Molecular Detection of *Helicobacter pylori in* the Atherosclerotic Coronary Plaques and Carotid Plaques: Is There Association with Development of Coronary Artery Disease?

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H. pylori is one of the novel risk factor of atherosclerosis that showed it might be contributed to atherosclerotic process. Precise understanding of atherosclerosis risk factors is of extreme importance to develop preventive and treatment strategies to minimize its consequences. Although epidemiologic studies have suggested a relationship between Helicobacter pylori (**H. pylori)** infection and atherosclerosis but this issue is still controversial. Fifty-five patients who underwent CABG and had the indication for coronary atherectomy (coronary group) and the other group was fifty-five patients who underwent cardiac surgery for non-coronary cause and had the carotid or aortic arch plaques then sampling from their carotid plaque was performed(carotid group). Ultimately, all plaques were analyzed by PCR method for **H. pylori** genome. **H. pylori** was detected in 9.1% samples of coronary artery plaques. In contrast, all of the carotid samples were negative for **H. pylori. The** binary logistic regression analysis was also showed no statistical significantly P value=0.45 (OR,5.5;CI,0.58-53.7)association between the presence of **H. pylori** and development of coronary artery disease. This study showed that the frequency of **H. pylori** positive plaques was significantly higher in coronary group than carotid group. On the other hand, it was not seen significantly association between **H. pylori** with development of coronary atherosclerotic plaques. Then it could not be an independent predisposing factor for coronary atherosclerosis in patients with chronic **H. pylori** infections in our study.

Key words: Atherosclerotic coronary plaques, Helicobacter pylori, polymerase chain reaction.

Despite several years of research, the exact mechanism of processes leading to initiation of atherosclerotic lesions has remained unclear. A precise understanding of risk factors of atherosclerosis is of extreme relevance to develop preventive and treatment strategies to minimize its consequences. Although the mechanisms of activation of inflammatory cells within atherosclerotic lesions have not been elucidated, there is evidence suggesting a role of autoimmunity as well as infections [1,2]. The role of inflammation in pathogenesis and progression of atherosclerosis has been increasingly discussed.

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Despite the sero-epidemiological studies have suggested a relationship between *Helicobacter pylori* (*H. pylori*) infection and atherosclerosis[3], the issue is still controversial and a potential link between *H. pylori* and atherosclerosis has been debated[4].

Although some opposed association between the presence of *H. pylori* as a pathogen of vessel walls and atherosclerosis[5], several studies have proposed an association between *Helicobacter pylori* infection and coronary artery disease[6-8].

Considering cardiovascular diseases, H. pylori infections may be associated with coronary artery disease because of the atherosclerotic changes in blood vessels that is due to direct effect of microorganism or its products such as cytotoxines and endotoxines on coronary endothelial cells, which can progress chronic inflammatory response[9]. In addition, H. pylori may have a role in promoting atherosclerosis by modifying lipid metabolism by elevating low density lipoprotein and decreasing high-density lipoprotein (HDL)[10,11]. Other underlying mechanism of H. pylori infection include: significantly increased acute phase reactants such as C-reactive protein and pro-inflammatory cytokines, activation of platelets and complement system, stimulation of monocytes to release inflammatory cytokines[7].

Molecular methods such as Polymerase Chain Reaction (PCR) are sensitive and accurate diagnostic tool for *H. pylori* infection. In comparison to other methods for diagnosing *H. pylori*, PCR yields high sensitivity and specificity for *H. pylori* and would be able to increase the probability of *H. pylori* detection[12,13]. Therefore, based on the above studies that mentioned the plausible role of *H. pylori in development path of atherosclerosis,* we aimed to survey the frequency of *H. pylori* infection in coronary atherosclerotic plaques and carotid plaques using molecular method and assess the probability of the infection on development of coronary artery disease.

MATERIALS AND METHODS

Ethics approval

The study was reviewed and approved by the University Review Board and hospital ethics committee and been performed in accordance with the ethical standards laid down in an appropriate version of the 2000 Declaration of Helsinki. Information about trial was given comprehensively both orally and in written form to the patients. All patients gave their written informed consents prior to their inclusion in the study according to University Hospital Ethics Board Committee.

Patient selection and study design

In a cross-sectional study, 180 patients who were candidate for cardiac surgery either coronary artery bypass surgery or other cardiac surgery such as congenital heart disease or valvular heart disease between July 2012 until September 2013 in department of cardiovascular surgery of Jundishapur university of medical sciences in Ahvaz Golestan hospital was included in our study. Eighty patients who were candidates for elective CABG based on coronary angiography included as a coronary group. Three patients were refused surgery and 22 patients did not meet the criteria for coronary atherectomy during CABG then they are excluded. 100 patients who were candidate for elective cardiac surgery to congenital heart disease or valvular heart disease and they did not have significant coronary artery disease documented by coronary angiography were included .45 patients were excluded because they did not have carotid artery stenosis or visible plaque in aortic arch during coronary angiography . Finally, 55 patients were cases (CABG or coronary group) who underwent coronary and aortic arch atherectomy and 55 patients (control or carotid group) who underwent cardiac surgery and had visible atherosclerotic plaque in carotid and aortic arch (see Figure 1) were included in our study.

In coronary group the mean age was (60.8 ± 8.5)year and in carotid group (57.5 ± 11.5)year (p=0.75).In coronary group there was (54.5%)male ,(45.5%) female and in the other group there was (58.1%) male ,41.9% female(p=0.66).

Inclusion and exclusion criteria

Inclusion criteria for case group were patients who underwent coronary angiography then candidate for elective CABG and simultaneously had indication for coronary atherectomy who are had significant and long standing coronary stenosis and surgeon could not performed graft anastomosis to formation of an appropriate lumen size . In addition they were

not old age (>80years) and they did not have congenital heart disease, valvular heart disease or contra indications for coronary atherectomy (extensive calcification of Left Main coronary artery or bifurcation lesion).

Inclusion criteria for control group were they had congenital or valvular heart disease required surgery and simultaneously they had symptomatic carotid artery stenosis (10 patient)documented by color Doppler sonography or had visible aortic arch plaque(45 patient) during coronary angiography . None of the patients had previously undergone CABG or percutaneous coronary intervention (PCI).

Data collection

A questionnaire consisted of demographic information, past and current medical history, socioeconomic status, and a systematized review of symptoms including acute chest pain, questions on diet, smoking habits (current smokers; defined as having smoked greater than or equal to 100 cigarettes in their lifetime and who smoked every day or on some days around the time of examination).

Body mass index (BMI) was calculated from weight and height and then categorized according to the modified World Health Organization (WHO) criteria. On the same day, after 12 hour fasting, we also measured blood pressure and performed laboratory tests for levels of total cholesterol, triglyceride; total serum cholesterol (TC) levels of higher than 200 mg/dL or serum triglyceride (TG) levels higher than 150 mg/dL were defined as hyperlipidemia.

Method of Tissue biopsy

Tissue samples were obtained from atherectomy of coronary artery or carotid atherosclerotic plaques by surgeon during CABG. Plaques of coronary and carotid artery were ranged from 0.5 to 2 cm long according to surgeons' clinical judgment. All specimens obtained during surgery were frozen at "70°C. Specimens were transferred to our experimental laboratory.

DNA Extraction from tissue specimen

DNA from 25 to 30 mg of tissue samples was isolated by DNeasy Tissue Mini kit (Qiagen, Germany) according to manufacturer protocol's recommendations. For H. pylori DNA amplification, we used 100 ng of isolated genomic DNA. The quality of isolated DNA from each specimen was analyzed spectrophotometrically. The DNA samples were stored at -80 C until further processed for experimental assays. Tissue processing, DNA extraction, PCR assay set-up, and post -PCR product analysis were performed in separate designated rooms and facilities to prevent cross-contamination.

Polymerase chain reaction (PCR) Analysis

For detection of *H. pylori*, complementary primers for glmM (phosphoglucosamine mutase) were used [3]. PCR analysis performed by primers as Forward: 5'-CGCTCTAACTTCCATAGGC-3' and Reverse: 5'-GCTCTCACCCACTTTATAG-3'.

Amplification conditions were as follows:

Initial denaturation: 94c-10min, Denaturation: 94c-1min, Annealing: 59c- 40 s, Extension: 72c- 30 s, Final extension: 72c- 5min and product was a 254 bp sequence. (Figure 2)

Statistical Analysis

Statistical calculations were conducted using SPSS 20 (Chicago, IL, USA). The quantitative variables were presented as mean±SD and were analyzed by student t-test. Statistical analysis was performed using Chi-Square or Fishers exact test and Spearman correlation coefficients for non-parametric samples. $P<0.05$ was considered as statistically significant. Sample size was estimated using sample size calculator software with 95% confidence interval and $p<0.05$.we use binary logistic regression to compare two groups.

RESULTS

One Hundred and ten (110) patients were enrolled in this analytic-observational study either of CABG group or control group. There were no significant differences between the groups in demographic data including age, sex and BMI (Table1). There were no significant differences between two groups in the prevalence of hypertension, diabetes mellitus, hyperlipidemia, smoking, and family history of coronary artery disease (Table2). Approximately 9.1% of patients in CABG group were positive for *H. pylori* genome in their coronary plaques. (Figure 2)

Lipid profile in two groups

The lipid profile (mean±SD) in coronary group was total cholesterol (131.4±36.2), LDL (118.72±31.2), HDL (40.3±11.5), TG (179.3±76.8);

and in group carotid were total cholesterol (142.6±34.2), LDL (131.2±32.5), HDL (39.1±8.2), TG (178.1±67.7). In Group coronary, the mean serum total cholesterol (p=0.077), triglyceride (p=0.37), LDL (p=0.065), and HDL (p=0.43) levels of patients were not signifi-cantly higher than in Group carotid (Figure 3A). In CABG group the patients who were *H. pylori* positive had significantly higher LDL and HDL levels but TG level was not significantly different (Figure 3B).

H. pylori in Atherosclerotic plaques

Positive *H. pylori* in coronary or carotid artery plaques of patients was tested by PCR analysis. Only 5 patients who underwent CABG(have ischemic heart disease) were positive for *H. pylori* in their plaques from coronary arteries, but all other specimens from carotid or aortic arch arteries were negative for *H. pylori* in both case and control group (Figure 4). Therefore, PCR test result was positive in 9.1% specimens from coronary artery atherosclerotic plaques. In con-trast, none of the carotid or aortic arch samples in coronary and carotid group was found to be positive (P=0.002).

Analyses of the presence of *H. pylori* in plaques and development coronary artery disease

We conducted a binary logistic regression analysis to test for possible associations between the presence of *H. pylori* genome and the coronary atherosclerotic plaques; the result showed P=0.45(OR,5.5;CI,0.58-53.7)there is no significant association between the presence of *H. pylori* and development of coronary artery disease.(table 3)

Table 1. Demographic characteristics of patients of Coronary group (had CABG), and Carotid group (no CABG). Data are presented as number or mean

	Coronary	Carotid	p-value
Age	60.8±8.5	57.5±11.5	0.75
Female/male	25/30	23/32	0.66
BMI	23.4±3.2	22.7±4.4	0.59

Table 2. Risk factors for atherosclerosis in patients of Coronary group and Carotid group

	Coronary	Carotid	p-value
Hypertension (yes/no)	38 (69%)	31 (56%)	0.058
Diabetes mellitus (yes/no)	24 (43%)	21. (38%)	0.082
Hyperlipidemia (yes/no)	39 (70%)	27 (49%)	0.051
Smoking (yes/no)	14 (25%)	18 (32%)	0.43
Family history (yes/no)	2 1(38%)	29 (52%)	0.055

Table 3. Analyses of presence of *H. pylori* genome and coronary atherosclerosis

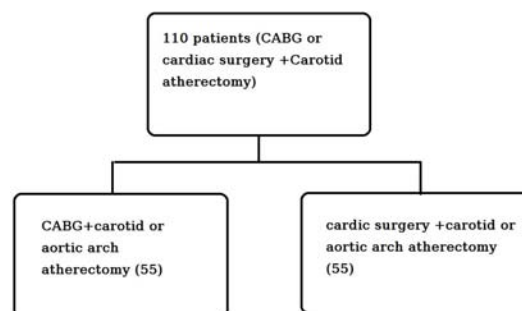| Coronary risk factors	Odds ratio (95% CI)	p-value
H. pylori	5.5(0.58-53.7)	0.46

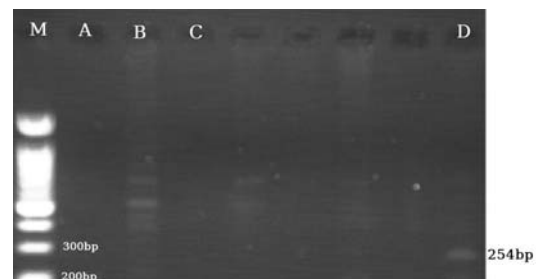**Fig. 1.** Patients' stratification in our study

Fig. 2. *H.pylori* DNA Detection, PCR method by using glmM (phosphoglucosamine-mutase) as a complementary primer: the final product is a 254 base pair sequence.
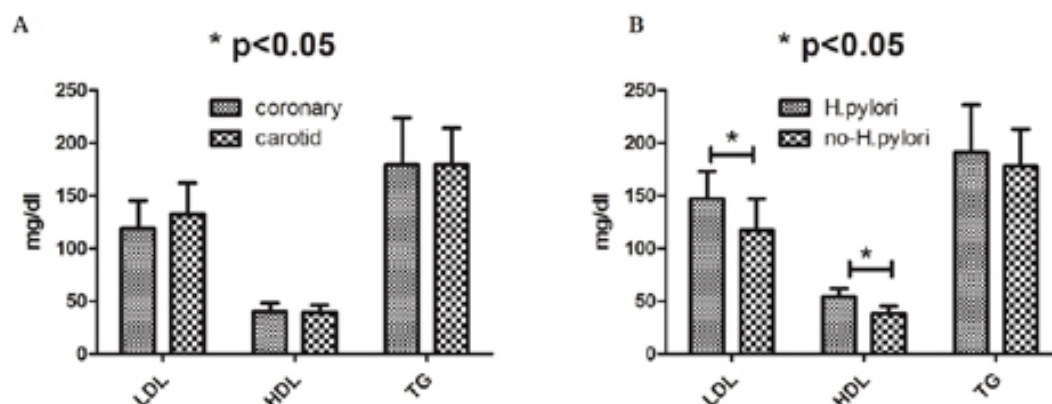
Fig. 3. Comparison of triglyceride (TG), Low-density lipoprotein (LDL), and high-density lipoprotein (HDL) of patients in coronary and carotid group (A: left panel); and patients with *H. pylori* positive and negative in coronary plaques (B: Right panel)
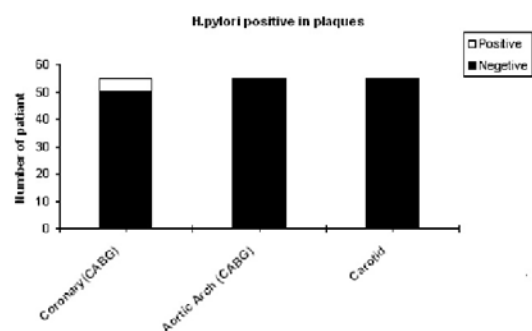

Fig. 4. Positive *H. pylori* in coronary artery, carotid or Aortic Arch plaques of case and control groups. Only 5 patients who had CABG were positive for *H. pylori* in their plaques from coronary arteries.

DISCUSSION

Atherosclerosis is a chronic inflammatory process accounts for coronary artery disease. Our study did not indicate that *H. pylori* is associated significantly with coronary atherosclerotic plaques in coronary artery wall in a meaningful manner in patients who have developed proved coronary diseases and underwent coronary atherectomy during CABG.

H. pylori may contribute to atherosclerosis either via direct infection of vascular cells or via the indirect effects of cytokines or acute phase proteins induced by inflammatory cells [14]. H. pylori lipopolysaccharide (LPS) might be implicated in chronic inflammatory responses induced by *H. pylori* [15]. More recently,

investigators reported that infectious burden of *H .pylori*, rather than the effects of a single organism, might contribute to atherosclerosis and its thrombotic complications [16]. *H. pylori* eradication can improve endothelial dysfunction [10]. Cautionary note is that the role of infection, as a pro-inflammatory cause of atherosclerosis, is still debated in the literature [17].

Our study provided data on presence of *H. pylori* with coronary atherosclerotic plaques but not carotids. Hypothetically, *H. pylori* presence should not be limited to coronary plaques; however, our findings showed that among 110 patients with or without atherosclerosis, none of them had *H. pylori* in carotid or aortic arch plaques, which were quite surprising to us. .Although the value of *H. pylori* detected only in coronary plaques is debatable, but we used PCR as a sensitive method that precludes these limitations. In addition, sensitivity is one aspect of *H. pylori* detection, but specificity is also important issue. PCR has been proved as a sensitive and specific method in detection of *H. pylori* in atherosclerotic plaques and there is no clearly proven superior method [18]. Altogether, coronary plaques appear to be more associated with *H. pylori* than carotid plaques. The negative *H. pylori* in non-coronary plaques suggests that *H. pylori* has specific affinity to coronary arteries because of diversity of endothelial function in different vascular bed. To establish this issue, further studies should be done. In accord to our findings Izadi and associates found that *H. pylori* PCR test result was positive

in 29.5% of coronary artery atherosclerotic plaques, but none of the mammary artery was positive for *H. pylori* genome in PCR [19].

H. pylori was positive in 9.1% of our patients with coronary plaques. Association of *H. pylori* and pathogenesis of atherosclerosis in coronary plaques of these patients may have therefore been called into question. In our study there is no significant association between the presence of *H. pylori* and development of coronary artery disease.

Our results depicted that other risk factors of atherosclerosis such as blood pressure, diabetes mellitus (DM), cigarette smoking, and family history had no significant differences between coronary and carotid groups. These results depicted that while underlying risk factors such as DM, hypertension have role in atherosclerosis, they are not distortion factors in our study. Results from human studies vary, reasonably explained by the complexity of the experiment, which causes several variables to change at the same time. As a matter of fact, atherosclerotic risk factors are various among different patients which can distort results of such epidemiologic studies and needs to be controlled to achieve genuine results regarding *H. pylori* association with atherosclerosis.

In our study Hyperlipidemia (defined as TC>200 or TG>150) generally as a risk factor had statistically significant difference between two groups (p=0.021), But when HDL, LDL, and TG separately analyzed had no significant differences among coronary and carotid groups which indicate that hyperlipidemia has a role in atherosclerosis and plaque formation in both coronary and carotid plaques. In a clinical study by Iriz and colleagues, they showed that patients who were positive for *H. pylori* DNA in carotid artery had significantly higher levels of total cholesterol, LDL, HDL cholesterol [6]. These are consistent with our study, when coronary group was subdivided into *H. pylori* positive and negative, then LDL and HDL but not TG were significantly higher between *H. pylori* positive and negative patients. Accordingly, in study of KIM HL and colleagues in Korea serum LDL level was elevated in patients who had *H. pylori* infection [11]. Other studies showed that serum triglycerides levels of *Helicobacter pylori*-positive subjects were significantly higher than in *H.*

pylori-negatives[20]. No mixed results are contradictory if seen in bigger picture. *H. pylori* may actually intervene with lipid synthesis in liver or endothelial cells. This distortion in lipid metabolism could happen through disruption or interference with several enzymes such as Carboxyl-esterase 3/triacylglycerol hydrolase (TGH)[21]. It may be that *H. pylori* is not a pure risk factor per se but infection produce inflammation and lipid peroxidation in atherosclerotic plaques [22].

There were also limitations to this study. This was a case-control study, we could not elucidate upon the mechanism by which *H. pylori* infection induced coronary plaque formation. To discover this mechanism, we need to conduct a prospective cohort model study.

In conclusion, *H. pylori* did not a predisposing factor for coronary atherosclerotic plaque formation in patients with chronic *H. pylori* infections. The presence of *H. pylori* in atherosclerotic plaque might be in accompanies with other common risk factors such as smoking, hypertension and diabetes mellitus are relative risk factors for plaque formation. Although there is no significant difference between coronary and carotid group in conventional atherosclerotic risk factors (such as diabetes mellitus, hypertension) but the patients in CABG group had more progressive atherosclerotic disease. Because in addition to aortic arch their coronary arteries also diseased, in fact *H. pylori* was found in the patient with more sever atherosclerotic disease.

In our study, the *H. pylori* positive patient had higher LDL levels, which showed the role of *H. pylori* in development of atherosclerotic in association with other atherosclerotic risk factors. However, the pathophysiologic process in development of atherosclerosis is similar in different arterial bed, but the affinity of *H. pylori* for coronary artery suggestive of the variable endothelial response in different arterial bed and importance of *H. pylori* on intensifying atherosclerotic process. The strength of our data for H. pylori with regard to atherosclerosis pathogenesis and their potential contribution by direct or indirect mechanisms still needs to be established. Further researches, should be focused on determining such association.

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