

**SURVEY, ISOLATION, BIOCHEMICAL CHARACTERIZATION, AND IDENTIFICATION OF  
*HELICOBACTER PYLORI* FROM GASTRIC PATIENT BIOPSY**THIRUNAVUKKARASU P<sup>1,3\*</sup>, ASHA S<sup>2</sup>, ROHITH KUMARREDDY<sup>1</sup>, RAMANATHAN T<sup>3</sup>, SUDHAKAR N<sup>1</sup><sup>1</sup>Department of Biotechnology, Dr. M.G.R. Educational and Research Institute University, Chennai - 600 095, Tamil Nadu, India.<sup>2</sup>Department of Biochemistry, DKM College for Women, Vellore, Tamil Nadu, India. <sup>3</sup>Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Cuddalore - 608 502, Tamil Nadu, India. Email: ppthirunacas@gmail.com

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**ABSTRACT****Objective:** This study was to isolate and identify the *Helicobacter pylori* from the biopsy samples of the gastric patient.**Methods:** Gastric biopsies were collected from the antral region of the gastric patient. Out of 96 patients, 59 males and 37 females in the age group between 11 and 70 years old were selected. A serial dilution of the sample was made. The bacterial colonies were examined on the basis of Gram staining, colony morphology, and biochemical reactions such as catalase, urease, oxidase, nitrate reduction, glycine utilization, growth (different media, different temperature, and salt tolerance), and antibiotic sensitivity.**Result:** From the findings, it was found that 75% (65% of male and 35% of female) have *H. pylori* infection remaining 25% were not infected. The rate of infection was found to be more in age group 55-65 and less in age group below 25. Among 75% of *H. pylori* infected patients, 72% are affected with ulcer, 19% with gastric cancer, and 8.3% found to be non-gastric inflamed. Gram staining result declared that the isolated bacteria from the biopsy sample observed to be Gram-negative, spiral shaped rod. Biochemical reports produced positive indication to all the tests.**Conclusion:** Based on the morphological, staining and biochemical test result, it was confirmed that the isolated bacteria was found to be *H. pylori*. Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related to death worldwide. In 1994, the international agency for research on cancer classified *H. pylori* as a Class I (definite) carcinogen, as *H. pylori* infection is considered as an important trigger in the process of carcinogenesis of both types of distal gastric cancer.**Keywords:** *Helicobacter pylori*, Gastric patient, Biopsy, Gram staining, Biochemical assay.©2017 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>) DOI: <http://dx.doi.org/10.22159/ajpcr.2017.v10i6.18670>**INTRODUCTION**

The *Helicobacter* genus belongs to a group of microaerophilic, nonsporulating, Gram-negative, spiral-shaped bacterium [1], which colonizes the mucous layer in the epithelial surface of the gastrointestinal tract and lymphoid tissue of human beings [2]. Earlier, some *Helicobacter* species such as *Helicobacter aurati*, *Helicobacter bilis*, and *Helicobacter muridarum* were isolated from both gastric and entero hepatic sites [3]. In world's population, approximately, 50% of colonized *Helicobacter pylori* varied by prevalence, age, country, ethnic background, and socioeconomic conditions [4]. The prevalence in developing countries can be as high as 80-90%, whereas it is lower in industrialized countries, ranging between 10% and 50% [5].

Initially Warren and Marshall [6], first isolated *H. pylori* bacterium from gastric biopsies patients with active chronic gastritis, duodenal ulcer or gastric ulcer, and gastric cancer [7]. They were later awarded the Nobel Prize in 2005 for their discovery of *H. pylori* and its role in gastritis and peptic ulcer disease. Several researchers confirmed that *H. pylori* eradication cured peptic ulcer [8-10] gastroduodenal disease [11,12]. Hence, this study was designed to isolate and identify the *H. pylori* from biopsy samples of the gastric patient.

**METHODS**

Preliminary standardization experiments were conducted and only those which gave reproducible results were adopted.

**Reagents**

Biopsy samples from gastric patients, Columbia agar, *Brucella* broth, heat infusion agar, serum supplemented plate agar nutrient agar, Gram's iodine, crystal violet, safranin and ethyl alcohol.

**Isolation of bacterium**

A total of 96 patients with a male-to-female ratio (59 males and 37 females), ranging from 11 to 70 years old with dyspeptic symptoms were studied from the M/s Maruthi Gastro Care Hospital, Cuddalore, Tamil Nadu, India. Gastric biopsies from the antral region were obtained through esophagogastroduodenoscopy from each patient. Biopsy samples were collected in pre-sterilized vials and stored them in an ice pot immediately until transfer to ultra-freezer at -20--40°C. Number or identification codes were given based on the samples collected. Each biopsy sample was ground in 1 mL of biological saline water using pre-sterilized tissue grinder.

About 1 mL of sample was serially diluted with sterilized distilled saline up to 10<sup>-1</sup>-10<sup>-10</sup> and 100 µL of each dilution was added to 20 mL of Columbia agar medium in 90 mm diameter sterile Petri dishes, then plates were incubated at 37°C for 10 days under the microaerophilic conditions (5% O<sub>2</sub>, 15% CO<sub>2</sub>, and 80% N<sub>2</sub>) with an anaerobic jar system using Anaerocult C (Merck, Germany) [3,13]. A high degree of humidity during incubation was obtained by placing a wet paper towel in anaerobe jars. Plates were routinely observed for bacterial growth and to check contamination; a suggestive growth of a *Helicobacter* spp. was identified from 3<sup>rd</sup> day, either as small (1 mm or less in diameter), clear, dome-shaped colonies or as a fine, translucent lawn [2]. Growing colonies were examined for *H. pylori* on the basis of colony morphology and positive biochemical reactions for catalase, urease, and oxidase (although many entero hepatic species are urease negative) and negative Gram-stain [14,15]. The growth of *H. pylori* after 3 days of incubation was confirmed using Bergey's Manual of Systematic Bacteriology [16], then it was subcultured on a selective medium and stored it below -20°C until further use.

### Colony and cell morphology

Exponentially overnight grown cultures in *Brucella* broth (Vincent, 1970) were spread on Columbia agar plates and incubated at 37°C for 72 hrs. After 72 hrs, the color and morphology of the colonies were noted. Cell motility and shape of single colony was observed under light microscopy (Nikon, Japan). The bacterial species isolated from gastric patient biopsy samples were identified based on the Bergey's Manual of Systematic Bacteriology [16].

### Motility

A loop full of culture was transferred to *Brucella* liquid medium and incubated on a rotary shaker operating at 200 rpm at 37°C. After 48 hrs of incubation 100 µL was tested for motility on cavity slide.

### Biochemical identification

#### Urea hydrolysis [17]

The hydrolysis of urea was detected by streaking the culture on Christensen agar medium containing peptone besides urea and a small amount of glucose, with phenol red as an indicator and incubated for 2 days.

#### Sterilized by filtration

After sterilization of Part-A in flasks (100 mL/flask), 5 mL of Part-B solution added and the medium was used to prepare plates.

#### Catalase production [18]

The catalase test was performed directly on colonies growing on Columbia agar. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) solution (3%) was used to flood colonies.

#### Test for oxidase [19]

This was performed directly on colonies growing on Columbia agar. A 1% aqueous tetramethyl-p-phenylene diamine-dihydrochloride (TMPPD reagent) impregnated paper on which a loopful of young culture was streaked.

#### Nitrate reduction [20]

A loop full of exponentially grown culture was inoculated into nitrate broth and incubated at room temperature for 2-3 days. After visible growth, 1 mL of the nitrate broth was transferred to a clean test tube, a 2-3 drop of sulfanilic acid solution and 2-3 drop of α-naphthylamine was added and allowed to stand for 5 minutes.

#### Glycine utilization

This test was performed with Columbia agar amended with 1% glycine. A loopful of young culture was streaked and incubated.

#### Growth on different media

A loop full of culture was transferred from purified slants and streaked on plates containing, Columbia agar, *Brucella* agar, blood agar, brain heart infusion agar, serum supplemented plate, and nutrient agar. These plates were incubated at 37°C for 72 hrs.

#### Optimum temperature for growth [18]

The optimum temperature was determined in nutrient broth with a loopful of the bacterium as the inoculum at different temperature in increments (25, 30, 35, 40, and 45°C).

#### Salt tolerance [18]

Salt tolerance was determined in *Brucella* broth with sodium chloride added at different concentrations 0.5, 0.75, 1.0, 1.25, and 1.5% (w/v).

#### Antibiotic resistance/sensitivity

Since resistance to certain broad-spectrum antibiotics is a common feature of most of the *H. pylori*, antibiotic resistance of *H. pylori* was checked with the different group of antibiotics such as nalidixic acid,

trimethoprim, vancomycin, penicillin, and tetracycline cephalothin. Then, minimum inhibitory concentration (MIC) was recorded.

## RESULTS

During the period of isolation of bacteria from (March 2011) patients attended the endoscopy unit at M/s Maruthi gastro care Hospital, Cuddalore, Tamil Nadu, India. Complaining of suggestive symptoms of peptic ulcer disease such as upper abdominal pain, acidity, nausea, and vomiting were reported from these patients through the endoscopic process.

The sample was collected in sterilized tube and immediately transferred to ice box within 2 hrs and brought to the laboratory. The samples were ground with the help of sterile tissue grinder and 1 mL of biological saline was added. The isolated single colony through serial dilution method will be carried out. 10<sup>-7</sup> was isolated better colonies. Further this microbe was confirmed based on morphological and microscope analysis. A total of 96 patient were checked as positive to endoscopic diagnosis during the study period. Out of 96 patients, 63 males and 33 females were aged 11-78 (Table 1). It was also found that a higher and lower level of *H. pylori* infection was observed in 55-65 age and below 25 age, respectively.

*H. pylori* infection was determined in 62 patients and non *H. pylori* infection in 34 patients. *H. pylori* infection in male patients was 43 and remaining 20 patients was non *H. pylori* infection in the same way, out of 33 female patients 22 have an *H. pylori* infection remaining 11 noninfections (Table 2).

In the present finding 65.5 and 33% of male and female patients were affected from gastric inflammation, respectively. 72% of patients affected with ulcer, 19% of patient affected with gastric cancer and 8.3% of patients found noninflamed. Higher cancer patients observed from the age between 35 and 45 age was 11.5% (Table 3).

### Morphological observation

The cells were straight, non-spore forming, motile, helical or curved, rounded ends and spiral periodicity, translucent colonies 1-2 mm in diameter, grows microaerophilically (Fig. 1). Gram's differential staining declared that the isolated bacterium was Gram-negative and spiral-shaped rods (Fig. 2).

**Table 1: Distribution of patients with *H. pylori* and non-*H. pylori* ulcer by age group**

Age (in years)	Number of patients tested	<i>H. pylori</i> ulcer	Non <i>H. pylori</i> ulcer
		Number of patients infected (%)	Number of patient infected (%)
Below 15	3	1 (33)	2 (66)
15-25	13	8 (61.5)	5 (38.4)
25-35	19	13 (68.4)	6 (31.5)
35-45	26	17 (65.3)	9 (34.6)
45-55	19	11 (57.8)	8 (42.1)
55-65	16	12 (75)	4 (25)
Total	96	62 (64.5)	34 (35.4)

*H. pylori*: *Helicobacter pylori*

**Table 2: Distribution of patients with *H. pylori* and non-*H. pylori* ulcer by sex group**

Sex	Number of patient	<i>H. pylori</i> ulcer	Non <i>H. pylori</i> ulcer
Male	63	43-68.2	20-31.7
Female	33	22-66.6	11-33.3

*H. pylori*: *Helicobacter pylori*

**Table 3: Determination of patient gastric ulcer, gastric cancer and non-gastric inflammation**

Age (in years)	Number of patients	Number of patients (%)				
		Sex		Ulcer	Cancer	Non gastric inflammations
		Male	Female			
Below 25	16	10 (62.5)	6 (37.5)	4 (25)	1 (6.2)	11 (68.7)
25-35	19	12 (63.1)	7 (36.8)	15 (78.9)	3 (15.7)	1 (5.2)
35-45	26	17 (65.3)	9 (34.6)	18 (69.2)	5 (19.2)	3 (11.5)
45-55	19	14 (73.6)	5 (26.3)	12 (63.1)	6 (31.5)	1 (5.2)
55-65	16	10 (62.5)	6 (37.5)	12 (75)	3 (18.7)	0 (0)
Total	96	63 (65.6)	33 (34.3)	70 (72.9)	19 (19.7)	8 (8.3)

**Biochemical assay***Urease test*

The hydrolysis of urea was detected by streaking the culture on Christensen agar medium containing peptone besides urea and a small amount of glucose, with phenol red as an indicator and incubated 2d. Formation of pink color indicated that urea hydrolysis and the pH changes were due to the formation of ammonia (Table 4 and Fig. 3).

*Catalase test*

The catalase test was performed directly on colonies growing on nutrient agar. H<sub>2</sub>O<sub>2</sub> solution (3%) was used to flood colonies. A brisk effervescence indicated a positive test for catalase production (Table 4 and Fig. 4).

*Oxidase*

This was performed directly on colonies growing on Columbia agar. A 1% aqueous *N,N,N',N'*-TMPPD reagent impregnated paper on which a loopful of young culture was streaked. Bacterial culture turned purple (bluish black) color within 1 min indicated oxidase presence (Table 4 and Fig. 5).

*Nitrate reduction test*

Development of noncolor formation indicated (red) negative to result (Table 4).

*Glycine utilization*

After 48 hrs of incubation, ninhydrin solution was flooded on the agar surface color formation occurred with ninhydrin solution, hence the bacterium was negative to test for glycine utilization (Table 4).

*Growth on different media*

The growth of bacterium was checked in different media, such as nutrient media, Columbia agar, *Brucella* agar, Brain heart infusion agar, and Serum supplemented plate. Among the media higher level of growth was observed in Columbia and *Brucella* agar plate. The plates with nutrient agar were devoid of any bacterial growth (Table 4).

*Growth at different temperature*

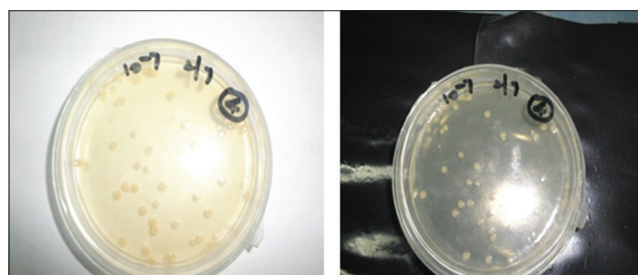
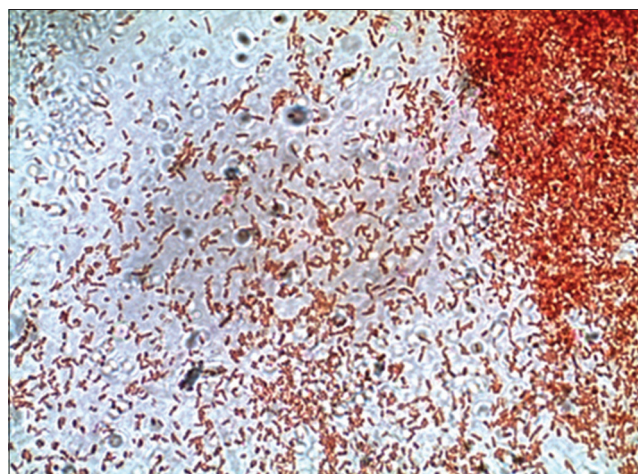
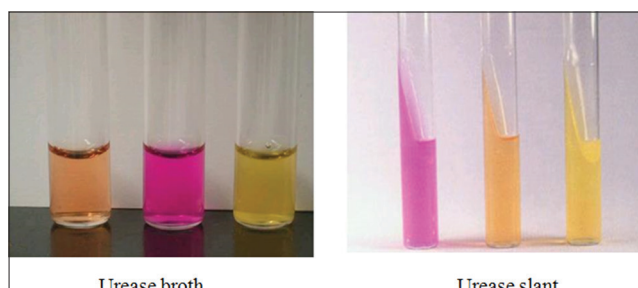
The optimum temperature for growth was determined in *H. pylori*. In this study, different temperature regiments were followed such as 25, 30, 35, 40, and 45°C. Among different temperatures, 25°C and 30°C had no growth but at 35 and 40°C good growth was observed whereas at 45°C no growth of *H. pylori* was observed (Table 4).

*Growth in salt tolerance*

Salt tolerance was determined in *Brucella* broth with sodium chloride added at different concentration (0.5, 0.75, 1.0, 1.25 and 1.5%, w/v). Good growth was observed up to 1.25% NaCl whereas at 1.5% NaCl, growth was completely arrested (Table 4).

*Antibiotic resistance/sensitivity*

The antibiotics resistance and sensitivities are an essential assay for the pathogen. In the present research, different kinds of antibiotics such

**Fig. 1: Isolation of Helicobacter pylori from gastric patient in petri plate****Fig. 2: Gram staining of Helicobacter pylori with microscopic observation****Fig. 3: Conformation of Helicobacter pylori through urease test**

as nalidixic acid, trimethoprim vancomycin, penicillin, tetracycline, and cephalothin were used. Then, the MIC was recorded. Among the tested antibiotics, the bacterium showed moderate sensitivity to nalidixic acid, trimethoprim, and vancomycin. These resistant characters are also very much useful in selecting marker for classical genetic experiments (Table 4).

Table 4: Biochemical characteristics by of *H. pylori*

Assay	Observation
Colony shape	Round, small, translucent, 2-3 mm
Cell shape	Spiral, helical or curved with blunt ends
Cell motility	+
Gram staining	-
Urease	+
Oxidase	+
Catalase	+
Nitrate reduction	-
Glycine utilization	-
Growth on	
Nutrient agar	-
Columbia agar	+
BruceII agar	+
Blood agar	+
Brain heart infusion agar	+
Serum supplement agar	+
Growth at	
25°C	-
30°C	-
35°C	+
40°C	+
45°C	-
Growth in NaCl (%)	
0.5	+
0.75	+
1.0	+
1.25	+
1.5	-
Resistance/sensitivity (50 µg/ml)	
Nalidixic acid	+
Trimethoprim	+
Vancomycin	-
Penicillin	-
Tetramycine	-
Cephalothin	-

*H. pylori*: *Helicobacter pylori*

## DISCUSSION

*H. pylori* infection is one of the most common infections worldwide. However, it is not known, how *H. pylori* are transmitted and wherein the natural environment the organism resides [21]. It is likely that raw sheep's milk could be an intermediate transmission vehicle of *H. pylori* infection [22]. It has been reported that *H. pylori* are almost always acquired in childhood [23]. In other hand, Sasaki *et al.* [24] reported that *H. pylori* were detected in cow's feces and soil and the bacteria could invade the teat channel of a cow when it was sprawled on the ground including cow's feces and soil [25]. As reported by Fujimura *et al.* [26] that *H. pylori* gene is frequently detected in cow's milk samples, the samples might have been contaminated with the organism from contaminated soil. Furthermore, they have shown the possibility that *H. pylori* survive in raw milk. This study was investigated to isolate, identify, and found the total count of bacterial colony and occurrence in gastric patients.

In this study, 43 males and 22 females were identified to have *H. pylori* infection out of 63 males and 33 females (totally 96 patients) and remaining were identified as non-*H. pylori* infectants. These results were comparable to Al-Sulami *et al.* [27] whose findings diagnosed 136 consecutive dyspeptic patients with peptic ulcer from mucosal antral biopsy specimens by endoscopy at Basra General Hospital, Iraq. Modified Columbia urea agar showed a higher isolation rate than classic Columbia agar (67.6% vs. 44.1% of patients), and the results were obtained faster (24 hrs vs. 5-7 days) with more clear-cut identification. Among the total 136 patients with the positive peptic ulcer, 92 patients (67.6%) showed positive evidence of *H. pylori* infection using bacterial



Fig. 4: Urease assay

Fig. 5: Conformation of *Helicobacter pylori* through oxidase test

culture and 81 patients (59.6%) by histopathological analysis. Among *H. pylori* ulcer patients, the highest detection rates of the bacterium (79.2%) were recorded in the age group 41-50 years, while no single case was recorded in the age group ≤20 years.

Several authors have been studied to evaluate the prevalence of *H. pylori* infection in peptic ulcer disease indicating a range from 60% to 70% at Iraq [28,29]. In this study, a bacterium *H. pylori* was isolated, purified, and identified by various biochemical tests such as catalase, urease and oxidase from the collected samples. Young *et al.* [30] have reported the culture of *H. pylori* was  $1.75 \times 10^{-1}$  CFU/ml from gastric juice collected from the patient in Royal London Hospital, Whitechapel East London. Milyani and Barhameen [31] have characterized the cytopathic effect of *H. pylori* using albino rats and Swiss mice, isolated from the gastric biopsy of a patient at King Khalid National Guard Hospital, Jeddah City, Saudi Arabia.

As similar to our results toward Gram-staining, morphology and biochemical findings, Al-Sulami *et al.* [26] also reported the similar results toward Gram-staining, morphology and biochemical staining. All the isolated *H. pylori* were subjected to Gram-staining, identified the characteristics of this bacterium as Gram-negative and spiral shaped rods and positive result to biochemical tests (oxidase, catalase, and urease).

A variety of media, selective and nonselective, or a combination of both, have been proposed for use in the primary isolation of *H. pylori*, but the optimal method of recovery still remains to be established [32]. Columbia blood agar is a nonselective medium used for many years

alone or in combination with other non-selective and selective media for culturing *H. pylori* from antral biopsy specimens taken from peptic ulcer patients during upper gastrointestinal endoscopy [33]. The isolation rate of *H. pylori* using this medium alone is very variable. Results as low as only 28.5% total isolation rate were reported by Piccolomini *et al.* [34]. The isolation rate of *H. pylori* using Columbia blood agar in the present study was 65%; the colonies were few in number and tiny in size. Bacterial contamination of the medium was frequent. The contaminant bacteria were *Pseudomonas* spp., *Proteus* spp., and *Klebsiella* spp., the source of which could be contaminated biopsy forceps and contamination during obtaining, transporting and preparing of the defibrinated sheep blood added to the classic Columbia agar. The growth rate of *H. pylori* on this medium was slow, as 5-7 days were needed for the colonies to appear. Efforts have been invested to improve the reliability of Columbia agar and allows change it to become selective for *H. pylori*. In this study, we have used Columbia agar and obtained good results. According to the previous report, Columbia agar was found suitable for *H. pylori* culture.

The antibiotics resistance and sensitivities are an essential assay for the pathogen. In the present research, different kinds of antibiotics such as nalidixic acid, trimethoprim vancomycin, penicillin, tetracycline, and cephalothin were used. Then, the MIC was recorded. Among the tested antibiotics, the bacterium showed moderate sensitivity to nalidixic acid, trimethoprim, and vancomycin. These resistant characters are also very much useful in selecting marker for classical genetic experiments.

In our present study, the isolated bacterium *H. pylori* showed only a moderate sensitivity against various tested antibiotic (nalidixic acid, trimethoprim, and vancomycin). Similarly, Kumar *et al.* [35] also found the resistance of *H. pylori* against clarithromycin. Hence, they successfully developed clarithromycin loaded mucoadhesive microspheres to completely eradicate the *H. pylori* in the stomach.

Gastric cancer is the second most common cause of cancer-related mortality worldwide. Detection of the disease usually occurs at an advanced stage and overall survival rates for gastric cancer are poor. The present model for gastric cancer progression clearly maintains *Helicobacter* infection as the primary inducer of gastric metaplastic and neoplastic disease. *H. pylori* are a ubiquitous organism, infecting more than half the world's population. It has been suggested that this infection directly contributes to the formation of gastric cancer in up to 80% of cases; however, gastric malignancy develops in only a subset (<1%) of infected patients. Predisposition to *Helicobacter* associated gastric cancer is most likely multifactorial, including the interaction of bacterial, host and environmental components. The development of intestinal-type gastric cancer is a multistep process [36]. It involves temporal progression from chronic gastritis to gastric atrophy, intestinal metaplasia, dysplasia, and finally gastric cancer. While a number of factors contribute to this transition, it has become clear that *H. pylori* are the primary trigger for neoplastic progression. The association between *H. pylori* and gastric cancer has been known for over a decade [21]. The early studies estimated the prevalence of *H. pylori* in patients with gastric cancer and recent work by Ekstrom *et al.* [37], Uemura *et al.* [38] using a combination of two or three methods to diagnose *H. pylori* infection have shown a much stronger association.

In this present investigation, in total, 96 patient's affected gastric ulcer, 19 patients have affected gastric cancer and 8 have non-gastric inflammation. The discovery of *H. pylori* by Warren and Marshall [6] changed the conventional concept of gastroduodenal ulcer disease. Studies, over the years, suggested a high correlation between *H. pylori* infection and peptic ulceration [39]. The prevalence of *H. pylori* infection shows a wide geographical variation [40]. Although it cannot be said that *H. pylori* cause ulceration, as half of the healthy population also harbors this organism, it had been shown that infection certainly makes the occurrence of ulcers more likely also, eliminating this bacterium reduces the rate of ulcer recurrence to <25%.

Childhood is the critical period for infection and transmission of disease. Most probably occurs from person to person. The iatrogenic route certainly exists but is considered relatively unimportant. Much debate surrounds the oral-oral and fecal-oral routes, which are probably more significant.

## CONCLUSION

The human stomach is the only known reservoir of *H. pylori*. However, the possibility that there are other reservoirs cannot be excluded, as the conditions required for growth are met in the gastrointestinal tract of all warm-blooded animals. *H. pylori* have only been isolated from primates, but other *Helicobacter* species have been isolated from other animals. This suggests the presence of host specific binding sites, although the techniques required for isolation of *Helicobacter* species might differ between various hosts.

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