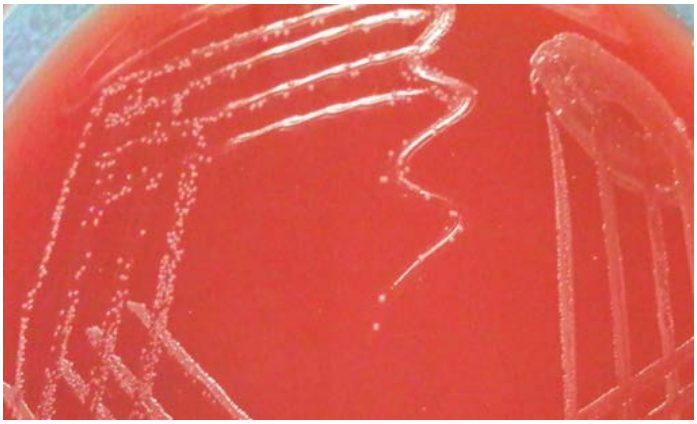

Figure 3: Tiny, grey colonies on Modified Thayer Martin Agar

Table 1. Direct Gram smear and culture positivity for *H. pylori* in cases of perforated peptic ulcers

Table 2. Isolation of *H. pylori* from combinations of transport and culture media

Combinations of Transport & culture medium (n=50)	Positivity for *H. pylori*		Growth other than *H. pylori*		No growth	
	No.	%	No.	%	No.	%
Physiological saline/ Blood agar	04	08	26	52	20	40
Physiological saline/ Chocolate agar	03	06	25	50	22	44
Physiological saline/ Modified Thayer Martin medium	05	10	06	12	39	78
Stuart's transport medium/ Blood agar	06	12	16	32	28	56
Stuart's transport medium/ Chocolate agar	04	08	18	36	28	56
Stuart's transport medium/ Modified Thayer Martin medium	08	16	02	04	40	80

Table 2 shows isolation of *H. pylori* from different combinations of transport and culture media. The best combination for isolation of *Helicobacter pylori* was Stuart's transport medium and Modified Thayer Martin medium in which 100% isolates grew (08) followed by the combination of Stuart's transport medium and blood agar (75%). Growth other than *H. pylori* was more commonly seen on non-selective transport and culture media (32-52%) as compared to that when selective Modified Thayer Martin medium was used (4-12%).

Table 3 shows the different combinations of transport and culture media and organisms other than H. pylori grown in these media. Maximum growth of organisms other than H. pylori was seen with the sterile saline and Blood agar combination (52%) while the least growth rates were seen with the Stuart's transport medium and Modified Thayer Martin medium combination (04%). Proteus mirabilis was the most common organism grown.

Table 3. Different combinations of transport and culture media and list of organisms other than H. pylori grown

Combinations of Transport & culture medium	Growth other than *H. pylori*	
	No. of organisms	Organism
1.Physiological saline/ Blood agar	26 (52%)	1. *Proteus mirabilis- 10* *2. Escherichia coli- 08* *3. Pseudomonas aeruginosa- 03* *4. Proteus vulgaris- 02* *5. *Micrococcus species- 02*
2. Physiological saline/Chocolate agar	25 (50%)	*1. Proteus mirabilis - 10* *2.Escherichia coli- 07* *3. Pseudomonas aeruginosa - 06* *4.*Micrococcus species- 02*
3. Physiological saline/Modified Thayer Martin medium	06 (12%)	*1. Proteus mirabilis- 05* *2. Pseudomonas aeruginosa-- 03*
4. Stuart's transport medium/ Blood agar	16 (32%)	*1.Pseudomonas aeruginosa-06* *2.Escherichia coli- 04* *3. *Micrococcus species- 02* *4. *Bacillus subtilis- 02* *5. *Diphtheroids- 02*
5. Stuart's transport medium/ Chocolate agar	18 (36%)	*1.Pseudomonas aeruginosa-08* *2. Proteus mirabilis- 07* *3. *Diphtheroids- 02* *4. *Bacillus subtilis- 01*
6.Stuart's transport medium/ Modified Thayer Martin medium	02 (04%)	*1.Proteus mirabilis- 02*

IV. DISCUSSION

Helicobacter pylori is a fastidious organism requiring an enriched medium and an appropriate environment for growth. The success in culture and isolation of this organism is affected by various factors related to the biopsy site preparation, collection and transport of the specimen and the culture media used for isolation. [(5)] Culture has been considered the gold standard for diagnosis of *H. pylori* infection. Moreover, isolation from mucosal biopsy samples is a pre-requisite for further studies of the organism including susceptibility testing, analysis of virulence factors and molecular epidemiological studies. [(4)]

Direct Gram staining revealed Gram negative bacilli suggestive of *H. pylori* in 22 out of the 50 samples (44%) (Table 1). This is similar to the findings in a study conducted by Sharma *et al* in 2012 where 44.2% biopsy samples showed positivity for *H. pylori* on direct Gram stain.[(15)] However, positivity in direct Gram staining varies from 40-90% as reported by various workers. [(16, 17, 18)]

In the present study, *Helicobacter pylori* was isolated from 16% specimens (08/50) (Table 1). This may be explained by presence of dead bacilli, overgrowth of contaminant bacteria from gut flora [5] or transformation into a non-cultivable coccoid form. [19] Isolation rates reported by Ayyagari *et al* [20] and Nanivadekar *et al* [21] in their studies are 23.9% and 24% respectively. However, isolation rates reported vary from 2% in a study by Gaval *et al* [22] to 63% by Akbar *et al* [23] in various Indian studies.

Maximum number of isolates of *Helicobacter pylori* (16%) grew on Modified Thayer Martin agar followed by Blood agar (12%). Of all the combinations of media used, the best combination was Stuart's transport medium & Modified Thayer Martin agar, in which all the 08 isolates grew (100%), followed by Stuart's transport medium & Blood agar combination, on which 06 of the total 08 isolates grew (75%) (Table 3). Sang *et al* also conducted a study in 1991 where Modified Thayer Martin agar showed best results for the primary isolation of *Helicobacter pylori*.[12] However, Cuchi *et al* did not report significant difference in yield of *H. pylori* from blood agar and Thayer Martin agar.[24] Growth of organisms other than *Helicobacter pylori* was found to be higher when non-selective transport medium (sterile, physiological saline) was used as compared to that seen on use of selective Stuart's transport medium. Non selective culture media like blood agar and chocolate agar also showed higher growth rates (32-52%). Use of selective transport medium with a selective medium for culture (Modified Thayer Martin agar with antibiotics) reduced the growth of organisms other than *H. pylori* to 4% (Table 3). This correlates with the study conducted by Cuchi *et al* in 2002 where almost 20% growth rates were seen with non-selective media as compared to around 5% with selective media.[24] While *Micrococcus species*, *Bacillus subtilis* and diphtheroids are normal gut flora, organisms such as *Pseudomonas aeruginosa*, *Proteus species* and *Escherichia coli* may have been isolated in the present study due to perforation of the peptic ulcer. Consequently, the efficacy of selective media is much more pronounced in this study because the added antibiotics inhibit the growth of this flora. Enriched media containing serum or lysed blood is seen to be better for primary isolation of *H. pylori*, as seen in the present study and also in the studies conducted by Sang *et al* and Cuchi *et al* [12, 24].

Most studies on the association of *Helicobacter pylori* with gastro-duodenal diseases have been done in cases of uncomplicated disease using endoscopically obtained antral biopsy samples. Very few studies are available to assess the association of *H. pylori* with complicated ulcer disease. Tokunaga et al in 1998 demonstrated a significant relationship between peptic ulcers and the density of *H. pylori* using immune-histochemical staining.[8] Mihmanli *et al* also demonstrated *H. pylori* in the wall and mucosa of peptic ulcers in 38.8% cases of chronic duodenal ulcer perforations through histo-pathological identification using Hematoxylin-eosin staining of biopsy specimens.[25] In the present study, Gram staining indicated presence of bacteria resembling *H. pylori* in 44% cases which is almost similar to the findings of Mihmanli *et al*. This indicates a causal relationship between *H. pylori* and perforated peptic ulcers. However, a larger study needs to be conducted to prove a more definitive association.

V. CONCLUSION

Correct biopsy site preparation, aseptic sample collection, rapid transport in appropriate transport medium and use of highly selective media are pre-requisites to ensure higher rate of primary isolation of *Helicobacter pylori* from biopsy samples in cases of perforated peptic ulceration. Using a combination of a selective transport medium with an enriched solid medium containing lysed blood and antibiotics helps in achieving higher yield of *H. pylori* with lower rates of contamination with organisms other than *H. pylori*.

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