Prevalence of *Helicobacter pylori* detected by PCR in saliva of male smokers and non-smokers with chronic periodontitis

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ABSTRACT

Aims: To assess the comparative prevalence of *Helicobacter pylori* (*H pylori*) in saliva of smokers and non smokers with chronic periodontitis.

Study design: Male individuals diagnosed with chronic periodontitis with and without smoking habits were enrolled in the study. The un-stimulated whole saliva was subjected to *H pylori* DNA detection using real time PCR. The percent prevalence 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 Method: A total of 48 males with chronic periodontitis 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 smoking habit. Healthy volunteers 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), and clinical attachment loss (CAL). Salivary samples were subjected to real time PCR for detection of *H pylori* DNA.

Result: Periodontal parameters were significantly changed 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 have shown presence of *H pylori*. Chi-square test have shown a significant change (P=.003) between Group I and Group III however there is a non significant change 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 relative higher risk of *H pylori* infection in oral cavity, than non smokers. The study needs validation on a larger sample size.

Keywords: *H pylori*, chronic periodontitis, smokers, PCR, saliva

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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 to its survival in the stomach (Skirrow, M. B. 2002). The association of *H pylori* with chronic type B gastritis and peptic ulcer disease has been demonstrated (Fergusson, Jr. *et al* 1993). In the western world, average *H pylori* infection rates in healthy adults are estimated to be between 10–40%, whereas patients with gastritis and duodenal ulceration have infection rates of 80–100%. Evidence exists for possible oral transmission from person to person and fecal-oral transmission is thought to be particularly prevalent in developing countries (Riggo, Lennon 1999). 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 (Eskandari, A. *et al* 2010). Various studies (Umeda, M. *et al* 2003, Gebara, E. C. *et al* 2004, Zarić, S. *et al* 2009) 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 species. Tobacco smoking has been regarded as a true risk factor for periodontitis. Smokers have both increased prevalence and more severe extent of periodontal disease, as well as higher prevalence of tooth loss and endentulism compared to non-smokers (Tonetti, MS. 1998). A reservoir of dental plaque exists in periodontal pockets in smokers with periodontitis and the potential for re-infection of the stomach by *H pylori* is obvious (Watts, TLP. 2006). Further (Suzuki T *et al* 2006) have demonstrated that smoking increases the treatment failure rate for *H pylori* eradication. Saliva contains an abundance of biomolecules that reflects physiological status. Salivary diagnostics offer an easy, inexpensive, safe and cost effective approach for disease detection (Patil, Patil, 2011). Saliva based diagnosis has been reported in periodontitis (Kaufman, Lamster 2000) and also in studies representing prevalence of *H pylori* in periodontitis (Gebara, EC. *et al* 2004, Souto, Columbo 2008). Hence the present
study was undertaken to comparatively evaluate the prevalence of \textit{H pylori} in saliva of chronic periodontitis patients with and without smoking habits.

2. MATERIAL 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 55\textsuperscript{th} 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, were constituted the study population and were divided into the following groups:

Group I: Smokers with chronic periodontitis; n=30 (mean age 44.2 ± 5.88 yrs)

Group II: Non-smoker with chronic periodontitis; n=18 (mean age 41.72 ± 4.36 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 (Armitage, G C 2000). 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. Subjects having past gastric illness six months prior to the study and undergoing any treatment, diabetics and alcoholics were excluded. The subjects in the smoker group were current smokers (predominantly cigarette smokers) with smoking habit of \( \geq 3 \) years and frequency of smoking \( \geq 5 \) cigarette / day. Volunteers from the same geographical region as those of the patients with apparently good oral and systemic health and without smoking habits were enrolled as controls. Group III: Non-smoker healthy controls; n=16 (mean age 39.64 ± 5.04 yrs)
2.2 CLINICAL MEASUREMENTS

The periodontal status of all individuals was evaluated by measurement of gingival index (GI) as developed by Loe H and Silness J (1963), plaque index (PI) as described by Silness P and Loe H (1964), (Soben, P. 2003) and clinical attachment loss (CAL). CAL is measured on six sites of each tooth (mesial, median and distal points at buccal and palatal aspects) and the individual scores were compared on a scale for characterization of periodontitis as slight, moderate or severe (John, MN. 2006). 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. 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, during which individuals were requested not to drink any beverages except water. They were given drinking water (bottled) and asked to rinse their mouth out well (without drinking the water). 5 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 and drop down the head and 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 -4°C 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 manufacturer’s instructions. Briefly, 200 µL saliva, 20 µL QIAGEN Protease and 200 µL buffer AL (lysis buffer), were added, mixed and incubated at 56°C for

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10 minutes. After brief centrifugation, 200 µL 96% ethanol was added and mixed. The obtained mixture is 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 -20°C till further analysis. The DNA samples obtained were subjected to real –time PCR, performed 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 as (forward: 5’-TGGCGTGTCTATTGACAGCGAGC-3’, reverse: 5’-CCTGCTG GCATACTTCACCATG-3’) (Lu, J.J. 1999, Ribeiro, M. 2007). The reaction mixture was composed of follows: 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 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 60 s and primer extension at 60°C 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 (SPSS 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. \( P \) – value <0.05 is considered to be statistically significant. The values on \( H \) pylori are n (%) and the \( P \) – value on \( H \) pylori is obtained using Chi – square test if cell frequencies were larger than 5, else Fisher’s exact probability test were used. \( P \) – value <0.05 is considered to be statistically significant.
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 (Akalin, F.A. 2005, Akalin, F.A. 2007, Wei, D. 2010.) Further smokers with chronic periodontitis have shown altered clinical parameters than non smokers with chronic periodontitis. (Erdemir, EO. et al 2004, Buduneli, N. 2006, Bulunet, K. et al 2007.). The present study has observed significantly higher PI \( (3.0 \pm 0.5 \text{ v/s } 2.3 \pm 0.6, P=0.001) \) and CAL \( (8.5 \pm 1.0 \text{ v/s } 7.7 \pm 0.9, P=0.021) \) in smokers with chronic periodontitis compared to non smokers. However, smokers with chronic periodontitis showed significantly lower GI \( (1.9 \pm 0.5 \text{ v/s } 2.4 \pm 0.5, P=0.001) \) compared to non smokers. (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. (Heasman, L. 2006).

Table 1: Mean values for the clinical parameters and the percent prevalence for \( H \) pylori in various study groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (n=30)</th>
<th>Group II (n=18)</th>
<th>Group III (n=16)</th>
<th>( P ) value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical GI</td>
<td>1.9 ± 0.5</td>
<td>2.4 ± 0.5</td>
<td>0.7± 0.08</td>
<td>.001</td>
</tr>
<tr>
<td>PI</td>
<td>3.0 ± 0.5</td>
<td>2.3 ± 0.6</td>
<td>0.5 ± 0.2</td>
<td>.001</td>
</tr>
<tr>
<td>CAL (mm)</td>
<td>8.5 ± 1.0</td>
<td>7.7 ± 0.9</td>
<td>1.9 ± 0.3</td>
<td>.021</td>
</tr>
</tbody>
</table>

\( H \) pylori (% prevalence) +ve 13.3% 5.5% 0.0% .186‡ .003‡ .132‡

† \( P \) values for mean ± SD are obtained using one way analysis of variance (ANOVA) with Tukey’s correction for multiple group comparisons. \( P \) value < .05 is considered to be statistically significant.

‡ \( P \) value for \( H \) pylori is obtained using Chi – square test if cell frequencies are larger than 5, else Fisher’s exact probability test was used. \( P \) value < .05 is considered to be statistically significant.

The infection by \( H \) pylori is widely accepted as an important cause of gastritis and is strongly associated with peptic ulcer disease and gastric cancer. 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. (Hu, W. *et al* 2002, Eskandari, A. *et al* 2010, Gao, J. *et al* 2011). Various studies (Dye, BA. *et al* 2002, Souto, Columbo 2008, Rajendran, R. *et al* 2009, Fernando, N. *et al* 2009) have observed the possible role of oral cavity as a reservoir for *H pylori* and have correlated *H pylori* colonization and periodontal disease. Asqah, M. Al, *et al* (2009) have suggested that patients with poor oral hygiene have a higher prevalence of *H pylori* in dental plaque and the oral cavity may be a reservoir for *H pylori*. Dionf *et al* (2011) have stated that periodontal-pathogens like *F nucleatum* and *Eikenella corrodens* could co aggregate with *H pylori* in the subgingival dental plaque. Saliva plays a significant role in maintaining a healthy oral environment (Javed, F. *et al* 2009). It has been suggested that microorganism in dental plaque can survive in saliva (Patil, Patil 2011). Studies (Gebara, EC. *et al* 2004, Souto, Columbo 2008,) have detected *H pylori* in saliva and gingival plaques of periodontitis patients. According to Tiwari SK *et al* (2005) PCR amplification of the DNA of the ulcer causing bacterium *H pylori* in saliva samples has been shown to have comparable sensitivity to DNA tests using gastric biopsy samples or histopathological analysis of gastric tissue. Thus detection of *H pylori* in saliva using sensitive methods like PCR may reveal the presence of pathogen in the oral cavity. The present study has not detected *H pylori* DNA in healthy controls (100% negative), whereas 5.5% of non smokers and 13.3% smokers with chronic periodontitis have shown presence of *H pylori* DNA. Smokers with chronic periodontitis showed significant percent prevalence (*P* = .003) than healthy controls, however the percent prevalence has not reached significance (*P* = .186) compared to non smokers with chronic periodontitis, though the percent prevalence values are relatively higher. The non significance 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 pro inflammatory cytokines, such as TNF α, IL1 and IL8.

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H pylori directly interact with host cells, induce pro inflammatory cytokines and stimulates production of free radicals leading to inflammatory alterations in GIT (Tsuji, T. et al 2003). On a similar paradigm, these inflammatory markers, which may be produced by H pylori in oral cavity, may add an additional burden on the pre existing inflammation due to chronic periodontitis in oral cavity. Thus H pylori in oral cavity may contribute to the inflammatory destruction of peridontium in chronic periodontitis.

The overall prevalence of periodontal disease is high in India because of several factors like huge population (app 1000 million), of which 72% reside in the rural. Poor oral health care system and para dental infrastructure, with the dentist: population ration of 1:2 lac are important factors leading to periodontitis. Also habits like smoking, pan (betel leaves) with tobacco chewing was shown to be significant risk factor for development of periodontal disease (Agarwal, V. et al 2010).

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 (Bagaitkar, J. et al 2008). Tobacco smoking is the main 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 sub-gingival calculus formation is more prevalent and severe in smokers compared to non-smokers (Bergstrom, J. 2004). 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 alterations, altered neutrophill functions (chemotaxis, phagocytosis and oxidative burst), increased prevalence of periopathogens and altered fibroblast attachment and functions. It can also increase secretion of TNF α, prostaglandin E2, neutrophil elastase and collaginase in gingival cervical fluid (Georgia, Margaret 2004, Agarwal, V. et al 2010,).

The present study has observed a relative increase in the percent prevalence of H pylori in smokers than non smokers with chronic periodontitis. Smokers appear to be at higher risk of...
becoming infected with *H. pylori* and this increased risk may be due to the adverse effects of smoking on antioxidants or the immune system that may interfere with the normal protection against *H. pylori*, also they have elevated levels of circulating inflammatory mediators, which may exacerbate the detrimental effects of *H. pylori* associated gastric inflammation (Parasher, Eastwood 2000, Kim, J. *et al* 2012). Peleterio B *et al* (2008) in the review article have showed that tobacco availability was positively associated with the prevalence of intestinal mataplasia among *H. pylori*–infected subject at the area level. In India prevalence of intestinal mataplasia in *H. pylori*–infected subjects is 8.2% and tobacco availability (cigarettes/day/adult) is 3.75. Shikata K *et al* (2008) have observed that the combination of cigarette smoking and *H. pylori* infection increased the risk of gastric cancer more than smoking alone or *H. pylori* infection alone. And 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. Also, the risk of intestinal mataplasia was higher in subjects infected with high virulence *H. pylori* strains and among smokers, and was further increased when both factors were simultaneously present (Peleterio B *et al* 2007). Similar to the above studies it may be possible that co occurrence of periodontitis and smoking habit may favor *H. pylori* infection in oral cavity. The significantly increased (*P* = .001) plaque formation among smokers may become a favorable niche for colonization of perio-pathogens and may help coaggregation of *H. pylori*, as observed in our study. The present study has enrolled only male subjects, considering urban Indian social customs with respect to smoking habits. Also their smoking status was based on the verbal autopsy. 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 (Gebara, E.C.E. *et al* 2006), still detection of *H. pylori* DNA in salivary samples confirms its presence in the oral cavity of periodontitis cases and smoking may contribute to the higher prevalence of *H. pylori* in smokers. Further investigations based on larger sample size are recommended for validation.

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of the present observations and also to elucidate if basic periodontal therapies would be employed to eradicate this pathogen from the oral cavity and prevent its reinfection in higher risk groups like smokers.

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 are at a higher risk of infection 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 implementation of preventive programs for smokers.

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COMPETING INTERESTS

Authors have declared that no competing interest exists.

AUTHORS' CONTRIBUTIONS

Dr. Rajiv Kishor Saxena: Performed PCR analysis and helped to wrote the first draft of manuscript

Mr. Abdul Samad Aziz: Managed literature searches, wrote protocol, data acquisition and manuscript preparation.

Dr. Madhav Govind Kalekar: Designed, guided and supervised the study

Ms. Milsee J. P.: Helped in PCR analysis, manuscript editing and manuscript review

Dr. Adinath Narayan Suryakar: Designed and guided the study

Dr. Tabitha Benjamin: Managed the evaluation of the clinical parameters

Dr. Ravi Vasudev Shirahatti: Helped in manuscript editing and manuscript review

Dr. Raghuvendra Shrishail Medikeri: Helped in manuscript editing and manuscript review

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All authors read and approved the final manuscript

CONSENT

All authors declare that written informed consent was obtained from the patient for publication of this study. A copy of the written consent may be made available for review by the editorial office/chief editor/editorial board members of this journal

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.

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Periodontal conditions, oral Candida albicans and salivary proteins in type 2 diabetes

Newman, M. G., Takei, H. H., Klokkevold, P. R., Carranza, F. A. (Eds.), Carranza’s Clinical


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**SDI Patient Consent Form 1.0**

Date: 29/09/2012  
Place: Mumbai

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<td>Prevalence of <em>Helicobacter pylori</em> detected by PCR in saliva of male smokers and non-smokers with chronic periodontitis</td>
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<td>Name of the Authors</td>
<td>Dr. Rajiv Kishor Saxena, Mr. Abdul Samad Aziz, Dr. Madhav Govind Kalekar, Ms. Milsee Mol J.P., Dr. Adinath Narayan Suryakar, Dr. Benjamin Tabita, Dr. Ravi Vasudev Shirahatti, Dr. Raghavendra Shrishail Medikeri</td>
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<td>Patient's name:</td>
<td>Javed Shaikh</td>
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I give my consent for this material to be published in the above mentioned journal and associated publications without limit on the duration of publication.

I understand that the material will be published in the above mentioned journal will be included in any reprints of the published article. I understand that my name will not be included in the published article, and that every effort will be made to keep my identity anonymous in the text and in any images. However, I understand that complete anonymity and control of all uses cannot be guaranteed.

I have been offered the opportunity to preview the material in the format it will eventually appear and I am satisfied with it.

Signed: [Signature]

Full Name of the Patient: Javed Shaikh

**Alternative:**

Signed: [Signature]

Full name of the relative: [Name]
Relation: [Relation]

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<th>Patient's Name and contact address</th>
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</table>

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<td>College Name and Address</td>
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To,
Mr. Abdul Samad Aziz,
Ph.D. Student, Dept. of Biochemistry,
Through HOD,
Grant Medical College and
Sir J.J. Gr. of Hospitals, Mumbai

Sub: Regarding your proposal of Study Topic 'Study of oxidative stress makers in Periodonitis'

Ref: Your letter dt. 24/7/2008

Dear Dr.

The above mentioned research proposal of Study topic was discussed in the Institutional Ethics Committee Meeting held on 29.8.2008 at Department of Pharmacology, Grant Medical College, Mumbai.

Institutional Ethics Committee has unanimously approved your Ph.D. Study topic. This work will be done under the guidance and supervision of your guide Dr. M.G. Kalekar.

[Signature]
(Dr. S.B. Patel),
Member Secretary, Institutional Ethics Committee, and Prof. and Head,
Department of Pharmacology,
Grant Medical College, Mumbai.

Dr. S. B. Patel
Member Secretary
Institutional Ethics Committee,
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