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# Comparative Study on Helicobacter Pylori Infection as A Causative Agent in Gall Bladder Tissue with Symptomatic Cholecystitis/Cholelithiasisand Incidental Cholelithiasis

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## I. Introduction

Marshall and Warren were the first to prove conclusively that *H. pylori* was the etiological factor for gastritis and peptic ulcer disease. Since then, *H. pylori* has been implicated in the development of gastric adenocarcinoma and MALT lymphoma in the stomach. The prevalence of infection in the digestive tract by Helicobacter species varies in the population studied, suggesting epidemiological differences in the distribution of the bacillus in various countries. So far, *H. cinaedi, H. fennelliae, H. canis, H. rappini, H. pullorum*, and *H. canadensi* have been isolated from human intestinal tracts.

*Helicobacter* species isolated from the bile, gallbladder, or liver tissue of some animals, such as *Helicobacter pullorum* from poultry , *H. canis* from dogs , *H. cholecystus* from Syrian hamsters , "*Helicobacter rappini*" from sheep fetuses , and *H. hepaticus* and *H. bilis* from mice have been associated with hepatobiliary diseases. In the past few years, the presence of DNA of species of *Helicobacter*, including the well-known human pathogen *H. pylori*, has been identified in the bile, liver, and biliary epithelium obtained from patients with hepatobiliary diseases. More recently, the group isolated (for the first time) a *H. pylori* strain from the liver of a patient with cirrhosis, demonstrating that bacteria of the genus *Helicobacter* may be viable in the human liver, as it is seen to be in animals.

In regard to the biliary diseases, few patients were evaluated in the first studies. In one of those studies, *ureB H. pylor*- specific DNA was detected in the gallbladder tissue of a Japanese patient with gallstone and cholecystitis. In another study evaluating the presence of *H. pylori ureA* genes in the bile by nested PCR, Lin et al. observed a positive result in three patients with primary or metastatic pancreatic tumor but not in four patients with biliary diseases.

In studies of the same subject that included a larger number of patients, discordant results have been observed. In some of them, the presence of DNA of enterohepatic *Helicobacter* or *H. pylori* has been detected. Fox et al. have found *H. bilis*, *H. pullorum*, or "*H. rappini*" DNA in bile or gallbladder tissue from Chilean patients with cholecystitis or cholelithiasis. More recently, the level of *H. bilis* DNA was seen to be higher in the bile of patients from Japan and Thailand with bile duct or gallbladder carcinoma than from those without malignant disease of the biliary tree. In another study from Yugoslavia, the presence of *H. pylori*-specific DNA in the bile was associated with biliary tract carcinoma but no association was seen between patients with gallstone and those without biliary disease.

Other studies from Germany and Mexico failed in detecting the presence of DNA of *Helicobacter* spp. in bile or gallbladder tissue from patients with biliary tree disease. In a Japanese study, furthermore, DNA of *Campylobacter* (rather than that of *Helicobacter*) was detected in the bile and biliary epithelium of patients with hepatolithiasis.

These discordant results may be explained by regional differences. However, it has to be emphasized that in most of the studies there was no control group or there were few patients included as controls. In other studies, patients that com- posed the control group had other disorders (such as pancreatic or gastric malignancies) that may have introduced bias (since the presence of *Helicobacter* DNA has been detected in the bile of patients with these diseases). Furthermore, in the studies aimed to investigate the presence of *Helicobacter* in the biliary tree as a risk factor for biliary disease, no adjustment for confounding factors was done.

So we did a comparative study on helicobacter pylori as a causative agent in gall bladder tissue with symptomatic cholecystitis / cholelithiasis and incidental cholelithiasis.

## II. Aim And Objectives

**Aim Of The Study**
The study was undertaken to determine the presence of H.pylori as a causative agent of cholelithiasis.
**Objectives**:
A comparison regarding SYMPTOMATIC CHOLECYSTITIS/CHOLELITHIASIS AND INCIDENTAL CHOLELITHIASIS by PCR and Giemsa staining
1. Study about gall stones
2. To identify H.pylori association

**Eligibility Criteria**
**A.Inclusion criteria**:
- Patients more than 25 years and up to 60 years of age groups in both sexes presenting with cholelithiasis in GRH Madurai.
- Patient with BMI between 20 to 27
- Patients consented for inclusion in the study according to designated proforma

**B.Exclusion criteria**:
- Patients less than 25 years of age
- Patients more than 60 years of age.
- Macroscopic malignancy and perforation.
- Patient with severe co morbidities.
- Patients with BMI >27.
- Patient not consented for inclusion in the study.

## III. Methodology

MATERIALS AND METHODS :

In this case-control study, patients who underwent cholecystectomy were divided into case and control groups. Case group consisted of patients who underwent cholecystectomy due to cholecystitis or cholelithiasis and the control group consisted of patients who underwent this procedure for incidental cholelithiasis . Participants included in this study were patients admitted to Govt Rajaji hospital at Madurai from November 2017 to September 2019.
Gallbladder tissue was taken from all patients immediately after cholecystectomy. The samples were immediately frozen at _80°C before processing for culture and DNA extraction was performed.

HISTOLOGICAL STUDY:

Gallbladder tissue specimens for histology were fixed in 10% buffered formalin immediately after cholecystectomy. The samples were then embedded in paraffin wax and 5-_m-thick histological sections were stained with hematoxylin and eosin for histological analysis.

The samples were examined by a pathologist who was unaware of their origin. The diagnosis of cholecystitis was based on the presence of mono- or mono- and olymorphonuclear inflammatory cells in the lamina propria, fusion of the mucosal folds giving rise to buried crypts of epithelium, and the presence of Rokitansky-Aschoff sinuses. Gallbladder specimen stained with giemsa for Helicobacter species.

DNA ISOLATION:

Gallbladder tissue or bile DNA was extracted with a QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations, with minor modifications. Briefly, approximately 25 mg of tissue and 500 _l of bile samples were suspended in 180 _l of lysis buffer (buffer ATL) and homogenized by vortexing.

A total of 20 ml of a proteinase K solution (20 mg/ml) was then added, followed by an overnight incubation at 56°C. A second lysis buffer (buffer AL) provided in the kit was added, and the sample was incubated at 70°C for 10 min. Next, 200 _l of ethanol was added; this mixture was then loaded on the QIAamp spin column and centrifuged at 6,000 _ g for 1 min.

The QIAamp spin column was placed in a 2-ml collection microtube, and the containing filtrate was discarded. The column material was washed twice (250 _l each time) with the first buffer (buffer AW1) and twice (250 _l each time) with the second washing buffer (buffer AW2) provided in the kit. Finally, the DNA was eluted with 100 _l of distilled water (2 _ 50 _l). The DNA concentration was determined by measuring the optical density at 260 nm.

PCR AMPLIFICATION WITH HELICOBACTER GENUS-SPECIFIC PRIMERS:

The 16S rRNA gene of the genus Helicobacter was amplified by a nested PCR assay. The outer primer pair (B37 and C70) (4) was used to generate 16S rRNA amplicons of approximately 1,500 bp. The nested inner primer pairs, which are specific for the Helicobacter genus, amplified fragments of 1,200 bp (primer pair C97 and C05) or 400 bp (primer pair C97 and C98) (3).

PCRs were performed in an Applied Biosystems thermal cycler in thin-wall tubes. A 10-_l amount of each DNA preparation was added to 100 _l of a reaction mixture containing 1% Taq polymerase buffer (50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl [pH 8.3]), a 0.5 _M concentration of each primer, a 200 _M concentration of each deoxynucleotide, and 2.5 U of Taq polymerase. The amplified product was identified by electrophoresis in a 1.0% agarose gel.

The DNA was stained with ethidium bromide and examined under UV light. In the second round, 1 _l of the PCR product was added to the reaction mixture. The sequences of the primers and PCR conditions are shown in Table 1. An Escherichia coli strain (clinical isolate) and a H. pylori strain (TX30A) served as negative and positive controls, respectively and distilled water was used as an internal reaction negative control.

16S rRNA GENE SEQUENCING:

The nested PCR products of 1,200 or 400 bp were purified using a Wizard PCR-Prep purification kit (Promega, Madison, Wis.) according to the manufacturer's directions. The purified amplicons were directly sequenced with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions by using sequencing primers B35, B36, C01, C31, and X91 for the amplicons with 1,200 bp or C97 and C98 for those with 400 bp (3, 4).

The sequences were determined in an Applied Biosystems DNA automated sequencer (ABI PRISM 310; Applied Biosystems). The sequences were aligned using the CAP program at the INFOBIOGEN web server and compared (using the Blast Program at the National Center for Biotechnology Information computer server) with sequences listed in the GenBank database.

1] Kuruvammal 54 female who diagnosed as acute cholecystitis underwent open cholecystectomy. GB specimen sent for giemsa staining and PCR .

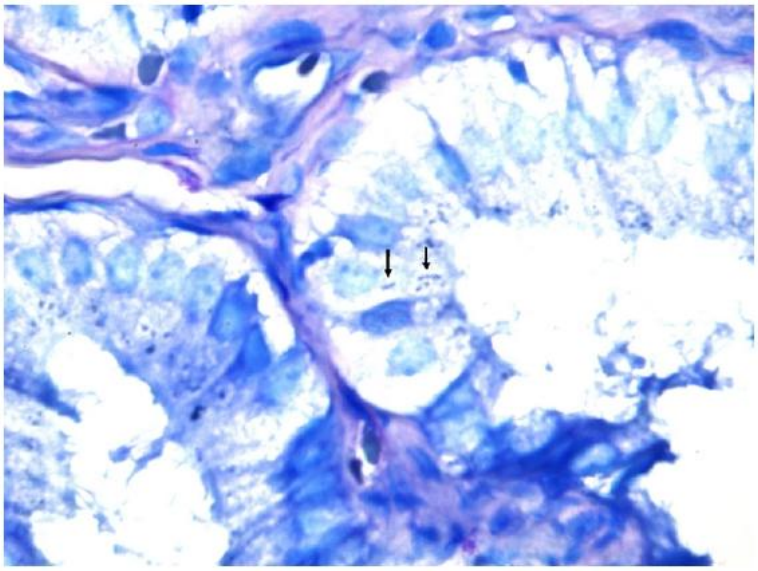

**Figure 1 -** Specimen positive for Helcibactor species with Giemsa stain.

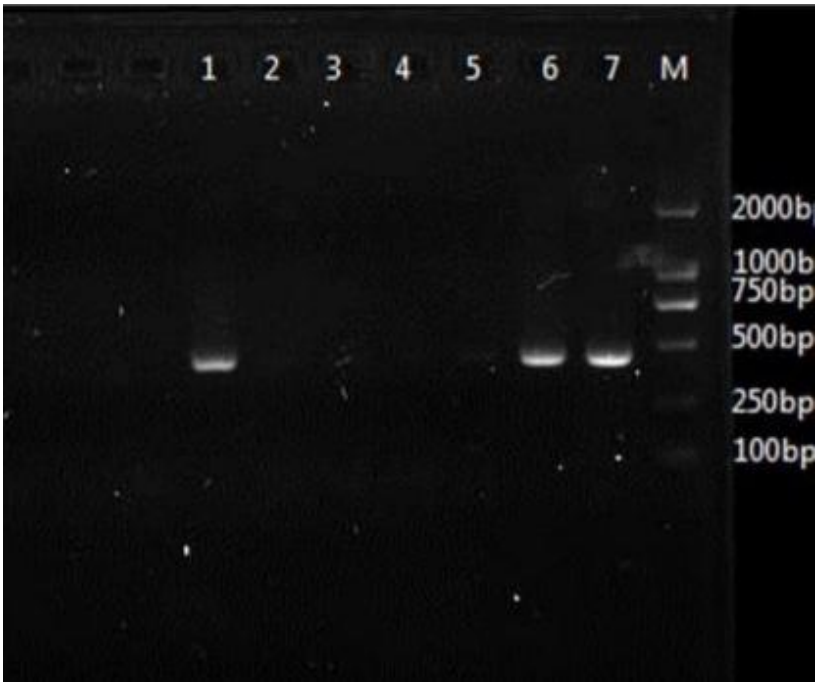


**Figure 2 – PCR result of Kuruvammal 54 yrs / F**

PCR product of helicobacter specific 16s rRNA gene from gall bladder and gastric mucosa sample. ( lame M – step ladder marker ; 1- positive control of gastric biopsy derived h.pylori DNA ;2- negative control of gastric biopsy; 3-negative 16s rRNA gene: 4,5-negative for 16s rRNA gene in gall bladder in one individual patient: 6,7- positive from 16s rRNA gene in the study patient )

## IV. Observation And Results

**Statistical analysis**

All analysis were done using SPSS version 16(SPSS Inc., Chicago, IL). The clinical, demographic, diagnostic variables were compared for symptomatic and asymptomatic groups. Chi square test or Fisher's Exact Test was used to compare categorical variables.

Descriptive statistics was computed. Continuous data were tested for normality using Shapiro wilks normality test. Since the data levels were not normally distributed, a non-parametric test, [the Mann-Whitney U test] was used to compare age and BMI between groups. The confidence interval was set at 95%.

**Table 1 - Comparison Of Variables Among Symptomatic And Asymptomatic Group**

| Variable | | Symptomatic group N | (%) | Asymptomatic group N | (%) | p value |
|---|---|---|---|---|---|---|
| **Diabetes** | Absent | 12 | 48.00% | 10 | 40.00% | 0.776 |
| | Present | 13 | 52.00% | 15 | 60.00% | |
| **USG Findings** | Cholelithiasis | 19 | 76.00% | 0 | 0 | |
| | Acute cholecystitis | 6 | 24.00% | 0 | 0 | |
| **Stone size** | < 2.5 | 12 | 48.00% | 16 | 66.70% | 0.252 |
| | > 2.5 | 13 | 52.00% | 8 | 33.30% | |
| **Wall thickness** | Absent | 17 | 68.00% | 25 | 100.00% | 0.004* |
| | Present | 8 | 32.00% | 0 | | |
| **GB polyp** | Absent | 25 | 100.00% | 21 | 84.00% | 0.035* |
| | Present | 0 | 0 | 4 | 16.00% | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **LFT** | normal | 16 | 64.00% | 19 | 76.00% | 0.538 |
| | Raised | 9 | 36.00% | 6 | 24.00% | |
| **OGD sopy** | No ulcer | 19 | 76.00% | 20 | 80.00% | 0.5 |
| | Ulcer present | 6 | 24.00% | 5 | 20.00% | |
| **Type of surgery** | Laproscopy | 19 | 76.00% | 23 | 92.00% | 0.247 |
| | Open surgery | 6 | 24.00% | 2 | 8.00% | |

Fisher's exact test; shows (*p<0.05)

***Demographic variables***

Among the total of 50 patients included in the study, 25 symptomatic patients with cholelithiasis (76%) and acute cholecystitis (24%) and 25 asymptomatic patients were evaluated. There was no significant difference in the age of the subjects in the 2 study groups. The mean age of the Symptomatic patients was found to be 44.84± 6.5 (SD) yrs while asymptomatic patients averaged 46.4 ± 7.9 yrs (p = 0.386). In which Gender distributions were equivalent, with male/female distribution of 12/13 for the patients presenting with symptoms and 13/11 for the subjects in the asymptomatic group (p = 0.547).Similarly BMI also found to be similar in both the groups with mean BMI of 24.96±1.2 in symptomatic and 24.92±1.4 in asymptomatic patients (p=0.902)

***Comparison of clinical and biochemical variables in study groups.***

In symptomatic group, 52% patients were presented with stone size more than 2.5 cm and 33% in asymptomatic group but the difference was not statistically significant (p=0.252).But 32% patient in symptomatic group had wall thickness whereas none of them in asymptomatic group (p=0.04*). Furthermore LFT was found to be raised in 36% of symptomatic group and 24% in asymptomatic group (P=0.5). Although there was no significant difference in patients presented with ulcer between study groups (p=0.5). Among 50 patients (76 %) underwent laparoscopic cholecystectomy for cholelithiasis/calculous cholecystitis in symptomatic group and only 8 patients underwent open surgery in which 6 of them were from symptomatic group. Among the asymptomatic group, 4(16 %) patients had gallbladder polyp but none of them in symptomatic group (p= 0.035*).

**Table 2 - Association of the Presence of *Helicobacter* in Gallbladder Tissue with Asymptomatic and symptomatic group**

| | variables | Symptomatic group N | (%) | Asymptomatic group N | (%) | p value |
|---|---|---|---|---|---|---|
| **Sex** | Female | 12 | 48.00% | 13 | 52.00% | 0.547 |
| | Male | 13 | 52.00% | 11 | 44.00% | |
| **GIEMSA staining** | negative | 17 | 68.00% | 23 | 92.00% | 0.074 |
| | positive | 8 | 32.00% | 2 | 8.00% | |
| **PCR** | Absence of Helicobacter DNA in gall bladder tissue | 15 | 60.00% | 22 | 88.00% | 0.06 |
| | presence of Helicobacter DNA in gall bladder tissue | 10 | 40.00% | 3 | 12.00% | |

Fisher's exact test; Not significant

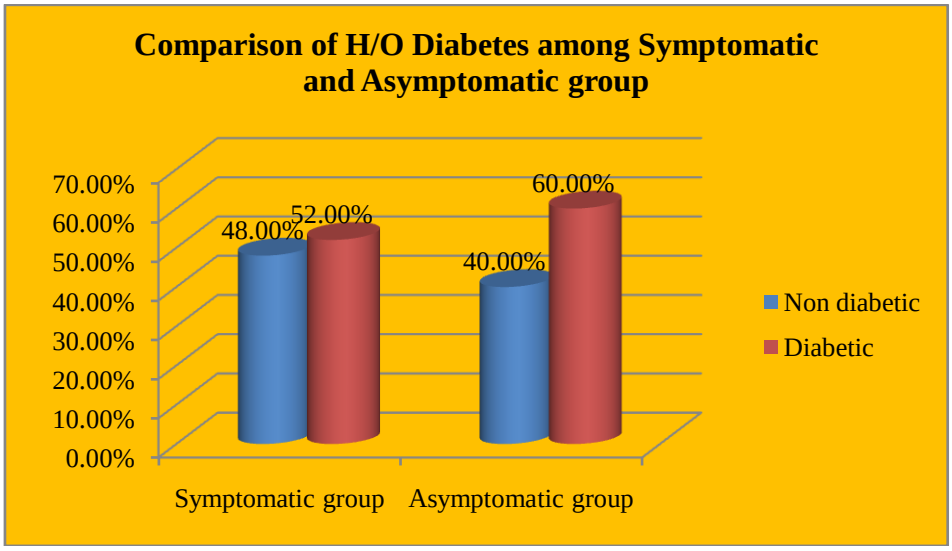


**Chart 1 - Comparison of H/O Diabetes among Symptomatic and Asymptomatic group**

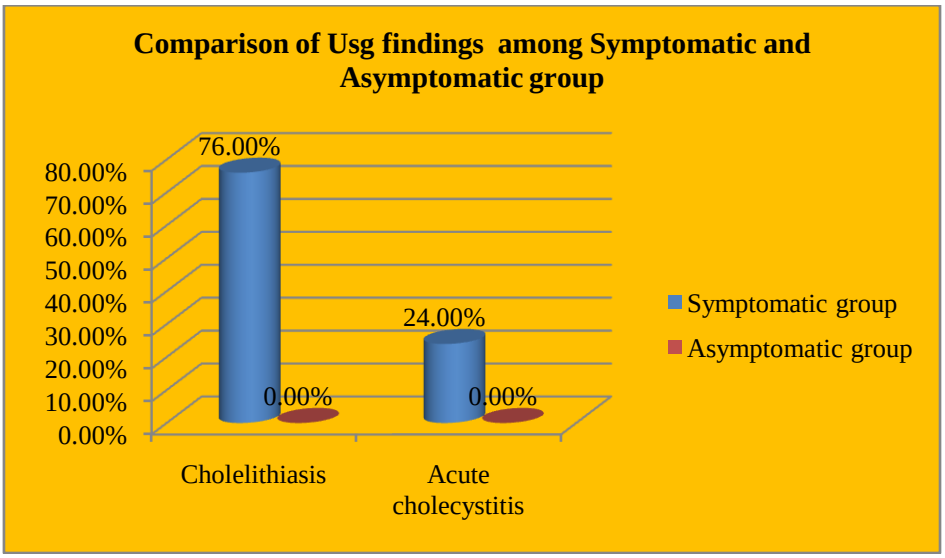


**Chart 2 - Comparison of USG findings among Symptomatic and Asymptomatic group**

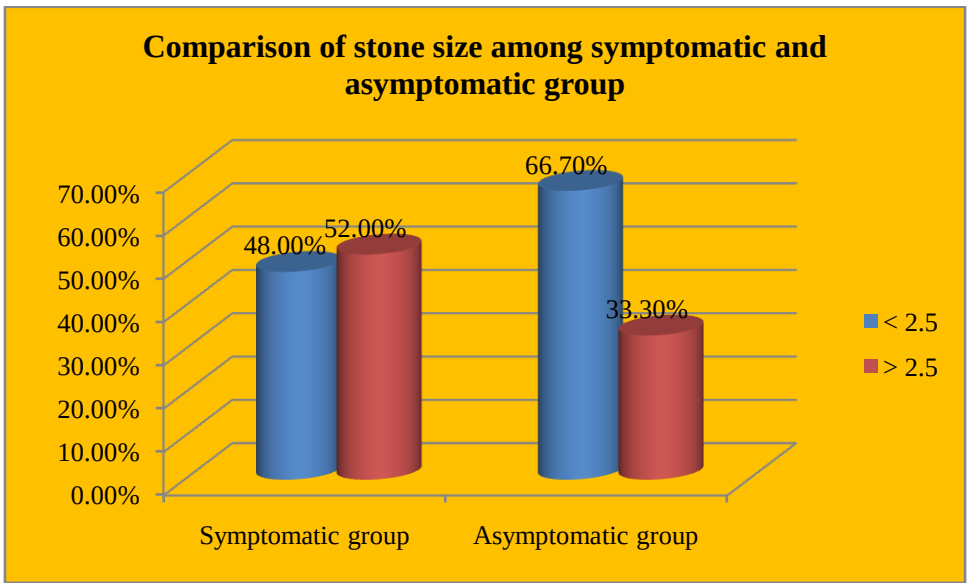


**Chart 3 - Comparison of stone size among symptomatic and asymptomatic group**

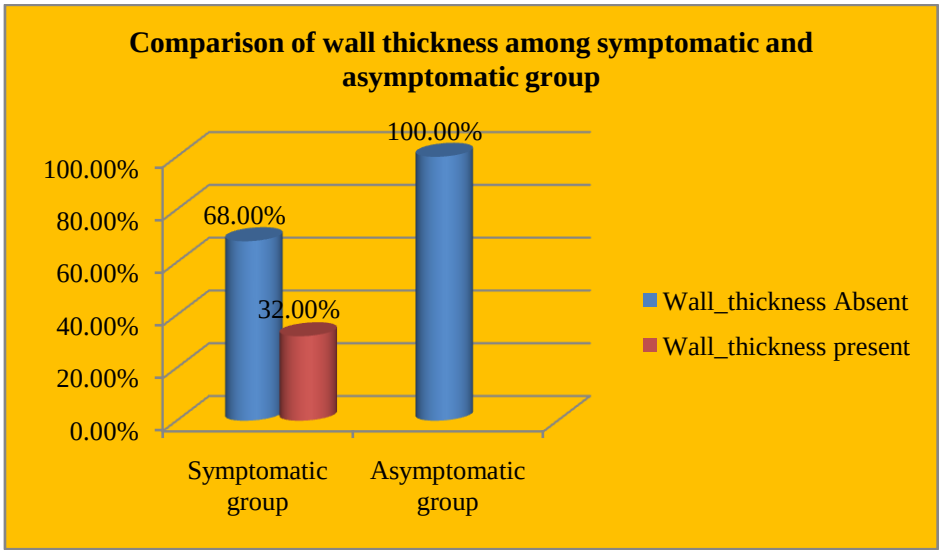


**Chart 4 - Comparison of wall thickness among symptomatic and asymptomatic group**

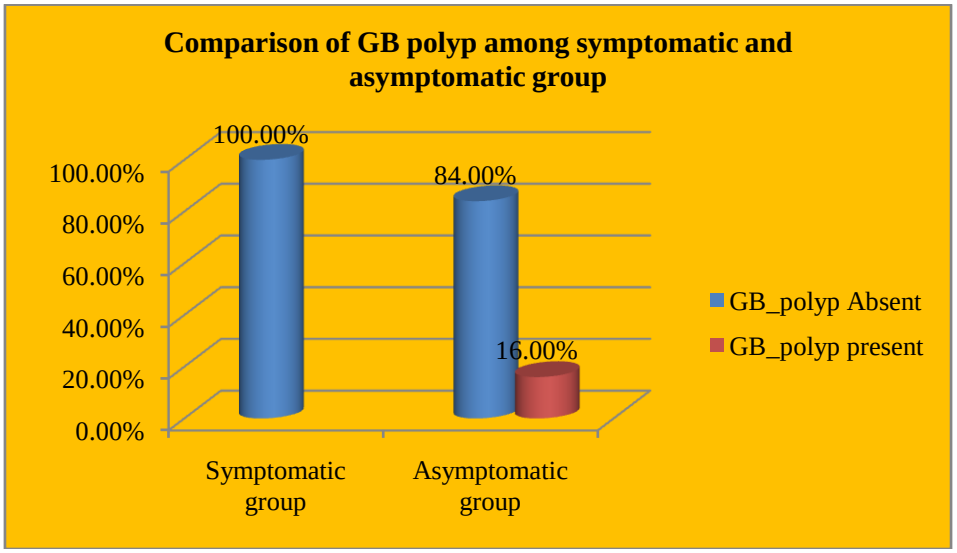


**Chart 5 - Comparison of GB polyp among symptomatic and asymptomatic group**

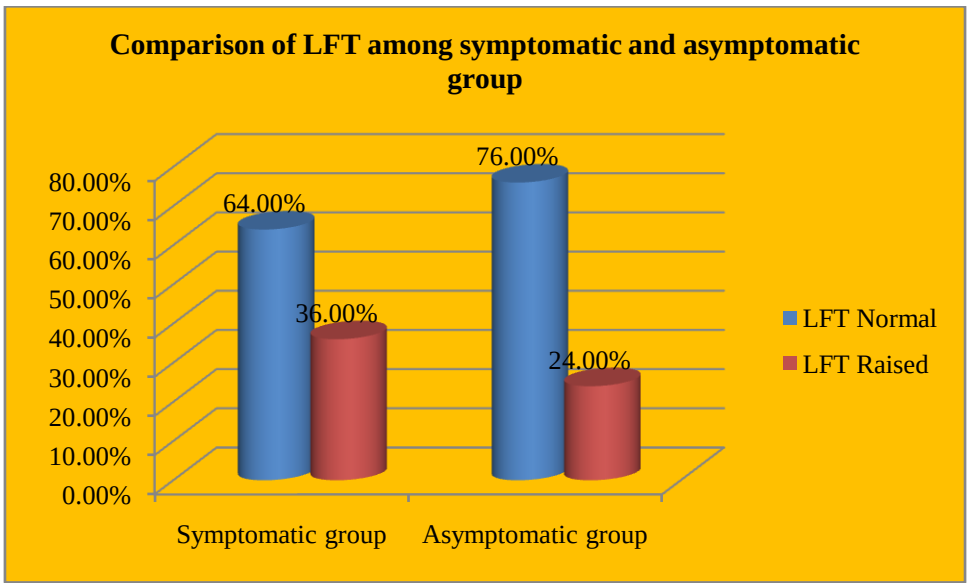


**Chart 6 - Comparison of LFT among symptomatic and asymptomatic group**

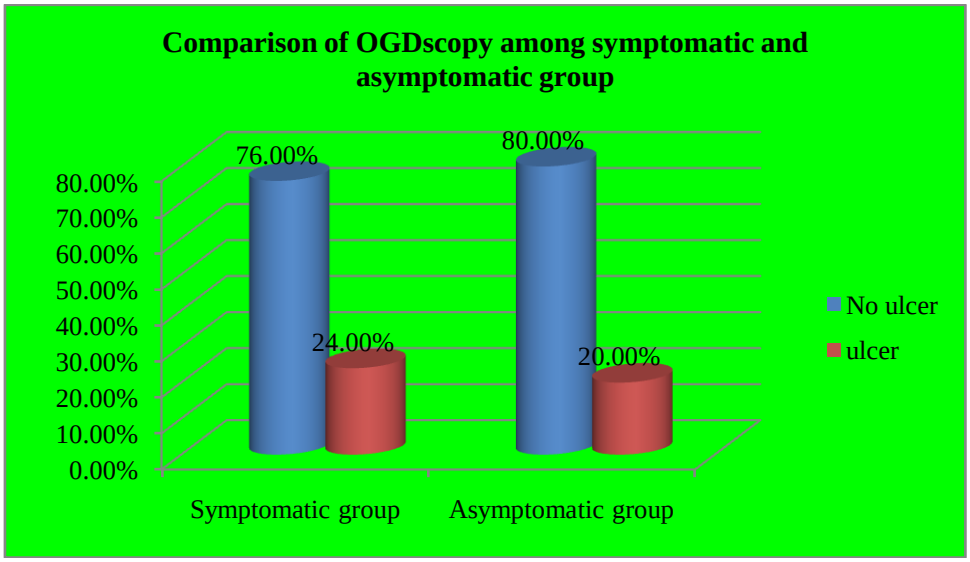


**Chart 7 - Comparison of OGDscopy among symptomatic and asymptomatic group**

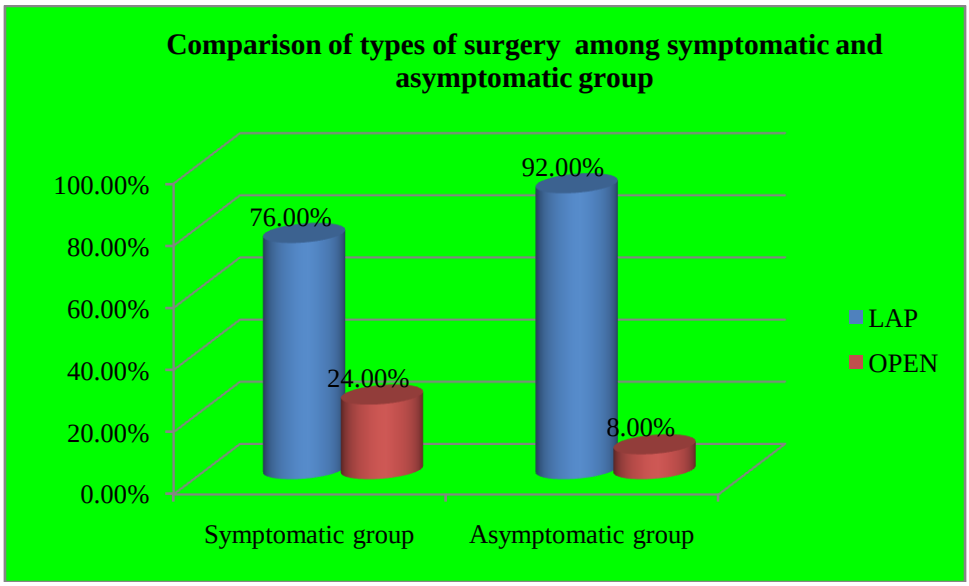


**Chart 8 - Comparison of types of surgery among symptomatic and asymptomatic group**

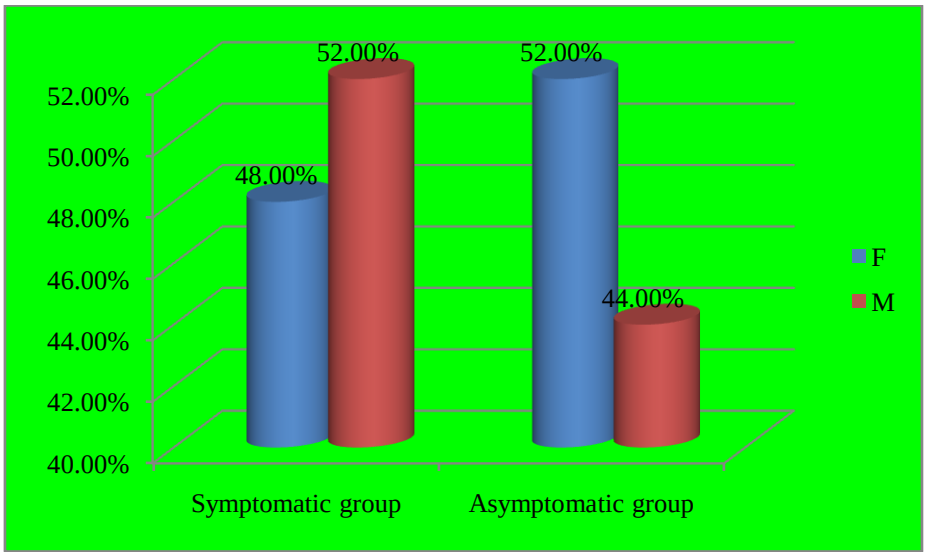


**Chart 9 – Age distribution among both groups**

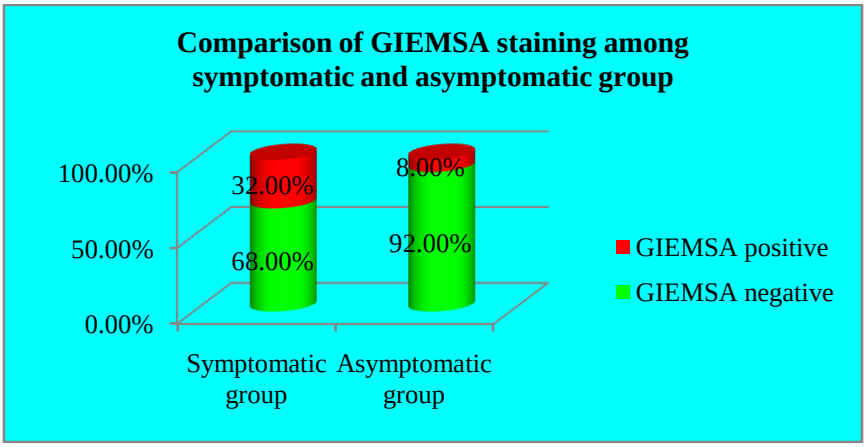


**Chart 10 - Comparison of GIEMSA staining among symptomatic and asymptomatic group**

Modified Giemsa staining detect *H. pylori* in 8 (32%) patients in symptomatic group and only 2 (8%) in asymptomatic group among the total 50 samples analyzed. Though the difference exist between the group which was not statistically significant (p=0.074).

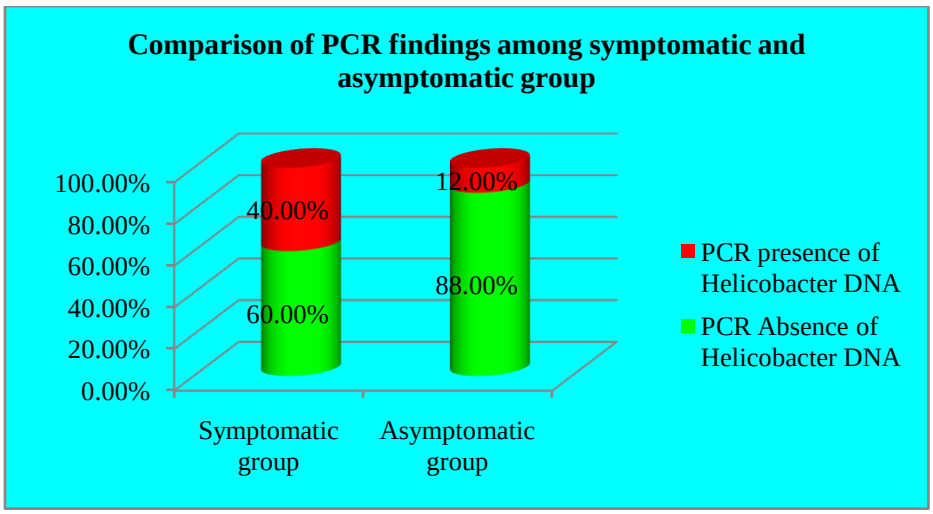


**Chart 11 - Comparison of PCR staining among symptomatic and asymptomatic group**

*Helicobacter* DNA was detected by nested PCR in the gallbladder tissue from 13 out of 50 patients in the study group. Among *Helicobacter* DNA-positive patients, 10 (33%) were from symptomatic group and 3 (12%) in asymptomatic group (p=0.06). Both the shorter (400-bp) and the longer (1,200-bp) amplicons were obtained in the samples of all positive patients.

**Table 3 - Comparison of age and BMI among study groups**

| | Group | N | Mean | Std. Deviation | Std. Error Mean | p value |
|---|---|---|---|---|---|---|
| **Age in yrs** | Symptomatic group | 25 | 44.84 | 6.562 | 1.312 | 0.386 |
| | Asymptomatic group | 25 | 46.64 | 7.926 | 1.585 | |
| **BMI** | Symptomatic group | 25 | 24.968 | 1.2628 | 0.2526 | 0.902 |
| | Asymptomatic group | 25 | 24.92 | 1.462 | 0.2924 | |

Mann whitney U test; Not significant

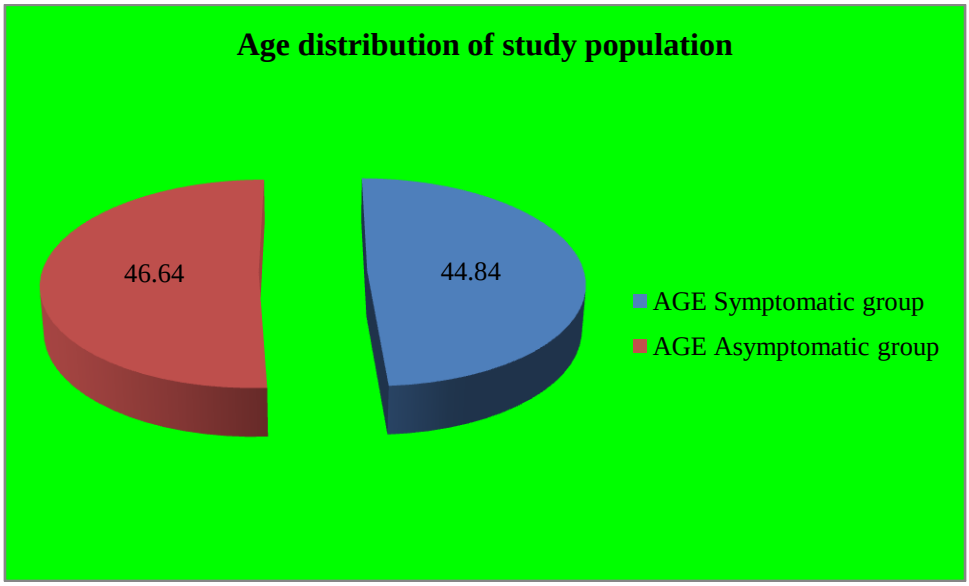


**Chart 12 - Comparison of age and BMI among study groups**

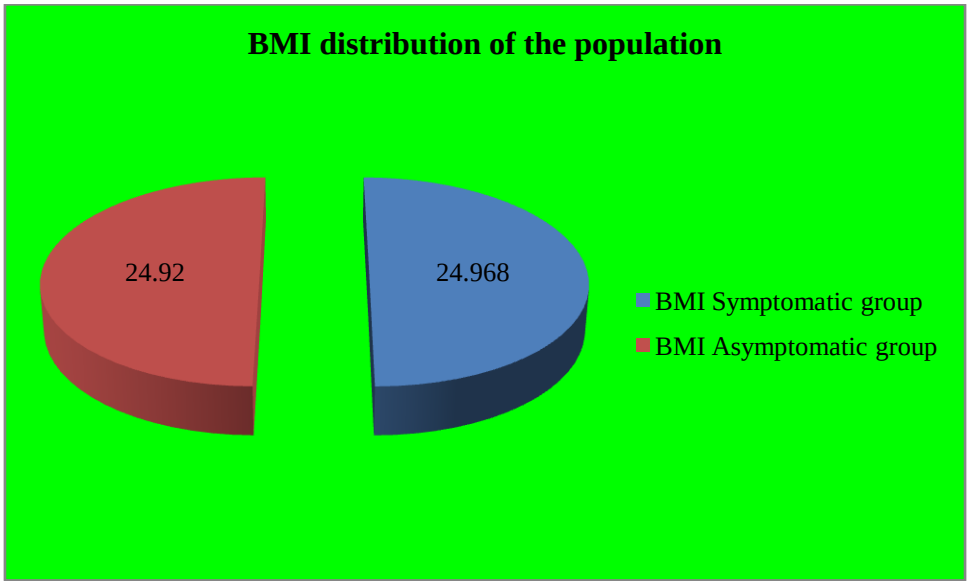


**Chart 13 - BMI distribution of the population**

## V. Discussion

1. H. pylori contributes to the formation of gallstones.

The relationship between H.pylori and gallbladder diseases, specifically gallstones, is still a controversial matter due to conflicting studies and inconclusive reports. However, there is enough evidence to show that bacterial population of H.pylori increases the risk of developing cholesterol-type gallstones. There are different mechanisms responsible for this condition but recent studies have highlighted the role of H.pylori.

According to a study published by the World Journal of Surgical Oncology, H.pylori releases a protein similar to that of an aminopeptidase enzyme which sets the stage for gallstone formation. This enzyme has cholesterol crystallization promoting abilities. Therefore, the presence of H.pylori can contribute to the formation of gallstones and serve as a starting point for infection around which a stone can develop. Aside from releasing proteins, it also produces soluble antigens that can lead to irregularities of the cycling of conjugated bile acids. This may result to abnormal transit time of bile acids.

Aside from the above-mentioned reasons, H.pylori's impact on overall immune system is said to contribute indirectly to lithiasis or stone formation.

2. H. Pylori aggravates gallbladder inflammation.

There are numerous known causes of chronic cholecystitis. One of them is the presence of bacterial infection in the biliary system. Various studies have shown that H. pylori is correlated with gallbladder inflammation, through the same mechanism that it contributes to the development of different gastrointestinal diseases.

Lab tests prove that in an H. pylori infected gallbladder, the cells lining the gallbladder are destroyed, with swollen mitochondria and dilated endoplasmic reticulum. These are crucial parts of the cell needed for energy, as well as the production and transport of proteins.

Rapid decrease in cell division, cell rupture, and cell death are all effects of H.pylori infection. The toxic factors in H pylori can activate factors inhibiting cell proliferation and ultimately lead to the death of cells.

Exposure of gallbladder cells to H. pylori also activates inflammatory cells in three different ways: via cellular immunity, humoral immunity, and autoimmunity.

3. H. pylori increases the risk of developing gallbladder tumors and gallbladder cancer.

H.pylori's role in inflammation leads to another gallbladder complication in which the bacteria contributes to the development of tumors and cancer of the gallbladder.

It is hypothesized that H.pylori plays a crucial role in the development of benign tumors and the higher prevalence of adenomyomatosis (GAM). GAM, also called adenomyomatous hyperplasia of the gallbladder, involves the wall thickening of the gallbladder wall, cholesterol accumulation, cholesterol crystallization, and/or enlargement of the gallbladder. Though GAM is usually asymptomatic, this condition can be an initial stage of a developing gallbladder cancer.

Gallbladder cancer, on the other hand, is characterized by chronic inflammation brought about by the presence of H.pylori. This leads to DNA damage, cell death and modulated enzyme activities. In 1994, the International Agency for Research on Cancer declared that Heliobacter pylori infection is associated with the development of stomach cancer. Aside from stomach and gallbladder cancer, H.pylori has been linked to non-Hodgkin's lymphoma.

Aside from the gastrointestinal tract and the gallbladder, other closely related organs within the biliary system like the liver and pancreas can also be severely affected by the proliferation of H. pylori.

Experiments in animal models have proven that the Heliobacter species can cause hepatitis, liver cancer, and severe damage to the immune system. H.pylori colonization is also a known culprit in pancreatic cancer.

Outside the biliary system, H.pylori has now been implicated in diverse conditions such as skin diseases, coronary artery disease, autoimmune diseases, and growth retardation in children. In this study, a total of 73 patients diagnosed with symptomatic gallstones have been admitted for laparoscopic cholecystectomy where a sample from stool and from bile were collected and tested for the presence of *H.pylori* antigens for all patients. There were 63 female (86.3%) and 10 (13.7%) males with age ranging from 28-63 years, mean age 41 (SD11.3) years.

Twenty three patients (31.5%) have positive *H. pylori* antigen in their stool samples, while 50 patients (68.5%) have negative test. Twenty one patients (28.8%) have positive *H. pylori* antigenin in their bile samples, while 52 patients (71.2%) have negative test. This shows the biliary colonization by *H. pylori* in patients with symptomatic gallstones.

Subgroup analysis revealed that sixteen patients (21.9%) have positive test for *H.pylori* antigen in their stool, but are bile-negative, and fourteen patients (19.2%) positive for *H.pylori* antigen in their bile, but are stool- negative. In contrast, only 7 patients (9.6%) revealed positive result in both specimens (stool and bile), with a P-value of 0.0002 which is highly significant

There was no correlation between the presence of *H.pylori* antigen in stool and bile with the sex of the patients with P- value =0.449.

This study showed the biliary colonization by *H. pylori* in patients with symptomatic gallstones was (28.8%), although it is an unusual anatomical site for H. pylori colonization. This is similar to Farshad *et al.,* (2004) who reported the presence of DNA but not antigen in 18.1% of gallstones and suggested that *H.pylori* infection may serve as initiating factor in development of gall stones (Farshad *et al*., 2004; Fox *et al*., 1998; Bulajic *et al.,* 1946; Sheta *et al*., Pandey, 2007; Figura *et al.,* 1998).

In our study,a total of 50 patients diagnosed with both symptomatic and asymptomatic gall bladder have been admitted for laparoscopic/open cholecystectomy and GB specimens were collected and tested for presence of H.pylori in GB wall with giemsa staining and PCR, with age group ranging from 25-60,with mean age 44.84 in symptomatic group and 46.6 in asymptomatic group. there were 26 females and 24 males.out of 50 patients 25 were symptomatic and 25 were asymptomatic. Out of 25 symptomatic patient 8 were positive for giemsa staining (32%) and 10 were positive for PCR (40%). In asymptomatic group,out of 25, 2 were positive for giemsa staining (8%) amd 3 were positive for PCR (12%). This study also shows that out of 50 patients 26 were female. So female were slightly more common to develop cholelithiasis.

In this comparative study,giemsa staining for H.pylori in gall bladder specimen was positive in 8 patient in symptomatic group and 2 patients in asymptomatic group. The test of significance is 0.074. PCR test concludes 10 patient were positive in symptomatic group 3 patient were positive in asymptomatic group. The test of significance is 0.061. as p value is more than 0.05, this study is concluded insignificant.

LIMITATION: small number of cases.

## VI. Conclusion

Gall bladder colonization by H.pylori infection might be a insignificant factor in development of gall stones and cholecystitis. Whether eradication therapy for H.pylori infection may or may not be helpful in gall stone formation is yet not settled down.

## VII. Recommendations

- Further studies with larger samples of patients are needed to confirm a causal relationship between H.pylori infection and gallstone formation and other hepatobiliary diseases, especially if held in prospective way in asymptomatic patients who are harboring H. pylori, yet have normal gallbladder.
- Although it is not cost-effective, use of PCR to detect H.pylori DNA in bile as well as in gallstones themselves is worthy to try in further studies.

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