Presence of *Helicobacter pylori* Detected by PCR in Saliva of Male Smokers and Non Smokers with Chronic Periodontitis

Rajiv Kishor Saxena[1], Abdul Samad Aziz[2*], Madhav Govind Kalekar[2], J. P. Milsee Mol[3], Adinath Narayan Suryakar[4], Benjamin Tabita[5], Ravi Vasudev Shirahatti[6] and Raghavendra Shrishail Medikeri[7]

[1]Department of Microbiology, Sinhgad Dental College, Pune, India.
[2]Department of Biochemistry, Grant Medical College and Sir J. J. Group of Hospitals, Byculla, Mumbai, India.
[3]Department of Biotechnology, Sinhgad College of Science, Pune, India.
[4]FACBI, Registrar, Maharashtra University of Health Sciences, Nashik, India.
[5]Department of Dentistry, Grant Medical College and Sir J. J. Group of Hospitals, Byculla, Mumbai, India.
[6]Department of Public Health Dentistry, Sinhgad Dental College, Pune, India.
[7]Department of Periodontics, Sinhgad Dental College, Pune, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author RKS performed PCR analysis and helped to wrote the first draft of manuscript. Author ASA managed literature searches, wrote protocol, data acquisition and manuscript preparation. Author MGK designed, guided and supervised the study. Author JPMM helped in PCR analysis, manuscript editing and manuscript review. Author ANS designed and guided the study. Author BT managed the evaluation of the clinical parameters. Authors RVS and RSM helped in manuscript editing and manuscript review. All authors read and approved the final manuscript.

Research Article

ABSTRACT

Aims: To assess the comparative presence of *Helicobacter pylori* (*H. pylori*) in saliva of smokers and nonsmokers with chronic periodontitis.
Study Design: Male individuals diagnosed with chronic periodontitis with and without

Corresponding author: Email: samadaziz79@gmail.com;

smoking habits were enrolled in the study. The unstimulated whole saliva was subjected to *H. pylori* DNA detection using real time PCR. The percent presence of *H. pylori* DNA among the groups, were statistically compared.

Place and Duration of the Study: Department of Biochemistry and Department of Dentistry, Grant Medical College and Sir JJ group of Hospitals, Mumbai and Department of Microbiology, Sinhgad Dental College, Pune, between January 2010 and June 2010.

Materials and Methods: A total of 48 males with severe chronic periodontitis (CAL 5mm) were divided into two groups, Group I (n=30, mean age=44.2±5.88 yrs) with smoking habit, Group II (n=18, mean age 41.72±4.36yrs) without any smoking habit. Nonsmokers with slight chronic periodontitis (CAL<3mm) were enrolled as controls, Group III (n=16, mean age 39.64±5.04 yrs). Periodontal status was evaluated by measuring gingival index (GI), plaque index (PI), probing depth (PD) and clinical attachment loss (CAL). Salivary samples were subjected to real time PCR for detection of *H. pylori* DNA.

Result: Periodontal parameters were significantly different between Group I and II compared to Group III (*P*=.001). Overall, *H. pylori* was not detected in Group III (100% negative), whereas 5.5% of Group II and 13.3% in Group I patients showed presence of *H. pylori*. Chi-square test showed a significant difference (*P*=.003) between Group I and Group III however it was seen that there was an insignificant difference between Group I and Group II (*P*=.312) and between Group II and Group III (*P*=.186).

Conclusion: The smokers with chronic periodontitis may be at a relatively higher risk of *H. pylori* presence in the oral cavity, than non smokers. The study needs validation on a larger sample size.

1. INTRODUCTION

H. pylori is a Gram negative, spirally shaped bacterium, 0.5 – 0.9 µm wide by 2 – 4 µm long. It is micro-aerophilic and requires carbon dioxide for growth. It produces an exceptionally powerful urease which is vital for its survival in the stomach [1]. *H. pylori* is an ancient member of the human microbiota which has co-evolved with humans at a moderate level of virulence [2]. Gastric *H. pylori* is involved in the physiological regulation of gut hormones involved in food intake, energy expenditure and body weight maintenance [3]. The association of *H. pylori* with chronic type B gastritis and peptic ulcer disease has been observed [4]. In the western world, average *H. pylori* carriage rates in healthy adults are estimated to be between 10–40%, whereas 80– 100% of patients with gastritis and duodenal ulceration harbor the microorganism. Evidence exists for possible oral transmission from person to person and fecal-oral transmission is considered to be particularly prevalent in developing countries [5]. Recently *H. pylori,* has been found in association with dental plaque, suggesting the oral environment may be one of the many potential pathways for transmission [6]. Various studies [7, 8, 9] have associated presence of *H. pylori* in oral environment of subjects with periodontitis suggesting that progression of periodontal pocket and inflammation may favor colonization by this bacterium. Tobacco smoking has been regarded as a risk factor for periodontitis. Smokers have both increased prevalence and severe extent of periodontal disease, as well as higher prevalence of tooth loss and endentulism compared to non-smokers [10]. A reservoir of dental plaque exists in periodontal pockets in smokers with periodontitis and the potential for reinfection of the stomach by *H. pylori* is obvious [11]. Further it has been demonstrated that smoking

increases the failure rate in treatment of for *H. pylori* eradication [12]. Salivary diagnostics offer an easy, inexpensive, safe and cost-effective approach for disease detection Saliva contains an abundance of biomolecules that reflect physiological status. [13]. Saliva-based diagnosis has been reported in periodontitis [14] and also in studies representing prevalence of *H. pylori* in periodontitis [8, 15]. Hence the present study was undertaken to comparatively evaluate the presence of *H. pylori* in saliva of chronic periodontitis patients with and without smoking habits.

2. MATERIALS AND METHODS

The study was undertaken as per the approval of the Institutional Ethics Committee (registration number: 391/CPCSEA) of Grant Medical College and Sir J. J. Group of Hospitals, Mumbai, following norms of the World Medical Association of Helsinki; ethical principles for medical research involving human subjects (amended by 55th WMA General Assembly, 2004). A written informed consent was obtained from all the subjects enrolled in the study. The subjects had the right to refuse to participate in the study or to withdraw consent to participate at any time without reprisal.

2.1 Study Groups

Individuals visiting the Department of Dentistry, Grant Medical College, Mumbai, constituted the study population and were divided into the following groups based on their smoking and periodontal status:

Group I: Current smokers with severe chronic periodontitis (CAL 5mm); n=30 (mean age 44.2 ± 5.88 yrs).

Group II: Never smokers with severe chronic periodontitis (CAL 5mm); n=18 (mean age 41.72 ± 4.36 yrs).

Group III: Never-smokers with slight chronic periodontitis (CAL<3mm), n=16 (mean age 39.64 ± 5.04 yrs).

The patients in the study groups were clinically evaluated for chronic periodontitis according to the criteria accepted by the American Academy of Periodontology in 1999 [16] and the periodontal examination was performed as documented by Armitage [17]. The patients were otherwise healthy, with no history of major illness and consumption of antioxidants, antibiotics, anti- inflammatory or any other drugs and had not received any periodontal therapy for at least six months prior to the inception of the study. Individuals, who had reported gastric illness, received any medical and periodontal treatment in the past six months prior to the study, diabetics and alcoholics were excluded from the present study. The subjects in the smoker group were current smokers (predominantly cigarette smokers) with smoking habit of   3 years and frequency of smoking   5 cigarette / day. This information was obtained during the clinical history taking of the subjects.

2.2 Clinical Measurements

The periodontal status of all individuals was evaluated with the measurement of gingival index (GI) as developed by Loe and Silness [18]. GI is used for assessment of gingivitis in individual dentition. The plaque index (PI) assesses the thickness of plaque at the gingival

area of the tooth, and was measured as described by Silness and Loe [19]. The probing depth (PD) is the distance from the gingival margin to the base of the probeable crevice. It gives information about periodontal pockets [17].The clinical attachment loss (CAL) is the distance from the cementoenamel junction to the base of the probeable crevice. CAL measurements are the best way to assess the presence or absence of additional periodontal damage. PD and CAL measurements are recorded at six sites around each tooth (i.e. mesiobuccal, buccal, distobuccal, meisolingual, lingual and distolingual) [17]. All clinical measurements were evaluated by a single investigator using University of North Carolina (UNC-15) probe (Hu-Friedy, Chicago).

2.3 Sample Collection

After clinical evaluation the individuals were referred to the department of Biochemistry, Grant Medical College, Mumbai, for saliva collection. The procedure for saliva collection was accessed from Molmeth (URL: http://www.molmeth.org/protocols/1BXQD00) as per the protocol derived from the WHO/IARC guideline "Common Minimal Technical Standards and Protocols". Fasting un-stimulated whole saliva samples were obtained in the morning, prior to which individuals were requested not to drink any beverages except water. They were given drinking water (bottled) and asked to only rinse their mouth out well (without drinking the water). Five minutes after this oral rinse, the individuals were asked to spit whole saliva in a sterile container. About 5ml of whole saliva was collected from each individual. They were asked to refrain from talking or lower their head down so as to let the saliva run naturally to the front of the mouth. They were also asked not to cough up mucus during the saliva collection. The salivary samples were collected and stored at -4ºC until DNA extraction. The salivary samples for PCR analysis were transported using cool bags to the Department of Microbiology, Sinhgad Dental College, Pune.

2.4 PCR Analysis

For PCR, DNA was extracted from saliva samples by using QIAGEN DNA extraction kit (Germany) according to the instructions of the manufacturer. Briefly, 200 µL saliva, 20 µL QIAGEN Protease and 200 µL buffer AL (lysis buffer), were added, mixed and incubated at 56ºC for 10 minutes. After brief centrifugation, 200 µL of 96% ethanol was added and mixed. The obtained mixture was applied to the QIAmp mini spin column and centrifuged at 8000 rpm for 1 minute. Pure DNA was eluted from the column using buffer AE (10 mM Tris.Cl; 0.5 mM EDTA; pH 9.0) and stored at -20ºC till further analysis. The DNA samples obtained were subjected to real-time PCR, using SYBR-Green PCR master mix (Applied Biosystem). The RT-PCR was targeted at the 26 KDa *Helicobacter* species-specific antigen (*SSA*) gene with primer sequence (forward: 5'-TGGCGTGTCTATTGACAGCGAGC-3', reverse: 5'-CCTGCTG GCATACTTCACCATG-3') [20, 21]. The reaction mixture composed of the following : 20 µL of 2X SYBR Green PCR Master mix (Qiagen), 50 nM of each primer and 1 µL of extracted DNA (200 ng).The reaction was cycled with initial PCR activation step at 95ºC for 10 min, followed by 40 cycles of denaturation at 95ºC for 30 s, annealing at 60ºC for 60 s and primer extension at 60ºC for 60 s. Applied Biosystems ABI 7500 SDS real-time thermal cycler was used for PCR data acquisition and analysis.

2.5 Statistical Analysis

The measured values for the clinical parameters were subjected to statistical analysis using Statistical Package for Social Sciences (SSPS software, version 11.5) for MS Windows. The

values were expressed as mean± SD. The underlying normality assumption was tested for each clinical parameter using percentile to percentile (PP) plot technique before applying any statistical test. Comparison of significance of difference of average of clinical parameters across the three study groups was done using analysis of variance (ANOVA) technique with Tukey's correction for multiple group comparison. The values on *H. pylori* are n (%) and the *P* – value on *H. pylori* was obtained using Chi – square test if cell frequencies were larger than 5, else Fisher's exact probability test were used. *P* – value for mean PD between *H. pylori* positive and *H. pylori* negative subjects in Group I was obtained using Mann Whitney U test. *P* = .05 is considered to be statistically significant, for all the statistical test employed.

3. RESULTS AND DISCUSSION

Chronic periodontitis is a chronic inflammatory disease of the periodontium and significantly higher periodontal clinical parameters like GI, PI, CAL compared to healthy controls have been documented [22,23,24]. Furthermore, smokers with chronic periodontitis showed altered clinical parameters than non smokers with chronic periodontitis [25,26,27].

Table 1. Mean values for the clinical parameters and the percent presence for *H. pylori* in various study groups

Parameters		Group I (n=30)	Group II (n=18)	Group III (n=16)	*P* value[†] Group I vs. Group II	Group I vs. Group III	Group II vs. Group III
Clinical	GI	1.9 ± 0.5	2.4 ± 0.5	0.7± 0.08	.001	.001	.001
	PI	3.0 ± 0.5	2.3 ± 0.6	0.5 ± 0.2	.001	.001	.001
	PD(mm)	5.7 ± 0.5	5.2 ± 0.5	1.6 ± 0.2	.141	.001	.001
	CAL(mm)	8.5 ± 1.0	7.7 ± 0.9	1.9 ± 0.3	.021	.001	.001
H. pylori presence (n %)	+ve	13.3 n %	5.5 n %	0.0 n %	.186[‡]	.003[‡]	.132[‡]

†P values for mean ± SD was obtained using one way analysis of variance (ANOVA) with Tukey's correction for multiple group comparisons. P = .05 is considered to be statistically significant.
‡P value for presence (n %) of H. pylori was obtained using Chi – square test if cell frequencies are larger than 5, else Fisher's exact probability test was used. P = .05 is considered to be statistically significant.

The present study has observed significantly higher PI (3.0 $\pm$ 0.5 v/s 2.3 $\pm$ 0.6, *P*=.001), CAL (8.5 $\pm$ 1.0 v/s 7.7 $\pm$ 0.9, *P*=.021) and relatively higher PD (5.7 $\pm$ 0.5 v/s 5.2 $\pm$ 0.5, *P*=.141) in current smokers with chronic periodontitis compared to never smokers with chronic periodontitis. However, current smokers with chronic periodontitis showed significantly lower GI (1.9 $\pm$ 0.5 v/s2.4 $\pm$ 0.5, *P*=.001) compared to never smokers with chronic periodontitis (Table-1). The lowered GI observed in smokers could be attributed to the smoking habit. Smoking associated periodontitis is characterized by limited gingival redness. Smoking leads to sustained peripheral vasoconstriction caused by chronic low dose of nicotine. This leads to reduced gingival bleeding [28].

Although, the infection by *H. pylori* is widely accepted as an important cause of gastritis and is strongly associated with the disease of peptic ulcer and gastric cancer, studies in the literature indicate that it is also commensal with important hunger regulation functions of

benefit to the host. *H. pylori* generally dominate the gastric niche and the response that it induces in the host affects the gastric hormones including gastrin and somatostatin. *H. pylori* positive individuals (especially individuals carrying *cag* positive strains) have lower risk of childhood asthma, allergic rhinitis and skin allergies than those without *H. pylori*. The disappearance of *H. pylori* might contribute to the current epidemics of early life obesity, type II diabetes and related metabolic syndrome [2]. *H. pylori* colonization is also involved in ghrelin and leptin expression; which are important hormones involved in regulation of appetite and energy expenditure, with consequent effects on body morphometry [3].

The human stomach was considered to be the only reservoir for *H. pylori* until this bacterium was discovered in the human oral cavity, saliva, dental plaque and in oral lesions or ulcers [6,29,30]. Through various studies [15,31,32,33] it was observed that there was a possible role of oral cavity as a reservoir for *H. pylori,* and these studies have correlated *H. pylori* colonization and periodontal disease. The oral cavity may be a reservoir for *H. pylori* and the patients with poor oral hygiene have a higher prevalence of *H. pylori* in dental plaque [34]. Among the various periodontopathogens, co-aggregation of *Fusobacterium nucleatum* and *Eikenella corroden* with *H. pylori* in the subgingival dental plaque have been reported [35]. In the oral cavity, saliva plays a significant role in maintaining a healthy oral environment [36] and it has been suggested that microorganisms in dental plaque can survive in saliva [13]. Detection of *H. pylori* in saliva and gingival plaques of periodontitis patients have been documented [8,15]. The PCR amplification of the DNA of the bacterium *H. pylori* in saliva samples have been seen to have comparable sensitivity to DNA tests using gastric biopsy samples or histopathological analysis of gastric tissue [37]. Thus detection of *H. pylori* in saliva using sensitive methods like PCR may reveal the presence of this bacterium in the oral cavity. The present study did not detect *H. pylori* DNA in Group III (100% negative), whereas 5.5% of Group II and 13.3% Group I individuals showed the presence of *H. pylori* DNA. Current smokers with severe chronic periodontitis showed significant percent presence ($P = .003$) than never smokers with slight chronic periodontitis, however the percent presence is not as significant ($P = .186$) when compared to never smokers with severe chronic periodontitis, though the percent presence values are relatively higher. The insignificant level of *H. pylori* presence observed may be due to smaller sample size of the study population.

It is well accepted that persistent *H. pylori* infection results in an inflammatory response in the stomach leading to high induction of proinflammatory cytokines, such as TNF , IL1 and IL8 [38]. *H. pylori* directly interacts with host cells, induces pro inflammatory cytokines and stimulates production of free radicals leading to inflammatory alterations in the epithelial cells of gastrointestinal tract [39]. On a similar paradigm, these inflammatory markers, which may be produced by *H. pylori* in the oral cavity, may add an additional burden on the pre existing inflammation due to chronic periodontitis in the oral cavity. Thus *H. pylori* in oral cavity may contribute to the inflammatory destruction of peridontium in chronic periodontitis.

The overall prevalence of periodontal diseases is high in India because of several factors like huge population (app 1000 million), of which 72% reside in the rural areas, poor oral health care system and para dental infrastructure, with the dentist: population ration of 1:2 lakh. Individuals with habits like smoking, consumption of pan (betel leaves) with tobacco chewing were also seen to be at a significantly higher risk for developing periodontal diseases [40].

Tobacco smoke exposure increases susceptibility to respiratory tract infections, sexually transmitted diseases, periodontitis, *H. pylori* infection, meningitis, otitis media and post

surgical and nosocomial infections [41]. Tobacco smoking is an important risk factor associated with chronic destructive periodontal disease which results in bone loss, pocket formation and premature tooth loss. There is an emerging evidence to suggest that subgingival calculus formation is more common and severe in smokers compared to non-smokers [42]. Various factors contribute to the deleterious periodontal effects of smoking, including alterations in both microbial and host response factors. The proposed mechanisms for the negative effects of smoking on periodontium may include vascular changes, altered neutrophill functions (chemotaxis, phagocytosis and oxidative burst), increased occurrence of periopathogens and altered fibroblast attachment and functions. It can also increase secretion of TNF , prostaglandin E2, neutrophil elastase and collagenase in gingival crevicular fluid [40,43].

Table 2. Mean values for the PD in *H. pylori* positive and *H. pylori* negative subjects in Group I

Clinical parameter	Group I (n= 30)		P value[§]
	H. pylori positive (n = 4)	H. pylori negative (n = 24)	
PD (mm)	6.3 ± 0.7	5.5 ± 0.5	.098

§ P values for mean ± SD was obtained using Mann-Whitney U test.
P = .05 is considered to be statistically significant.

Among the periodontal clinical parameters, the PD measurements give a good approximation of the principal habitat of the periodontal pathogens (i.e. periodontal pockets) [17] and the smokers show higher PD values than nonsmokers [25]. In the present study a statistically significant difference was not found in the percent presence of *H. pylori* between current smokers with chronic periodontitis and never smokers with chronic periodontitis. This may be correlated to the PD values observed in these groups (Table 1). The current smokers showed relatively higher periodontal pockets than never smokers, however the average PD values did not reach statistical significance (*P*= .141). When PD values of *H. pylori* positive subjects from Group I was compared to *H. pylori* negative subjects, an insignificant difference was observed (*P*= .098), however, the average PD value was relatively higher in *H. pylori* positive subjects (Table 2).It may be possible that subjects with deep periodontal pockets may provide a suitable intraoral habitat for *H. pylori*. Individuals with smoking habits are at a higher risk of harbouring *H. pylori* and this increased risk may be due to the adverse effects of smoking on antioxidants or the immune system, besides they have elevated levels of circulating inflammatory mediators, which may exacerbate the detrimental effects of *H. pylori* associated gastric inflammation [44, 45]. The tobacco availability was positively associated with the prevalence of intestinal metaplasia among *H. pylori* –infected subjects at the area level. In India prevalence of intestinal metaplasia in *H. pylori* –infected subjects is 8.2% and tobacco availability is 3.75 (cigarettes/day/adult) [46]. The combination of cigarette smoking and *H. pylori* infection increases the risk of gastric cancer more than only smoking or only *H. pylori* infection. It has been observed that, when their independent and joint influences on gastric cancer are taken into account, the impact is large, and a large part is due to their co-occurrence [47]. It was also seen that the risk of intestinal mataplasia was higher in subjects infected with high virulence *H. pylori* strains and among smokers, and was further augmented when both factors were simultaneously present [48]. Similar to the above studies it may be possible that co- occurrence of periodontitis and smoking habit may favor *H. pylori* colonization in oral cavity. The significantly increased plaque formation, clinical attachment loss and relatively higher periodontal pockets among

smokers may become a favorable niche for colonization of periodontopathogens and may help co-aggregation with *H. pylori*, as observed in our study.

Many PCR methods have been developed to detect the *H.pylori* in clinical specimens. The 16S rRNA gene PCR has been the most widely used method for the detection of *H.pylori* in clinical specimens due to its high sensitivity; however it has poor specificity and nonspecifically amplifies human DNA in test samples. The 26-kDa SSA gene PCR is less sensitive than 16S rRNA gene PCR, but this PCR does not amplify any other bacterial DNA. In the comparative study by Lu JJ et al [20], the culture positive specimens (n=24) were positive by both 16S rRNA and 26-kDa SSA gene PCR, however 50% and 42.3% of culture negative specimens (n=26) were positive by 16SrRNA gene PCR and 26-kDa SSA gene PCR respectively. Thus 16S rRNA gene PCR showed relatively more false positive results than 26-kDa SSA gene PCR [20]. The high specificity and availability of 26-kDa SSA gene PCR primer at our research place were the reasons for employing 26-kDa SSA gene PCR method for the current study.

The present study has enrolled only male subjects, considering urban Indian social customs with respect to smoking habits. Besides, their smoking status was based on the verbal information given by the subjects. The non-recruitment of healthy smokers (smokers without periodontitis) in the study puts a limitation for better inter and intra group comparison. It should be pointed out that detection by PCR does not implicate cell viability or pathogenicity [49], still detection of *H. pylori* DNA in salivary samples confirms its presence in the oral cavity of periodontitis cases and the obtained result demonstrates that smoking may contribute to the relatively higher presence of *H. pylori* in the oral cavity of smokers, though the association did not reach statistical significance. Furthermore investigations based on larger sample size inclusive of individuals of different smoking status and different smoking habits are recommended for validation of the present observations.

4. CONCLUSION

The detection of salivary *H. pylori* DNA in chronic periodontitis and further relative increase in its presence in smokers with chronic periodontitis indicates that smoking may provide suitable environment for colonization of *H. pylori* in the oral cavity. Smokers with chronic periodontitis may be at a relatively higher risk of colonization by *H. pylori*. This information may help in understanding the deleterious effects of smoking with respect to oral health and may contribute in designing and implementing preventive programs for smokers.

CONSENT

All authors declare that written informed consent was obtained from the patient for publication of this study.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

ACKNOWLEDGEMENTS

COMPETING INTERESTS

Authors have declared that no competing interest exists.

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