

Detection of *H. pylori* By PCR Method using UreA and UreC Gene in Gastric Biopsy Sample

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Gastric cancer is forth leading cause of death world wide. Among various causative agents one of them is *H.pylori* and is one of the world's most common bacterial infections, its natural habitat is the gastric mucosa PCR is a rapid, sensitive and accurate method for the specific detection of *Helicobacter pylori* in a variety of specimens. We compared the sensitivity of PCR that amplifies the highly conserved regions of *ureA* and *ureC* genes of *H.pylori* to detect the presence of *H.pylori* in the gastric biopsy specimens. A total of 50 gastric astral biopsy specimens were collected from dyspeptic patients of different age group. Presence of *H.pylori* in gastric mucosa was investigated by PCR. Twenty-five out of 50 samples were positive for *ureA* gene and 10 out 50 samples were positive for *ureC* gene with the positive predictive value 50% and 20% respectively. The prevalence rate was recorded 72%. amongst these males and females ratio were 77.2:67.8% by PCR method. The incidence of infection is high in 10-30 years of age as compare to old age group. It is concluded from the study that the *ureA* gene is more sensitive for the detection of *H. pylori* than *ureC* gene. We therefore recommend the use of *ureA* gene based PCR for clinical diagnosis.

Key words: *H. pylori*, PCR, Biopsy and Prevalence.

Nearly hundred years ago a well known anatomist, Bizzozero, reported that the spirochetes inhabit the gastric glands and the canaliculated of the parietal cells. The work of Bizzozero's was further extended by other scientists Salomon and Doengen who discovered the presence of these organisms in the stomach of mouse and other mammals such as rat, cat, dog, monkey, and human. The organisms were referred as gastric spirals due to their morphological characteristics¹. In 1940,

Freedberg and Baron found spirochetes like organism in 40% of gastric specimens. In 1950, Fitzgerald and Murphy observed the production of urease in patient with gastric ulceration. Then in the same year the work of Doenges and Freedberg were challenged by Palmer, who investigated 1,000 gastric biopsies taken with a blind suction biopsy instrument but claimed that no bacteria could be seen. In 1967 Susumu Ito of Harvard medical school while observing gastric mucosa under electron microscope observed spirals like organism within a parietal cell gland and publish photograph of one of these organisms².

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They cultured the positive biopsy samples under a variety of conditions but mainly micro aerobic incubation similar to that used for *Campylobacter*. However after 48h of incubation, the biopsies were discarded due to the overgrowth of normal flora gastrointestinal tract. The organism was grown because of lucky accident in which the culture were left the incubator for several days over the long Easter holiday and after four to five days transparent colony were observed. The longer than usual incubation resulted in the isolation of an organism and was termed as Campylobacter-like organism. The organism resembled to *Campylobacter* in several aspects including curved or spiral morphology, growth on enriched media under microaerophilic condition, similar G+C content. It was initially name as Campylobacter pyloridis and then it was corrected to Campylobacter in 1987³. Later it was found that the organism does not belong to the genus *Campylobacter* and thus a new genus was suggested in 1989⁴.

Helicobacter pylori is a non-spore-forming, Gram negative, spiral-shaped, curved rod shaped, or fusiform bacterium with 1 to 3 turns. The spiral wavelength may be varying with age and growth condition. Size ranges from 0.2-1.2 micrometer diameter and 1.5-10 micrometer in length⁵.

Electron dense granule bodies have been observed in *H.pylori* and it these granule are mainly poly phosphate granules, localized to three different regions in the cytoplasm: the cytoplasm, the flagella pole and the associated with the cell membrane and it may serve as source of energy⁶. External to the cell wall present a 40nm thick 9 to 11 periplasmic fibers or electron dense glycocalyx or capsule like layer. *Helicobacter pylori* were motile with a rapid corkscrew like or slower wave like motion due to the presence of bipolar tuft of 10 to 14 sheathed flagella⁷. *H.pylori* is microaerophilic in nature. It requires 5-10% oxygen, and 5 to 10% carbon dioxide for optimum growth in culture medium. *H.pylori* grow well at a temperature of 30-37°C all these requirement is fulfill in the gastrointestinal tract of mammals⁸.

Glucose is not necessary for growth but its presence enhances cell viability. Certain amino acids like arginine, histidine, isoleucine, leucine, methionine, phenylalanine and valine is necessary

for its growth. It is a fastidious organism therefore it does not grow on ordinary laboratory media and nutrient broth, unless growth medium is supplemented with either bovine serum or fetal calf serum, however (2, 6-dimethyl)-Beta-cyclodextrin (CD) also support the growth of *H.pylori*. Bovine serum, fetal calf serum and CD bind to the toxic metabolite produced by the bacteria which inhibit the growth of bacteria. Although the blood and serum may also contain other growth-stimulating factor required by the bacteria^{9, 10}. *H.pylori* is considered as the causative agent of gastro duodenal diseases such as chronic gastritis, peptic ulcer, duodenal ulcer, and gastric cancer. Although the gastric mucosal environment do not favor the growth of microorganisms, *H.pylori* is able to grow in this harsh condition with great adaptability and capacity to colonize the gastric epithelium. In most of the cases this colonization lead to asymptomatic carrier state that persist for longer period of time however in a subset of population (~10%) this colonization lead to pathological conditions¹⁴⁻¹⁵. The urea breath test is a non-invasive and fast mean for the detection of active *H.pylori* infection. This test is non-quantitative and it determines current infection. The test is based on the principal that the orally administered urea is hydrolyzed by the urease enzyme produced by the *H.pylori* in large quantity. Urea is hydrolyzed to ammonia and carbon dioxide, which then is absorbed from the stomach and eliminated in the breath. False negative result can arise if there are too few bacteria in the stomach of infected host to produce detectable urease especially during or after a treatment regiment, also in the case of infection with different bacteria that also produce urease. Generally, either ¹³C or ¹⁴C is used. The labeled urea is hydrolyzed by the urease enzyme in the stomach of an infected host, and the resulting CO₂ is absorbed across the gastric mucosa into the blood circulatory system, and then excreted through the lungs as expired air⁴⁰. The serological tests are based on the principal that infection with *H.pylori* induces both local and systemic antibody responses. The systemic response comprises a transient rise in IgM, it is followed by a specific rise in IgA and IgG. The serological test detect specific anti-*H.pylori* immune response mostly IgG antibodies in serum, whole blood, urine and saliva⁴². The circulating

antibodies to *H. pylori* can be detected by enzyme linked immunosorbent assay (ELISA) or western blotting and latex agglutination tests. The serological test has many advantages, it is inexpensive, essentially non-invasive, quick and easy to perform, and little specialized equipment is required. Its sensitivity is 91-100% and its specificity 50-90%^{43,44}. However, serum antibody persists even after *H. pylori* infection is eradicated, therefore serological test has limited application in *H. pylori* eradication⁴². This test is based on the principle that the urease enzyme produced by *H. pylori* hydrolyses urea to ammonia and carbon dioxide, which consequently raise the pH of the medium detected by phenol red indicator³⁸. The test is performed with gastric biopsy samples. The CLO test and rapid urease test are of similar sensitivity and specificity i.e. 90% and 100% respectively³⁹. As postulated in Koch's postulates that for every disease some agent is responsible which can be isolated from the patient and reintroduced in a susceptible body but unfortunately disease cases with strong clinical evidence of a bacterial etiology are not supported by laboratory isolation and identification of responsible pathogen. So the isolation centered approach has been in question. Thanks to the blessings of new molecular techniques used in microbiology the association of bacteria in disease can be accomplished without culturing in lab. One of them is PCR. PCR is a rapid, sensitive and accurate method for the specific detection of *H. pylori* in a variety of clinical specimens. It can detect low number of organisms in the sample. The sample used in PCR assay for the detection of *H. pylori* includes gastric biopsies, saliva, dental plaque and stool. However, gastric biopsies are more commonly used due to higher chances of bacterial presence in gastric mucosa.

MATERIALS AND METHODS

The study was based on the PCR evaluation of gastric biopsy samples collected from February 2010 to November 2011. The patients who reported to Medical Unit 1, Civil Hospital Karachi for upper gastro duodenal endoscopy and with the gastroscopic evidence of ulceration or gastritis were included in this study. The most commonly observed symptoms were epigastric pain, nausea,

vomiting, heart burn and anemia. (table – 6) Antral gastric biopsy samples were obtained from 50 patients with different age groups. Written consents were obtained from each patient and the study protocol was approved by the local ethical review board.

Collection and transport of the biopsy samples

Samples were collected using Olympus GIF XQ 10 gastroscope in the endoscopy unit of Civil Hospital Karachi. Patients who were recommended for endoscopy were asked to stop eating for at least six hours earlier and at the time of endoscopy they were asked to gargle with 4% Xylocain. The biopsy forceps were sterilized with 70% Alcohol. From each biopsy site specimens were collected in pairs and immediately one specimen was immersed in liquid thioglycollate and the other specimen was stabbed in an urease vial. Since *H. pylori* cannot survive in aerobic conditions so thioglycollate broth was used which provides anaerobic conditions till processing. After collection, the specimens were transported to the Immunology and Infectious Disease Research Laboratory in the Department of Microbiology, University of Karachi within two hours.

DNA was extracted from these samples for PCR. It was freeze at zero degrees centigrade. PCR was performed by following procedure.

PCR for detection of *H. pylori*

DNA extraction from biopsy

Following method was used to extract DNA from gastric biopsies. The biopsy samples were crushed and added to a test tube containing 240 µl D/W. Followed by the addition of 20-30 µl 20% SDS, then 80 µl PK buffer and then 40 µl proteinase K were added to each test tube. All the contents were mixed well. After mixing all the test tubes were incubated at 55°C overnight if it was a biopsy and for 1 hour if it was a culture. After incubation all the test tubes were placed at room temperature. Then to each test tube 100 µl 6M NaCl were added and mixed well. After mixing all the test tubes were centrifuged for 1 minute at 14,000 g. After centrifugation the supernatant was separated in a separate tube. To the supernatant 1 ml 100% chilled ethanol was added and centrifuged as mentioned above. The supernatant was discarded. Then 1 ml 70% ethanol was added and centrifuged as mentioned above. Supernatant was discarded.

and the mixture was suspended in 100µl TE buffer.

Preparation of PCR Mix

PCR mixture was prepared as follows. To each test tube 14.7µl Nuclease free water was added. Followed by the addition of 2.5µl PCR Buffer, 1.0µl MgCl₂ and 0.5µl dNTPs. Than to each test tube 0.5µl forward primer and 0.5µl reversed primer were added. Finally 0.3µl Taq pol and 3.5µl of DNA sample were added to each test tube.

Amplification conditions

Beta-Globulin gene

The primer sequence of Beta-Globulin forward is 5' ACACA ACTGTGTTCACTA GC 3' and reversed is 5' CAACTTCA TCCACG TTCACC 3'. Product were amplified under the following condition: initial denaturation at 95°C for 5min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec and polymerization at 72°C for 30 sec with final round of polymerization at 72°C for 10min, in Bio-metraThermocycler.

ureA gene

The primer sequence of *UreA* gene forward 5' GCCAATGGTAAATTAGTT-3' and reverse 5' CTCCTTAATTGTTTTTAC-3'. Product were amplified under the following condition: initial denaturation at 95°C for 5 min followed by 36 cycle of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec and polymerization at 72°C for 30 sec with final round of polymerization at 72°C for 10 min, in Bio-metraThermocycler.

UreC (glmM) GENE

The primer sequence for *UreC* gene

Forward 5' AAGCTTTTAGGGGTGTTAGGGGTTT 3' and Reversed 5' AAGCTTAC TTTCTAA CACTAACGC-3'. Product were amplified under the following condition: initial denaturation at 95°C for 5 min followed by 36 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and polymerization at 72°C for 30 sec with final round of polymerization at 72°C for 10 min, in Bio-metraThermocycler.

GEL electrophoresis

PCR product were analyzed by gel electrophoresis using 1.7% (W/V) agrose, stained in 0.2µl of ethidium bromide, and examined by ultraviolet transilluminator. Using 100bp marker

RESULTS

A total of 50 biopsy samples from different age groups were investigated for *H. pylori* infection by PCR. All the sample were initially investigated for Beta-Globulin gene, All sample show positive result for Beta-Globulin gene which confirmed that DNA was extracted from biopsy specimen. *ureA* gene based PCR was found to be positive in 25/50 (%). The positive predictive value is found to be 50% and negative predictive value is also found to be 50% (table3).

The second PCR used the glmM (*ureC*) as the target. Out of the 50, 10 samples showed positive results for *ureC* gene. The positive predictive value was determined to be 20% and negative predictive value was found to be 80% (table 3). Out of 35 positive sample amplify by two

Table 1. Oligonucleotide sequence of primers and PCR product sizes

Amplified gene	Primer	Sequence (5'-3')	PCR product size
UreaseA gene	HPU1	59-GCCAATGGTAAATTAGTT-39	411 bp
	HPU2	59-CTCCTTAATTGTTTTTAC-39	
<i>glmM</i> gene	Forward primer	59-AAGCTTTTAGGGGTGTTAGGGGTTT-39	294 bp
	Reverse primer	59-AAGCTTACTTTCTAACACTAACGC-39	
<i>Beta-Globulin</i> gene	Forward primer	5' ACACA ACTGTGTTCACTAGC 3	168bp
	Reverse primer	5' CAACTTCATCCACGTTCCACC 3'	

Table 2. Prevalence and positivity of *H. pylori*

Total patient	<i>H. pylori</i> +ve	Prevalence of <i>H. pylori</i>
50	36	72%

Table 3. Results of PCR in terms of predicted value

Value	<i>ureA</i> gene	<i>ureC</i> gene
Positive predicted value	50 (25/50)	20 (10/50)
Negative predicted value	50 (25/50)	80 (40/50)

Table 4. Incidence of *H.pylori* infection with respect to age

Group	Age range years	No of patient	H.P.+ve.n-% age		H.P.-ve.n-% age	
I.	10-20 years	3	3	100	1	0
II.	21-30 years	14	8	57.14	7	4285
III.	31-40 years	20	17	85.0	7	15.0
IV.	41-50 years	6	2	33.33	4	66.66
V.	51-60 years	5	2	40.0	3	60.0
VI.	61-70 years	2	0	0	2	100

Table 5. Prevalence of *H.pylori* in different sex

Sex	No of patient	<i>H.pylori</i> +ve	<i>H.pylori</i> -ve
Male	28	19 (67.8%)	9 (32.14%)
Female	22	17 (77.2%)	5 (22.72%)

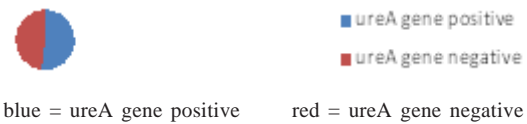
Table 6. Symptoms of *H.pylori* patients

Symptoms	<i>H. pylori</i> positiveN (%)	<i>H. pylori</i> negativeN (%)
Gastric pain	80%	80%
Burning	74%	20%
Fever	15%	25%
Nausea	10%	90%
Vomiting	12%	28%
Lower GI tract symptoms	90%	50%
Others	30%	40%

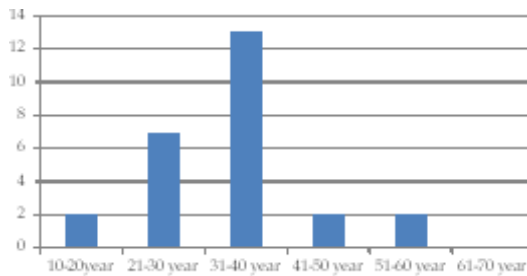
different PCR method 35 sample were positive for both *ureA* and *ureC* gene in PCR.

A total of 50 patient with endoscopically proved duodenal ulcer were included in this study. Out of 50 patients, 36 showed evidence of *H.pylori* infection so prevalence of *H.pylori* recorded was 72 %.(table-2)

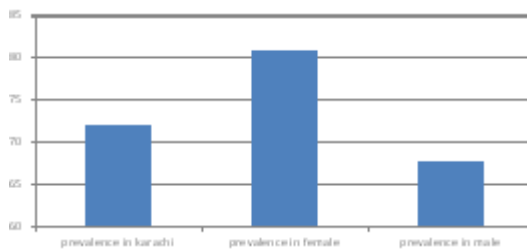
Regarding *H.pylori* positivity 19 out of 28 (67.8%) males were *H.pylori* +ve and 9 out of 28 (32.14) were *H.pylori* -ve. Incase of females 17 out of 22 (77.2%) were *H.pylori* +ve so positivity was 77.2:67.8% i.e. 1.14:1 (table-5) Maximum incidence of *H.pylori* was 100% recorded in age group I (10-20 years) and then 85% recorded in age group III (31-40 years) while the minimum incidence was zero percent recorded in age group VI (60-70 years) Age of the patient range from 14-65 years (meanage). Maximum number of patients



Graph 1. Prevalence of ureA gene in the *Helicobacter pylori* infection



Graph 2. Incidence of *Helicobacter pylori* infection with respect to age



Graph 3. Prevalence of *Helicobacter pylori* in Karachi and in male and female

were in age III (31-40 years) next were in age group II (21-30years) then age group I V (41-50 years) and then age group V (51-60 years) and then age group I (10-20 years) and then age group VI (61-70 years).(table 4) Data about prevalence showed that its prevalence is high among the age group I (10-20 years) which is 100% and then second most susceptible group is age group 3(30-40 years) which is 85%.



Fig. 1. Gel photograph illustrates the specific detection of *H. pylori* using *ureC* gene by Praline number 1, 4, 5 and 10 indicated amplified product of 294bp.



Fig. 2. Gel photograph showing specific detection of *H. pylori UreC* gene by PCR method and examined by gel electrophoresis in Gastric biopsy specimens. Paraline 5, 6, 7 and 8 indicate amplify product of 294bp while the rest are negative

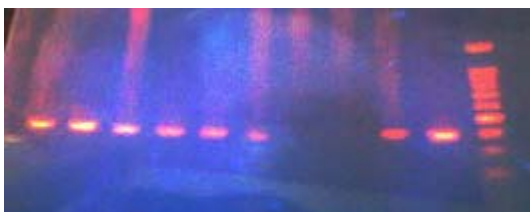


Fig. 4. Gel photograph showing specific detection of *H. pylori UreA* gene by PCR method and examined by gel electrophoresis in Gastric biopsy specimens. Paraline 1, 2, 3, 4, 5, 6, 9 and 10 indicate amplify product of 411bp while the rest are negative

DISCUSSION

The diagnosis of *H. pylori* infection is necessary to understand gastroduodenal pathologies and to decide the exact regime required for the treatment and control.

Although culture isolation has been the standard method for detection of organism, but it may not be the most appropriate method for

detection of *H. pylori* like organism due to cost, the special conditions required for specimen transport and growth, and the long interval between specimen harvest and test results, which delay treatment decision. Among the other method, serological test which detect specific antibody against *H. pylori* has disadvantage that it cannot distinguish between active infection and previous exposure to *H. pylori*³. The rapid urease test and histology may not prove to be specific tests since the presence of other urease producing bacteria with *H. pylori* like morphology in stomach cannot be denied. Detection of *H. pylori* DNA from the gastric tissue by the polymerase chain reaction (PCR) is quite satisfactory, can yield high level of sensitivity (93%) and specificity (100%) can detect as few as 10 *H. pylori* colony forming units. The potential advantage of PCR includes high specificity, quick results, and the ability to type bacteria without the requirement for special transport condition.

This study used *ureA* gene and *ureC* (*glmM*) genes as the PCR target. Both genes are highly conserved for *H. pylori*, and a number of researchers have used them previously⁶⁸.

All the sample were initially investigated for Beta-Globulin gene PCR, in order to confirm the extraction of DNA. All the sample were found to be positive with Beta-Globulin gene PCR. Than after confirmation of DNA extraction from biopsy all the sample were investigated for *ureA* and *ureC* gene. 25 out of 50 samples were found to be positive with *ureA* gene based PCR with positive predictive value 50%. While the *ureC* (*glmM*) gene amplified only 10 samples with positive predictive value 20%. Our results are contradictory with the previously published report by Lu *et al* since the same primers are used in this study. compared five different PCR methods targeting different segment of the *H. pylori* genome. Of 5 different PCR methods, they found *ureC* (*glmM*) gene PCR as the most sensitive and specific for the detection of *H. pylori* in gastric biopsy specimens⁶⁹. There could be several reasons behind the contradictory results; first there may be a chance of polymorphism in *ureC* gene in Pakistani population. However further study is required to confirm the *ureC* gene polymorphism in Pakistani strains of *H. pylori*.

According to the present study the prevalence of *H. pylori* in gastritic patient belonging

to Karachi is 72% (36 out of 50 patients are PCR positive for either *UreA* gene or *UreC* gene). This result is comparable with various previous studies which have recorded a consistently high prevalence.

The reason for variation in prevalence could be due to low sensitivity of diagnostic tests, age group of patients, ethnic difference and socioeconomic status of the patients. High prevalence rate of *H. pylori* in this study may be due to the age factor (as majority of the patients were in 3rd to 4th decades of life at which age *H. pylori* infection is generally high), crowded environment, low sanitation, poor hygiene which predispose to the spread of *H. pylori* infection, and selection of high sensitivity diagnostic tests i.e. PCR

Regarding to the sex, this study showed predominance of male patients over female patients of gastritis (M/F ratio – 28/22 i.e. 1.2:1) which is comparable to other studies showing predominance of male gender. Lam S. K, and Org G.B. documented a M/F ration of 4:1 [72]. A study conducted by W. Ahmed et al in 1990 revealed a ratio of 5.7:1. In the literature it is reported that gastritis was lower in young women until the onset of menopause and this led to the idea that somehow female hormones protect against the development of gastritis.

Contrary to the result of this study M/F ratio is different in developed countries like United States where it is reported to be 1:1⁷³, and United Kingdom where it is reported to be 2:1⁷⁴. This wide geographical differences in the sex ratio support environmental factor theory and the changing habits of females in the developing vs. developed countries.

Distribution of the patient according to the age has revealed that majority of patients in the present study were in their 3rd -4th decades of life. This figure is consistent with that reported in the literature. High prevalence of *H. pylori* is reported in 1995 in age group 31-50 years⁷⁵. In addition the prevalence of *H. pylori* showed an increase with the increasing age. Difference in the age suggest two possibilities. Either risk factors for infection in adults differ from those acting during childhood or most infections may be acquired before childhood and the observed increase in seroprevalence with age could be predominantly a

cohort effect⁷³.

There are numerous reasons that why sometime the diagnostic test failed to detect *H. pylori* from sample. Firstly the use of different drugs prior to the endoscopy which are the known suppressors of *H. pylori*, reduce the number of organism and as they are few in number they can't survive in the environment or in other words in vitro. Secondly the biopsy specimens in some cases have not been taken from the correct area or the area colonized by *H. pylori* may have been missed. Third may be due to delay in processing of the biopsy specimens. Goodwin *et al* recommended that the biopsy specimens should be kept at 4°C if more than 2 hours are required before processing. Since Civil hospital Karachi is quite far from university, it took some time to transport the biopsy specimens and due to insufficient facilities, specimen could not be kept at low temperature during transport. It also indicates that *ureA* gene has sufficient sensitivity for clinical application than *ureC* gene.

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