Detection of *Helicobacter pylori* in Gastroduodenal Diseases by Real Time PCR

Ashok Kumar[1]*and Imran Khan[2]
[1]Department of Biotechnology, H.N.B.G. University, (A Central University), Srinagar- Garhwal (Uttarakhand)
[2]Dept. of Microbiology and genetics, S.G.PG Institute of Medical Sciences Rai Bareily Road Lucknow- India

Abstract:
We have investigated in the present study that *H. pylori* as causative agent of GC remain controversial; therefore, we planned to study the presence of *H. pylori* infection in patients with GC. We also investigated the presence of *H. pylori* infection in patients with peptic ulcer disease (PUD) and non-ulcer dyspepsia (NUD). The subjects of this study comprised of 64 patients (age 24 to 71 yrs, 36 male). Out of 64, 21 with GC and 12 with PUD were included as symptomatic groups (Group I and II) and 31 patients with NUD were included as disease control group (Group III). A total 8 (38.0%) patients with GC, 2 (16.6%) patients with PUD and 16 (51.6%) patients with NUD were positive by RUT. A total 6 (28.5%) patients with GC and 10 (32.2%) patients with NUD were positive by culture while none of the patients of PUD was positive by culture. By using specific primer sequences of *ureA*, 411 bp PCR product of *H. pylori* was detected in 33 (51.3%) patients and the distribution in different groups of patients was as follows: 12 (57.1%) in patients with GC, 5 (41.6%) in PUD and 20(64.51%) in NUD. *ureA* was detected in 43 (67.1%) patients and the distribution in different groups of patients was as follows: 13 (61.9%) in patients with GC, 8 (66.6%) in PUD and 22 (70.9%) in NUD. Real-time PCR is 76.74% sensitive and 80.95% specific than *ureA* PCR. There was no significant difference of *ureA* gene expression among disease population and control population
Keywords: *H. pylori, Gastrc ulcer, peptic ulcer dyspepsia, urea, Real time PCR.*

Introduction:

H. pylori infection is common worldwide and it is estimated that almost half of the World's population is infected with this organism [Ernest and Gold 1999]. Acquisition of *H. pylori* infection in children results in adulthood disorders that may vary from asymptomatic state to serious illnesses. It is estimated that life time risk of gastritis, PUD, MALT lymphoma and GC in *H. pylori* infected population is above 100%, 10%, <1% and 1-2% respectively. On a global scale, GC is the second commonest cancer in the world. There is substantial international variation in GC incidence with the highest rates reported from China, Japan and other East Asian countries [Singh and Ghoshal 2006].

Helicobacter pylori is a gram-negative fastidious bacterium that resides on the human gastric epithelium. It grows under microaerophilic environment. Subsequent to the first isolation of *H. pylori* in 1982 [Marshall and Warren 1984], its association with gastritis, peptic ulcer disease (PUD) and gastric cancer (GC) and mucosa associated lymphoid tissue (MALT) lymphoma in human.

Diagnosis of *H. pylori* infection is an important issue. At present, there are at least seven diagnostic assays for *H. pylori*: bacterial culture, a rapid urease test, a urea breath test, histology, PCR, serology, and a stool antigen test [Crotty et al 1994; Blaser 1992]. The sensitivities of bacterial culture, the rapid urease test, the urea breath test, histology, and the stool antigen test are limited when few organisms are present or when patients are taking acid-suppressing agents (proton pump inhibitors)[Ho et al 1991]. More importantly, none of these techniques accurately quantifies the number of *H. pylori* present in test samples. Because *H. pylori* is a fastidious, slow-growing bacterium, it requires 4 to 5 days to grow in rich media and requires specific culture conditions [Hachem et al 1995]. Because normal stomach flora can cause serious interference with the culture of *H. pylori*, highly restricted culture plates containing several antibiotics are required. Generally, however, the different types of antibiotics used in agar culture plates suppress the growth not only of normal stomach flora but also of *H. pylori* [Lee and Mitchell 1994; Marchetti et al 1995]. All of these characteristics of *H. pylori* culture make accurate quantification of the bacteria unreliable. The urease assays are not sensitive [Coudron and Kirby 1989;

Rauws et al 1989] and may not be specific in the presence of other urease-positive bacteria [Mobley and Hausinger 1989]. Serology may not differentiate active from past infection and cannot be used to indicate the clearance of *H. pylori* from the stomach because antibodies may stay at the same level even after eradication of the bacteria. Like these techniques, PCR also has drawbacks. PCR detects only specific gene fragments, not viable bacteria.

However, all of the methods mentioned here, are qualitative assays. To monitor the effectiveness of therapy or a vaccine, a quantitative assay is required. A more accurate quantitative assay for detecting *H. pylori* could facilitate monitoring of therapy, enable more accurate epidemiological studies on the acquisition and spread of *H. pylori* infection, and provide a standard by which to measure the effectiveness of vaccines against *H. pylori*. To address this issue, an accurate and reproducible technique for the detection of minute amounts of *H. pylori* mRNA within the gastric mucosa was needed. For this purpose, we developed a quantitative real-time reverse transcriptase PCR (RT-PCR) assay that allowed us to determine the levels of *H. pylori* genes within human gastric mucosae. We also have demonstrated the specificity, sensitivity, and reproducibility of this method for detecting *H. pylori* in clinical samples. The bacilli are found under the mucus layer of the stomach adhering to the glycolipids on the surface of gastric environment; allow colonization, the pathogenesis and virulence factors of H. pylori have been subjects of extensive research. Several virulence factors including motility, enzymes, toxins, adhesions, lipopollysaccharides and surface and cytoplasmic proteins have been proposed. A number of these are pro-inflammatory and lead to secretion of inflammatory cytokines including IL-8 leading to local inflammation and also induce systemic immunity. The most prominent virulence factor is the surface exposed urease enzyme which by its alkalizing property helps to combat the extreme Ph 2.0 of the gastric environment. The enzyme production is regulated by a complex of nine genes (*ureA- ureI*) which constitute structural and functional genes. Loss of ure*A* or ure*B* results in urease negative phenotype. Infection with strains which produce vacuolating cytotoxin (vacA^+) and possess cytotoxin associated protein gene (cagA^+) is more common among people with peptic ulcer and gastric carcinoma. Serological detection of infection with cagA^+ strains is at present the best practical test for virulence.

Non-ulcer or functional dyspepsia is defined as the presence of symptoms of upper gastrointestinal distress without any identifiable structural abnormality during diagnostic work-up, in particular including upper gastrointestinal endoscopy. Dyspeptic symptoms may have a reflux-like character, with heartburn and regurgitation as predominant signs; may appear dysmotility-like, with early satiety and nausea; or may be ulcer-like, with pain and vomiting. Together, these symptoms are very common; they are frequently experienced by 20 to 40% of the adult population of the Western world. Thirty to 60% of patients with functional dyspepsia carry *H. pylori*, but this prevalence is not much different from that in the unaffected population. The role of *H. pylori* infection in dyspepsia not associated with ulcer remains controversial. *H. pylori* play a role in the etiology of dyspeptic symptoms; thus, *H. pylori* test-and-treat strategies are effective for a subgroup of patients with dyspepsia. For patients with uninvestigated dyspepsia, an *H. pylori* test-and-treat strategy is an appropriate option, although empirical acid-suppressive therapy can be more efficient in populations with a low *H. pylori* prevalence. Also, in patients with investigated non-ulcer dyspepsia, *H. pylori* eradication is a relevant option. In both situations, patients must be aware that symptom resolution may take months after completion of therapy.

Gastric or duodenal ulcers (commonly referred to as peptic ulcers) are defined as mucosal defects with a diameter of at least 0.5 cm penetrating through the muscularis mucosa. Both gastric and duodenal ulcer diseases are strongly related to *H. pylori*. In initial reports from all over the world in the first decade after the discovery of *H. pylori*, approximately 95% of duodenal ulcers and 85% of gastric ulcers occurred in the presence of *H. pylori* infection. Serological confirmation of *H. pylori* infection has, however, verified an *H. pylori* seroprevalence of 60-100% in gastric ulcers [Kuipers et al 1995].

Materials and Methods:

1. Study population

A total of 64 adult patients aged 24 to 71 years who underwent upper gastrointestinal endoscopy between Oct 2008 and Jul 2010 at Sanjay Gandhi postgraduate Institute of Medical Sciences were included in the study. Patients were divided broadly into 3 categories:

Group I – Patients with gastric cancer (GC): Total 21, 15 males

Group II - Patients with peptic ulcer disease (PUD): Total 12, 7 males

Group III - Patients with non-ulcer dyspepsia (NUD): Total 31, 14 males

Patients with NUD were considered disease controls throughout the study.

The diagnosis of gastroduodenal diseases was based on clinical, endoscopical and histopathological examinations. The ethics committee of the institute granted approval for the study and the consents were obtained from all the patients. Patients of all three groups who had received anti-microbial therapy, H2 receptor blockers, proton pump inhibitors and non-steroidal anti-inflammatory drugs in the last 4 weeks before endoscopy were excluded from the study.

2. Specimen

2.1 Antral biopsies: During each endoscopic (Olympus, Japan) examination, 5 antral biopsies were obtained; each biopsy was used for isolation and identification of *H. pylori* by different methods.

3. Isolation and Identification of *H. pylori*

3.1 Rapid urease test (RUT): One piece of antral biopsy was inoculated in 10% urea broth with phenol red as the indicator. The presence of urease was indicated by colour change from yellow to pink [Aydin et al 2004].

3.2 Microscopy: Smears were prepared from the biopsy tissue by crushing it between the slides. Crushed smear was air-dried, heat fixed and stained by modified Gram method. The slides were over layered with crystal violet for 30 sec followed by over layering with iodine solution for 1 min. The two staining steps were interspaced by washing with distilled water. The smears were then decolorized with pure acetone and counterstained by dilute carbol fuchsin (1 in 10 dilution of 1% carbol fuchsin) for 30 sec. The stained smears were dried on filter paper and screened for *H. pylori* under oil immersion lens (100X) of light microscope (Olympus, Japan).

3.3 Culture- Antral biopsies from all patients were transported to the laboratory in 200 µl of Brucella broth (Difco, USA) containing 20% glycerol. Biopsies were cultured within an hour of collection on Brucella agar (Difco, USA) supplemented with 7.5% sheep blood, chocolate [Morgan et al 2003] and vancomycin 5 mg, polymyxin B 2500 units and amphotericin B 3 mg per liter. The plates were incubated at 37^{o}C under microaerophilic conditions provided by a candle jar technique. *Escherichia coli* growth was used to maintain microaerobic conditions and a sterile cotton wool soaked in sterile distilled water was also put to provide the high humidity. Plates were examined after 72 hours, there after 48 hours for 7 days.

H. pylori colonies were identified by following tests:

a) **Colony characteristic**: Grey translucent tiny (0.5- 1 mm) colonies were observed on chocolate agar plates.

b) **Microscopy:** Few bacterial colonies were smeared and stained by modified Gram's stain and screened for typical *H. pylori* morphology under oil immersion lens of light microscope.

c) **Oxidase**: Few bacterial colonies were smeared with wooden stick on filter paper strip impregnated in tetramethyl-p-phenylenediamine dihydrochloride (1% w/v in water). Positive reaction was indicated by the appearance of violet colour in the smeared area within 30 sec of impregnation.

d) **Catalase:** Loopful of hydrogen peroxide (10% v/v in water) was over layered on the colonies. Prompt evolvement of effervescence indicated the presence of catalase enzyme.

e) **Urease:** Presence of urease enzyme was tested by rapid urease test (RUT). Few colonies of *H. pylori* were inoculated into 10% urea broth with phenol red indicator. The presence of urease was indicated immediately by colour change from yellow to pink.

4. PCR based detection of *H. pylori*: *H. pylori* specific *ureA* gene PCR was done on extracted DNA from Culture and biopsy.

4.1 Extraction of genomic DNA from antral biopsy

DNA from biopsies was extracted using QIAamp DNA mini kit (QIAGEN, Germany) as per manufacturer's instruction.

4.2 Polymerase chain reaction:

Amplification was carried out in thermocycler (Perkin Elmer Cetus, USA) in 50 µl reaction volume containing 200 ng of genomic DNA, 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide, 0.5 µM each specific primer and 1.25 U of Taq polymerase. Following primer pair was used for amplification of *ure*A gene of *H. pylori*:

5/-GCCAATGGTAAATTAGTT- 3/

5/-CTCCTTAATTGTTTTTAC-3/

35 PCR cycles for amplification were carried out and cycles were as follows- 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 7 min.

DNA extracted from the type stain (ATCC 26695), and without template DNA was used as the positive and negative control, respectively in each batch of PCR assay.

The amplified product (411 bp) was electrophoresed in 2% agarose containing 0.5 µg/ml of ethidium bromide and examined under transilluminator (UVS System, USA).

4.3 PCR condition

PCR was performed in a 50 µL reaction volume containing 100 ng of genomic DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide, 0.5 µM each specific primer and 1.25 U of Taq polymerase.

4.4 Analysis of the PCR-amplified products

The amplified product (6 µl) was electrophoresed in 2% agarose (Sigma, USA) containing 0.5 µg/mL ethidium bromide and examined under Trans-illuminator (UVS Systems, USA). Electrophoresis was performed in TBE buffer.

5. Statistical analysis

The data was analyzed by SPSS 12.0 statistical package (Chicago, IL, USA) while a P-value < 0.05 was considered significant. For analysis of relative gene expression, REST (Relative expression software tool) is used. This software work on the formula given below-

$$\text{Ratio} = \frac{(E_{target})^{\Delta CP_{target\ (control-sample)}}}{(E_{ref})^{\Delta CP_{ref\ (control-sample)}}}$$

6. RNA extraction. Total RNA was extracted from antrum biopsies using an RNeasy mini kit (QIAGEN, Hilden, Germany). To remove trace amounts of genomic DNA, the preparations were treated with RNase-free DNase I (QIAGEN). The RNA samples were quantified by measuring the absorbance at 260 and 280 nm, and the integrity was assessed by agarose gel electrophoresis.

Table 1: sequence of primer and probe

Gene	Primer Sequence	Probe
ureA	5'-CGTGGCAAGCATGATCCAT-3' 5'-GGGTATGCACGGTTACGAGTTT-3'	(6FAM)TCAGGAAACATCGC TTCAATACCCACTT-(TAMRA)

7. cDNA formation. cDNA was synthesized by using 5 µg of total RNA by RevertAid^{TM} H Minus First Strand cDNA synthesis kit.

8. Real Time RT PCR. *ureA* gene amplification was accomplished by real time monitoring of fluorescence intensity during PCR using TaqMan probes. We prepared 1.1 µg of cDNA per reaction and reactions were run in triplicate for samples, standards and controls. Amplification was performed in a total of 20 µl containing 2× Universal TaqMan PCR master mix (QIAGEN, Hilden, Germany), 300 nM of *ureA*), 250 nM of Probe and 1.75 µl of water. The sequence of primer and probe are given in Table2. A clinical isolate of *H. pylori* was used to prepare the standards. Standard curve was plotted by serially diluted the DNA of isolate from 10^{10} to 10^{1}. Human GAPDH gene was used as a reference gene. With help of GAPDH, relative expression of ureA gene was determined in Different disease population. Quantization of GAPDH was performed using specific kit (Genome Diagnostics). All the runs were performed in Real time instrument (corbett Research, Australia).

Results:

1. Study Population

Twenty one patients with GC (15 males) were included in group I.

Twelve patients with PUD (7 males) were included in group II.

Thirty one patients with NUD (14 males) were included in group III. (Disease control)

2. Isolation and Identification of *H. pylori*:

Gastric biopsies from all adult patients included in the study were examined by various methods for the presence of *H. pylori*.

2.1 Rapid urease test (RUT): This test was conducted immediately after obtaining the biopsy from the patient. Urea solution became pink in the presence of *H. pylori* urease (Figure 1). The time taken for the positive reaction was one minute to 1 hour. A total 8 (38.0%) patients with GC, 2 (16.6%) patients with PUD and 16 (51.6%) patients with NUD were positive by RUT (Table 2).

2.2 Culture: Colonies of *H. pylori* were small, convex and translucent on Brucella chocolate agar after 72 h of incubation (Figure 2). These colonies were confirmed by *H. pylori* specific biochemical tests (described in material and methods) and also by microscopically using modified Gram's staining (Figure 3).

A total 6 (28.5%) patients with GC and 10 (32.2%) patients with NUD were positive by culture while none of the patients of PUD was positive by culture (Table 4).

***3. ureA* PCR**: By using specific primer sequences of *ureA*, 411 bp PCR product of *H. pylori* was detected in 33 (51.3%) patients and the distribution in different groups of patients was as follows: 12 (57.1%) in patients with GC, 5 (41.6%) in PUD and 20(64.51%) in NUD (Table 2). PCR amplified product of *ureA* (411 bp) is shown in figure 4.

4. Real time RT PCR

By using specific primer and probe, *ureA* was detected in 43 (67.1%) patients and the distribution in different groups of patients was as follows: 13 (61.9%) in patients with GC, 8 (66.6%) in PUD and 22 (70.9%) in NUD (Table 2). The standard graph was obtained by serial dilution of H. pylori culture DNA by 10^{10} to 10^{1} fold. (Figure 5 and 6)

On comparing the Real- time Data with *ureA* PCR, we found Real-time PCR is

A **B**

Figure 1: Rapid urease test (RUT) -urea broth with phenol red indicator before inoculation of antral biopsy (yellow colour); urea broth after inoculation of *H. pylori* positive antral biopsy (pink colour).

Figure 2: Typical colonies of *H. pylori* on chocolate agar.

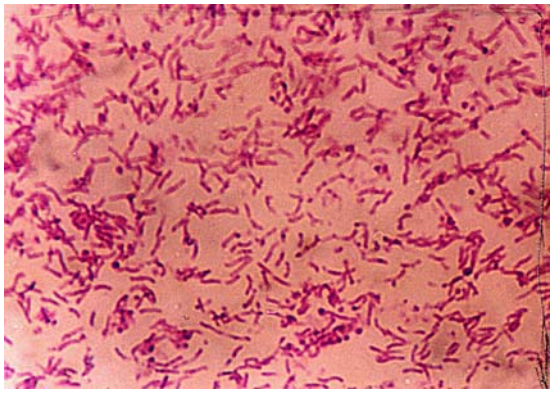

Figure 3: *H. pylori* in culture smear (modified Gram's stain x 1000)

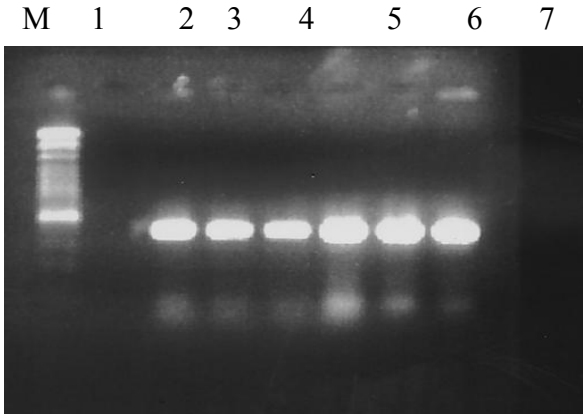

Figure 4: Specific primers based *ureA* PCR of *H. pylori* showing 411 bp amplified product. M is molecular weight marker (100 bp ladder), lane 1 is negative control, lanes 2 to 4 are *H. pylori* amplified product from biopsy, lanes 5 to 7 are *H. pylori* amplified product from culture.

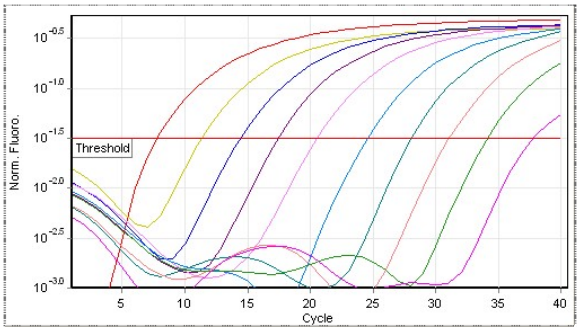

Figure 5: Standard curve of *H. pylori* with copy number 10^{10} to 10^{1}. This curve was obtained by serial dilution of *H. pylori* culture DNA

76.74% sensitive and 80.95% specific than *ureA* PCR. (Table 3)

On comparing the relative expression between control and disease population we found no significant difference of *ureA* gene expression among disease population and control population.

Discussion:

In this study we showed that real-time PCR was routinely capable to accurately detect *H. pylori* directly from gastric biopsies and also to determine the level of *ureA* gene expression with disease outcome. We found that the performances

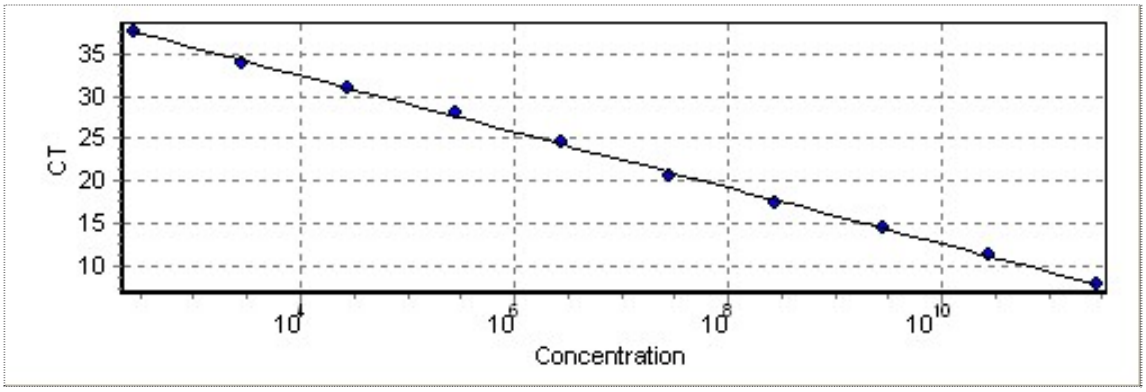

Figure 6: Standard plot of *H. pylori* serial diluted DNA

Table 2: *H. pylori* positivity by different diagnostic tests in patients with gastric cancer (GC), peptic ulcer disease (PUD) and non-ulcer dyspepsia (NUD)

Total Patients (n =64)	**RUT**	**Culture**	***ureA* PCR**	**Real-time PCR**
GC (n =21)	8(38.0%)	6(28.5%)	12(57.1%)	13(66.6%)
PUD (n =12)	2(16.6%)	0(0.0%)	5(41.6%)	8(61.9%)
NUD (n =31)	16(51.6%)	10(32.2%)	20(64.51%)	22(70.9%)

RUT, Rapid urease test;

Table 3: Sensitivity and Specificity of Real-time RT PCR

		***ureA* Real-time**		**Total**
		positive	**negative**	
ureA PCR	positive	33(a)	4(b)	37
	negative	10(c)	17(d)	27
Total		43	21	64

Table 4: Relative expression of *ureA* gene with comparison to control population

Gene	**Type**	**Reaction Efficiency**	**Std. Error**	**95% C.I.**	**P value**	**Result**	**Median expression ratio**
ureA	PUD	1.0	0.000 - 124.066	0.000 - 1,970.992	0.196	Not significant	.043
ureA	GC	1.0	0.000 - 244.895	0.000 - 95,013.200	0.225	Not significant	.25

Sensitivity =a/ (a+c) ×100
Specificity = d/(b+d)× 100

of real-time PCR (sensitivity and specificity) were significantly better than those of *ureA* PCR.

On comparing the real- time data with *ureA* PCR, we found real-time PCR is 76.74% sensitive and 80.95% specific than *ureA* PCR. One limitation of our study is that discrepancies between *ureA* PCR and real-time PCR were classified as positive or negative according to the result of only

two methods, culture and RUT. We did not performed histology, which is one of the reliable methods for *H. pylori* detection. We also did not perform serology because it encounters past infection rather than current infection.

In some non routine studies, conventional PCR was found to be a very sensitive method. The superiority of conventional PCR over other routine tests has already been reported [Goodwin and Armstrong 1990; Gulbis and Galand 1993; Goodwin et al 1987]. We found that real-time PCR is more sensitive specific than normal *ureA* PCR. To the best our knowledge this is the first study in India which determines the sensitive and specificity of real-time PCR over normal PCR for the *H. pylori* detection. However, an American study proved that the real-time PCR is more sensitive than normal PCR.

The feasibility of this real-time PCR assay was good, with a duration of two to three hours and its cost was reasonable, twice the price of normal PCR. It can be performed on any Light Cycler apparatus available in the laboratory. Further we also wanted to see that whether there is any difference of *ureA* gene expression between disease and control population is occurring or not. However, we did not found any significant difference between disease and control population. A possible explanation of this is that patients with disease group might have taken the antibiotics previously. Small sample sizes also have a major factor for finding of this result.

Conclusion:

This work shows that the real-time PCR technique that we have introduced in routine in the hospital is easy to perform and more accurate than *ureA* PCR for the detection of *H. pylori*. Furthermore, it permits a concomitant easy and reliable determination of expression level of *ureA* gene, which we think is now mandatory for the prevention of *H. pylori* and its associated disease.

Acknowledgement:

The authors are very much thankful to all those patients who participated in this study. The authors also are grateful to Mrs. K.P. Rathoure for valuable comments and technical help.

References:

[1] Aydin F, Kaklikkaya N, Ozgur O, Cubukcu K, Kilic AO, Tosun I, Erturk M. Distribution of vacA alleles and cagA status of *Helicobacter pylori* in peptic ulcer disease and non-ulcer dyspepsia. Clin Microbiol Infect 2004; 10: 1102 - 1104.

[2] Blaser, MJ. Hypotheses on the pathogenesis and natural history of *Helicobacter pylori*-induced inflammation. Gastroenterology1992; 102:720–727.

[3] Coudron, P. E., and D. F. Kirby Comparison of rapid urease tests, staining techniques, and growth on different solid media for detection of *Campylobacter pylori*. J. Clin. Microbiol.1989; 27:1527–1530.

[4] Crotty, P L, R. A. Staggs, P. T. Porter, A. A. Killeen, and R. C. McGlennen.. Quantitative analysis in molecular diagnostics. Hum. Pathol.1994; 25:572–579.

[5] Ernest PB and Gold BD. *Helicobacter pylori* in children: new insights into the immunopathogenesis of gastric disease and implications for managing infection in children. J Pediatr Gastroenterol Nutr 1999; 28: 462 – 473.

[6] Goodwin CS, Armstrong JA. Microbiological aspects of *Helicobacter pylori* (*Campylobacter pylori*). Eur J Clin Microbiol 1990; 9: 1-13

[7] Goodwin CS, McCulloch RK, Armstrong JA, Wee SH. Unusual cellular fatty acids and distinctive ultrastructure in a new spiral bacterium (*Campylobacter pyloridis*) from the human gastric mucosa. J Med Microbiol 1987; 19: 257 – 267

[8] Gulbis B, Galand P. Immunodetection of p21-ras products in human normal and preneoplastic tissues and solid tumors. Hum Pathol 1993; 24: 1271 - 1285.

[9] Hachem, C. Y., J. E. Clarridge, D. G. Evans, and D. Y. Graham. Comparison of agar based media for primary isolation of *Helicobacter pylori* J. Clin. Pathol1995; 48:714–716.

[10] Ho, S. A., J. A. Hoyle, F. A. Lewis, A. D. Secker, D. Cross, N. P. Mapstone, M. F. Dixon, J. I. Wyatt, D. S. Tompkins, and G. R. Taylor. Direct polymerase chain reaction test for detection of *Helicobacter pylori* in humans and animals. J. Clin. Microbiol.1991; 29:2543–2549.

[11] Kuipers EJ, Uyterlinde AM, Pena AS, Roosendaal R, Pals G, Nelis GF, Festen HP

and Meuwissen SG. Long-term sequelae of *Helicobacter pylori* gastritis. Lancet 1995; 345: 1525 - 1528.

[12] Lee, A., and H. Mitchell Basic bacteriology of *H. pylori*: *H. pylori* colonization factors, p. 59–72. *In* R. H. Hunt and G. N. J. Tytgat (ed.), *Helicobacter pylori:* basic mechanisms to clinical cure. Kluwer Academic Publishers, Dordrecht, The Netherlands.1994;

[13] Marchetti, M., B. Arico, D. Burroni, N. Figura, R. Rappuoli, and P. Ghiara. Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. Science1995; 267:1655–1658.

[14] Marshall BJ and Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1984; 1: 1311-1315

[15] Mobley, H. L., and R. P. Hausinger. Microbial ureases: significance, regulation, and molecular characterization. Microbiol. Rev.;1989; 53:85–108.

[16] Morgan C, Jenkins GJ, Ashton T, Griffiths AP, Baxter JN, Parry EM, Parry JM. Detection of p53 mutations in precancerous gastric tissue. Br J Cancer 2003; 89: 1314 - 1319.

[17] Rauws, E. A., E. A. Royen, W. Langenberg, J. V. Woensel, A. A. Vrij, and G. N. Tytgat 14C-urea breathe test in *C. pylori* gastritis. Gut 1989; 30:798–803.

[18] Singh K, Ghoshal UC. Causal role of *Helicobacter pylori* infection in gastric cancer: an Asian enigma. World J Gastroenterol 2006; 12: 1346 – 135