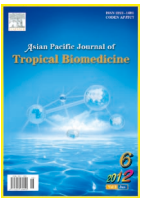
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage:www.elsevier.com/locate/apjtb



Document heading doi:10.1016/S2221-1691(12)60080-X

Association of smoking, alcohol and NSAIDs use with expression of cag A and cag T genes of *Helicobacter pylori* in salivary samples of asymptomatic subjects

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ARTICLE INFO

Article history:
Received 20 October 2011
Received in revised form 15 November 2011
Accepted 10 December 2011
Available online 28 June 2012

Keywords:
Smoking
Alcohol
NSAIDs
Helicobacter pylori
Cag A
Cag T
16S rRNA
PCR
Agarose gel electrophoresis
Virulence
Prevalence
Infection
Asymptomatic

ABSTRACT

Objective: To determine the association of smoking, alcohol and nonsteroidal anti-inflammatory drugs (NSAIDs) use with presence and virulence of *Helicobacter pylori* (*H. pylori*) infection in a representative sample of a random adult population of asymptomatic subjects. **Methods:** Non virulent 16S rRNA and virulent cag A and T genes from salivary samples of 854 asymptomatic subjects were determined using polymerase chain reaction. The presence and absence of virulent and non virulent infection was statistically compared with consumption of smoking, alcohol and NSAIDs. **Results:** The prevalence of infection in male and female subjects was found to be 69.25% and 66.90%, respectively. The prevalence of infection in the population of asymptomatic subjects with respect to consumption of alcohol was as follows: current (31.22%), former (52.20%) and never (43.58%). The prevalence of infection in the population of asymptomatic subjects with respect to smoking of cigarettes was as follows: current (88.80%), former (57.14%) and never (33.33%). The prevalence of infection in the subject population consuming NSAIDs and not consuming NSAIDs frequently was found to be 82.75% and 21.16%, respectively. Virulence in male and female subjects was found to be 60.00% and 50.00%, respectively. The presence of virulent infection in the population of asymptomatic subjects with respect to consumption of alcohol was as follows: current (28.57%), former (40.15%) and never (50.00%). The prevalence of virulent infection in the population of asymptomatic subjects with respect to smoking of cigarettes was as follows: current (79.32%), former (75.00%) and never (50.00%). The prevalence of virulent infection in the subject population consuming NSAIDs and not consuming NSAIDs frequently was found to be 88.23% and 66.66%, respectively. **Conclusions:** It can be concluded that smoking and NSAIDs consumption are aggravating factors for virulence of *H. pylori* and alcohol can inhibit *H. pylori* infection in asymptomatic subjects.

1. Introduction

Helicobacter pylori (*H. pylori*), termed as class I carcinogen by World Health Organization, is a worldwide menace with the ability to transform gastric lesions into a carcinogenic lymphoma[1-3]. It has been studied in depth by many workers proving its disease causing potential to be immense[4,5]. An array of factors like gender, age, smoking, chronic nonsteroidal anti-inflammatory drugs (NSAIDs) consumption and habitual alcohol have been studied by various researchers[6]. However, the difference between presence of virulent *H. pylori* and its presence in its dormant or viable but nonculturable (VBNC) form has been implicated[7].

The present investigation was designed to understand the association of NSAIDs use, smoking, alcohol and presence of virulent *H. pylori*. *H. pylori* strains possess a varied genetic diversity and many markers of virulence in *H. pylori* have been identified. Polymerase chain reaction (PCR) based methods have been used for the detection of *H.*

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Foundation Project: This work was financially supported by India Council of Technical Education as National Doctoral Fellowship (grant No. RID/NDF-37/2009/10).

pylori DNA in gastric mucosa and gastric juice, as well as in feces, saliva, dental plaque, and environmental samples. Determination of *H. pylori* in saliva serves as an excellent non invasive tool to detect *H. pylori* in human subjects. A number of target genes have been proposed as candidates for the PCR detection of *H. pylori*[8].

Cag A and cag T have been implicated to play an important role in the disease progression and can be used as biomarkers of virulent disease causing state of *H. pylori*[8]. In the present investigation, 16S rRNA, cag A and T have been targeted to determine the presence and virulence of the *H. pylori* in salivary samples of asymptomatic subjects, respectively.

2. Materials and methods

2.1. Chemicals

All the chemicals for DNA extraction were procured from S.D. Fine Chemicals, India. The reagents for PCR, gel preparation, and visualization were purchased from Vivantis India, Thane. The forward and reverse primers for 16S rRNA and cag A, E, T genes were synthesized at Ocimum Biosolutions, Hyderabad, India. Gel electrophoresis unit (Bangalore genie, Bangalore) was used to perform gel electrophoresis and gel documentation unit (Alpha Innotech Inc. USA) was used to visualize and capture the gel image.

2.2. Sample collection

A total of 854 healthy subjects were included in the present study. The sampling for the study was undertaken during May to October 2010. An informed consent was obtained from each individual. The study protocol was approved by Institutional Human Ethics Committee of Bharati Medical College, Bharati Vidyapeeth Deemed University, Pune. The study population consisted of men and women of more than 18 years of age. A questionnaire in local language or English was filled up by each participant to determine that none of the participants had symptoms suggestive of acid peptic diseases. The medication history of each subject was recorded and it was ascertained that they did not consume proton pump inhibitors, H_2 blockers and antibiotics before one month of saliva sampling. Saliva samples were collected by visiting homes, colleges and villages. Unstimulated saliva in the volume of 1.5 mL was collected in presterilized microcentrifuge tube and stored at −80 ℃ until processed. Approximately 1.5 mL of non-stimulated saliva was collected in a 2 mL microtube. After collection saliva was homogenized by vigorous shaking with the use of a vortex mixer and clarified by centrifugation (10000 g, 4 ℃, 4 min).

2.3. Collection of data

The questionnaire was available in local language and English for data collection that included gender, history of cigarette smoking, alcohol consumption, and NSAIDs use by the asymptomatic subjects. All the subjects who consumed NSAIDs more than 10 day per month were considered as NSAIDs users[9].

2.4. Preparation of genomic DNA for PCR

DNA isolation from salivary samples was performed according to phenol chloroform C-TAB method[8]. All the steps were performed in aseptic conditions to minimize contamination using cryocentrifuge (Eppendorf). The DNA was extracted and preserved at −20 ℃ until polymerase amplification by chain reaction was performed. Amplification of the DNA template was carried out using forward and reverse primers[8] as mentioned in Table 1. 16S rRNA (534 base pair fragment) was amplified in a programmable thermal cycler (Eppendorf). DNA sample from the same subject was used to amplify cag A using specific primers[8]. At each amplification, *H. pylori* DNA from strain ATCC 26695 was used as a positive control, while sterile water for injection instead of DNA served as a negative control. The products were analyzed by agarose gel electrophoresis unit (Bangalore Genei, India) and the gel image was captured using gel documentation unit (Apha Innotech Inc. USA).

2.5. Statistical analysis

Statistical analysis was carried out to examine the association between the various study variables with

Table 1
Primer sequences and respective product sizes of *H. pylori* specific genes.

Primer	Sequence	Amplicon size (base pair)
16S rRNA-F	5′ TAAGAGATCAGCCTATGTCC3′	534
16S rRNA-R	5′TCCCACGCTTTAAGCGCAAT3′	
Cag A-F	5′ CCATGAATTTTTGATCCGTTCGG3′	349
Cag A-R	5′ GATAACAGGCAAGCTTTTGAGAGGGA3′	
Cag T-F	5′ATGAAAGTGAGAGCAAGTGT3′	301
Cag T-R	5′TCACTTACCACTGAGCAAAC3′	

F: forward primer; R: reverse primer.

saliva PCR positivity for *H. pylori* using Fischer exact test. Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA), odds ratio, 95% confidence interval of odds ratio, and relative risk.

3. Results

The DNA isolated from all the samples was amplified to get a 534 base pair fragment amplicon corresponding to 16S rRNA gene in the subjects who had *H. pylori* infection. The same template was used to amplify cag A and T genes to find out expression of virulence factors in the detected *H. pylori* (Figure 1). The amplicons obtained were of 349 and 301 base pairs, respectively.

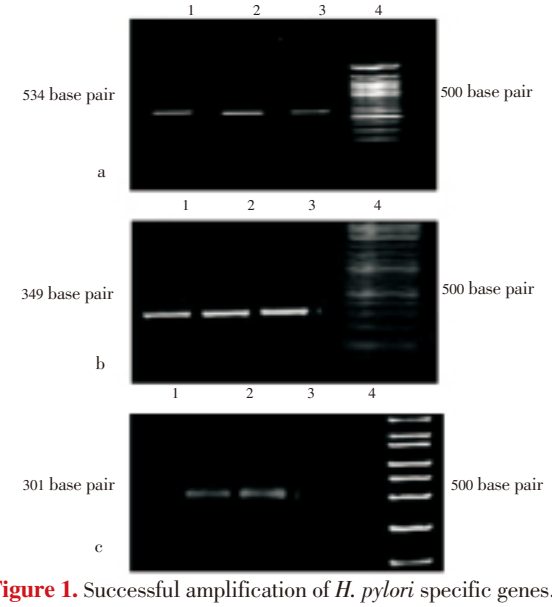

Figure 1. Successful amplification of *H. pylori* specific genes.
a: 16S rRNA (534 base pair); b: cag A gene (349 base pair); c: cag T gene (301 base pair).

3.1. Prevalence

The results in Table 2, indicated that the prevalence of infection in male and female subjects was found to be 68.25% and 66.90%, respectively. The *P* value was found to be equal to 0.698 1 and hence the prevalence was not dependent upon gender. The prevalence of infection in the population of asymptomatic subjects with respect to consumption of alcohol was as follows: current (31.22%), former (52.20%) and never (43.58%). The infection status in the people who had never consumed alcohol was not significant (P=0.144 0) whereas it was found to be significant in the people who were former alcohol consumers (P<0.000 1). The prevalence of infection in the population of asymptomatic subjects with respect to smoking of cigarettes was as follows: current (88.80%), former (57.14%) and never (33.33%). The differences of infection status in the people who were former smokers and current smokers were statistically significant (P=0.043 4 and P<0.000 1, respectively). The prevalence of infection in the subject population consuming NSAIDs and not consuming NSAIDs frequently was found to be 82.75% and 21.16%, respectively. Frequent consumption of NSAIDs was associated with *H. pylori* infection (P<0.000 1).

The odds ratio and 95% *CI* of odds ratio in the population of asymptomatic subjects with respect to gender were (0.94, 0.69-1.27) in males when females subjects were taken as referent. The odds ratio and 95% *CI* of odds ratio in the population of asymptomatic subjects with respect to consumption of alcohol were as follows: former (1.70, 0.86-3.38) and never (2.41, 1.57-3.70) when current consumers of alcohol were taken as referent. The odds ratio and 95% *CI* of odds ratio in the population of asymptomatic subjects with respect to smoking of cigarettes were as follows: current (15.86, 7.19-35.01) and former (2.67, 1.06-6.73) when people who were never smokers were taken as referent. The odds ratio and 95% *CI* of odds ratio in the population of asymptomatic subjects who frequently consumed NSAIDs were (17.88, 10.72-29.79) when subjects who do not consume NSAIDs frequently were taken as referent.

Table 2
Various risk factors with *H. pylori* infection status (16S rRNA) [*n* (%)].

Variables		No. of subjects	*H. pylori* positive	*H. pylori* negative	Odds ratio	95% *CI* of odds ratio
Gender	Male	570 (66.74)	389 (68.25)	181 (31.75)	0.94	0.69-1.27
	Female	284 (33.25)	190 (66.90)	94 (33.10)		Referent
Consumption of alcohol	Current	506 (59.30)	158 (31.22)	348 (68.78)		Referent
	Never	78 (9.00)	34 (43.58)	44 (56.41)	1.70	0.86-3.38
	Former	272 (31.77)	142 (52.20)	130 (47.80)****	2.41	1.57-3.70
Smoking	Current	768 (79.50)	682 (88.80)	86 (11.20)****	15.86	7.19-35.01
	Never	30 (3.50)	10 (33.33)	20 (66.66)		Referent
	Former	56 (17.00)	32 (57.14)	24 (42.86)*	2.67	1.06-6.73
NSAIDs use	Yes	580 (67.91)	480 (82.75)	100 (17.25)****	17.88	10.72-29.79
	No	274 (32.09)	58 (21.16)	10 (78.84)		Referent

Statistical analysis between groups was carried out using Fischer exact test. The values in paranthesis indicate the percentage of the number of subjects. *: P<0.05, ****: P<0.000 1 comparing with *H. pylori* positive individuals.

Table 3

Various risk factors with virulent *H. pylori* infection status (cag A and cag T positive) [*n* (%)].

Variables		No. of HP positive subjects	Virulent HP	Non virulent HP	Odds ratio	95% *CI* of odds ratio
Gender	Male	389	233 (60.00)	156 (40.00)	1.50	0.44-5.10
	Female	190	95 (50.00)	95 (50.00)		Referent
Consumption of alcohol	Current	158	46 (28.57)	112 (71.43)		Referent
	Never	34	17 (50.00)	17 (50.00)*	2.44	1.14-5.18
	Former	142	57 (40.15)	85 (59.85)	1.63	1.01-2.64
Smoking	Current	682	541 (79.32)	141 (20.68)*	3.84	1.10-13.44
	Never	10	5 (50.00)	5 (50.00)		Referent
	Former	32	24 (75.00)	8 (25.00)	3.00	0.69-13.12
NSAIDs use	Yes	480	423 (88.23)	57 (11.76)****	3.91	2.13-7.18
	No	58	38 (66.66)	20 (33.33)		Referent

Statistical analysis between groups was carried out using Fischer exact test. The values in paranthesis indicate the percentage of the number of subjects. HP: *H. pylori*; *: $P<0.05$, ****: $P<0.0001$ comparing with virulent *H. pylori* infection individuals.

3.2. Virulence

The results in Table 3 indicated that, the virulence in male and female subjects was found to be 60% and 50%, respectively. The P value was found to be equal to 0.5481 and hence the virulence was not dependent upon gender. The presence of virulent infection in the population of asymptomatic subjects with respect to consumption of alcohol was as follows: current (28.57%), former (40.15%) and never (50.00%). The virulent infection status in the people who had never consumed alcohol was significant ($P=0.0261$) and non significant in the people who were former consumers of alcohol ($P=0.0516$) when it was compared with people who were current consumers of alcohol. The prevalence of virulent infection in the population of asymptomatic subjects with respect to smoking of cigarettes was as follows: current (79.32%), former (75.00%) and never (50.00%). The virulent infection status in the people who were former smokers was not significant ($P=0.2383$) whereas it was found to be significant in the people who were current smokers ($P=0.0394$) when compared with people who had never smoked. The prevalence of virulent infection in the subject population consuming NSAIDs and not consuming NSAIDs frequently was found to be 88.23% and 66.66%, respectively. Frequent consumption of NSAIDs was associated with virulent *H. pylori* infection ($P<0.0001$).

The odds ratio and 95% *CI* of odds ratio in the population of asymptomatic subjects with respect to gender were (1.50, 0.44-5.10) in males when females subjects were taken as referent. The odds ratio and 95% *CI* of odds ratio in the population of asymptomatic subjects with respect to consumption of alcohol was as follows: never (2.44, 1.14-5.18) and former (1.63, 1.01-2.64) when current consumers of alcohol were taken as referent. The odds ratio and 95% *CI* of odds ratio in the population of asymptomatic subjects with respect to smoking of cigarettes were as follows: current (3.84, 1.10-13.44) and former (3.00, 0.69-13.12) when people who were never smokers were taken as referent. The odds ratio and 95% *CI* of odds ratio in the population of asymptomatic subjects who frequently consumed NSAIDs were (3.91, 2.13-7.18) when subjects who do not consume NSAIDs frequently were taken as referent.

4. Discussion

H. pylori is the causative agent of chronic gastritis, peptic ulcers, gastric adenocarcinoma, and lymphoma of the stomach[10]. Saliva has been a source to detect *H. pylori* from asymptomatic subjects as well as patients[13]. *H. pylori* can migrate from an infected individual to an uninfected individual *via* oral oral or fecal oral route. Oral cavity provides a harbor for the residence of *H. pylori*. Thereafter, it migrates to the gastric mucosa and resides in the acidic environment[14]. This concept has been used by various research workers to evaluate the infection status of *H. pylori* using the saliva from the oral cavity of *H. pylori*[13,14]. *H. pylori* may exist in the oral cavity of such asymptomatic individuals without showing its pathological manifestations. It may exist in a dormant VBNC form. It is worth considering that other diagnostic techniques than PCR are unable to detect this form of *H. pylori*.

In the present study, 16S rRNA, cag A and T genes were amplified to detect the presence and virulence of *H. pylori* in the oral cavity. 16S rRNA gene is present in a highly conserved region of *H. pylori* genome and serves as a gold standard in the detection of *H. pylori*. The cag pathogenecity island is the hallmark of virulence of *H. pylori*[5]. The cag PAI is an important virulence component for infection and it gives shape to the pathological manifestations of *H. pylori* infection. A considerable proportion of the population in Asia is infected with *H. pylori*[13]. In brief, cag pathogenecity island comprises the various components of type IV bacterial secretion system which acts as a molecular needle and syringe to inject pathogenic proteins into the gastric epithelial cells[13,14]. Injected cag A modulates signal transduction leading to detrimental host cell responses like up regulated proliferation of cells, apoptosis, and necrosis. Cag A positive strains of *H. pylori* significantly increase the risk for severe gastritis, peptic ulceration, and distal gastric cancer compared to strains that lack the cag island[15]. Hence, this gene was targeted in the present investigation to unravel the infection status and virulence in the infected asymptomatic subjects. Cag T has been implicated to be an important facet of the genetic make up of *H. pylori* and

can be implicated to serve as an identification mark for the pathogenic state of *H. pylori*[8].

Smoking has been implicated as a decisive factor promoting the infection of *H. pylori*[17]. Our results are in concert with the previous workers who have examined the effect of smoking in the promotion of *H. pylori* infection[16-21].

Chronic NSAIDs consumption leads to decrease in mucin synthesis and promotes aggravation of ulcers and *H. pylori* colonization. It is evident that chronic NSAIDs consumption is a risk factor in *H. pylori* infection as the salivary samples of subjects on chronic NSAIDs therapy were found to be infected with *H. pylori*. Cag A gene was also successfully amplified in some of these subjects. This reflects that NSAIDs consumption is a risk factor for virulent *H. pylori* infection.

The present investigation shows that a small population of asymptomatic subjects possesses a virulent strain of *H. pylori* and needs to take precautionary measures. These subjects were asymptomatic during sampling but it may be hypothesised that they may have a higher chance of acquiring an active gastric malady in the near future.

Recently, alcohol consumption has been studied in detail with the disease status and it has been elucidated in other parts of world that alcohol consumption has a negative relation with *H. pylori* infection[22].

The detection of non virulent and virulent *H. pylori* from saliva of the asymptomatic subjects indicates that *H. pylori* exists in a non pathogenic form in a group of subjects. This form is called VBNC bacterial form. This refers that bacteria are in a state of very low metabolic activity and do not divide, but are alive and have the ability and become culturable once resuscitated[23].

Hence, a large population in western Indian population is a carrier of non virulent strain of *H. pylori* out of which a small population carries virulent (cag A positive) *H. pylori*. Hence, the individuals have a risk of acquiring active infection if the VBNC form transforms back to its helical form. In this form *H. pylori* can exist in a plethora of substrates like food, water or biofilm[24-26]. Thereafter, it can easily be transmitted to an uninfected person.

PCR is the technique that detects low levels of *H. pylori* as well as determines the virulent and non virulent strains. This form of *H. pylori* can't be detected by culture and PCR provides a perfect method for the detection of VBNC form. In this state, *H. pylori* is detectable but non virulent. Hence, cag A gene could not be amplified in most of the salivary samples of asymptomatic subjects.

The present study demonstrates that people need to abstain from smoking and habitual consumption of NSAIDs which pose a serious threat to the asymptomatic subjects of acquiring active infection and are aggravating factors for *H. pylori* infection. NSAIDs consumption has been previously demonstrated to be a significant risk factor for *H. pylori* infection[16-20].

The study shows that *H. pylori* exists in the oral cavity of a considerable portion of the asymptomatic subjects. However, the cag pathogenecity island genes cag A and T were not detected in a substantial portion of population in which 16S rRNA gene was amplified. Hence, it could be deduced that *H. pylori* is present in the asymptomatic subjects but the virulent factors are expressed in a fraction of them. However, virulent strains may give rise to a plethora of gastric diseases when the suitable pathobiological conditions culminate in the gastric environment of the patients[27-37].

Hence, there is a need to ascertain that smoking and NSAIDs use are limited in the population to halt the progression of the *H. pylori* mediated gastritis and lymphomas. Our investigation also confirms that the *H. pylori* inhabiting the oral cavity is non virulent and if the virulent genes, like cag A and T corresponding to cag PAI are targeted in the oral cavity, it may serve as a tool to monitor disease progression in asymptomatic subjects.

Our investigation will help epidemiologists and physicians to understand the pattern of disease in this geographical domain and its implications on public health. The outcome of this study is the finding that a considerable portion of population is under constant threat of being affected with fulminant manifestations of *H. pylori* infection if rapid measures are not initiated to eradicate this menace from food, water and other contamination sources.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors would like acknowledge Dr. SS Kadam, Vice-Chancellor and Dr. KR Mahadik, Principal, Poona College of Pharmacy, Bharati Vidyapeeth University, Pune, India, for providing necessary facilities to carry out the study. The authors are thankful to Dr. Aleem A Khan, Dr. Santosh K Tiwari and Dr. Manoj Gopi of Centre for Liver Research and Diagnostics, Owaisi Hospital, Hyderabad for valuable guidance in molecular biology techniques in the investigation. The authors acknowledge all India Council of Technical Eductaion for financial support as National Doctoral Fellowship to Pinaki Ghosh (RID/NDF-37/2009/10).

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