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# Immunohistochemical Detection of *Helicobacter pylori* in Oral Squamous Cell Carcinoma

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**Original Research Article**

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**Abstract:** Head and neck carcinomas are biological heterogeneous group of cancers, of which oral cancer is the most common. Ninety percent of oral cancers are squamous cell carcinomas originating from the mucosal epithelium. Oral carcinogenesis is a multifactorial process where numerous risk factors are involved. A connection between bacterial infection and carcinogenesis is convincing and is of increasing interest. Helicobacter pylori (H.pylori) are flagellated, gram negative, spiral, microaerophilic bacteria and deemed by the World Health Organization and the International Agency for Research on Cancer as a class I human carcinogen. In addition to its role in causing gastritis, gastric ulcer, duodenal ulcer and adenocarcinoma of stomach, H.pylori has been found in the oral cavity in patients with halitosis, apthous stomatitis and periodontal diseases. However, there are limited studies available in literature of H.pylori in oral squamous cell carcinoma (OSCC) and its possible role in oral carcinogenesis. Therefore the aim of this study is to detect and establish a relationship between H.pylori and OSCC. Thirty paraffin embedded tissue blocks of clinically diagnosed and histopathologically confirmed cases of OSCC and ten of normal buccal mucosa were sectioned and stained with Hematoxylin and Eosin. The one subsequent serial section is immunohistochemically stained with anti human helicobacter pylori antibody. The presence of H.pylori is assessed and any association between presence of H.pylori and OSCC was analyzed. The study data was analysed using SPSS (Statistical Package for Social Sciences) software V.22, IBM, Corp. Statistical Analysis was done using Chi Square test and Mann Whitney U test. The level of significance [P-Value] was set at $P<0.01$. Chi Square test was used to find the association between the prevalence of H.Pylori between OSCC & Control group and it was found to be stastically significant (at $P<0.001$). Chi Square test showed significant differences between *H. pylori* positivity and different tissue types i.e in epithelium, lamina propria, inside the blood vessels and salivary gland duct and in the muscle layer of OSCC. There is a positive relation between the presence of *H. pylori* and OSCC. Therefore, early detection and eradication of *H. pylori* in high-risk patients are suggested.
**Keywords**: Oral squamous cell carcinoma, Helicobacter pylori, Immunohistochemistry.

## INTRODUCTION

H.pylori is a gram negative, motile, microaerophillic, spiral shaped bacteria colonizing the human gastric mucosa, it affects almost half of world's population. It is designated as a type I carcinogen by World Health Organization with approximately affecting 80% of Indian adults [1]. H.pylori is found to be associated with the development of gastric adenocarcinoma ,gastritis, gastric ulcer, duodenal ulcer but its presence is also detected in patients with halitosis, apthous stomatitis and periodontal diseases [2]. Oral squamous cell carcinoma is the most common malignancy of the oral cavity and is particularly common in developing world like India. The etiology of OSCC is found to be multifactorial with various factors contributing to its pathogenesis. Various studies have revealed an emerging role of bacteria in development of OSCC however they generally failed to conclusively establish any association. In this study we tried to find out the presence of H.pylori in OSCC and to establish if any association exist between them immunohistochemically.

## MATERIALS AND METHODS

Thirty formalin fixed, paraffin embedded biopsy specimens with clinically and pathologically confirmed OSCC cases and 10 cases of normal oral mucosa (NOM) samples were obtained from the Department of Oral and maxillofacial Pathology. One endoscopic biopsie of patient positive for H.pylori is taken as positive control. The study has been approved by our institutional ethical committee review board (Ref No.218 /2014-2015).

**Hematoxylin and eosin staining**

Formalin fixed, paraffin embedded specimens were cut into 4 micron meter sections and were stained with hematoxylin and eosin for histologic confirmation of clinical diagnosis and to detect H.pylori presence in OSCC. Additional sequential sections were prepared for immunohistochemical studies.

**Immunohistochemistry protocol**

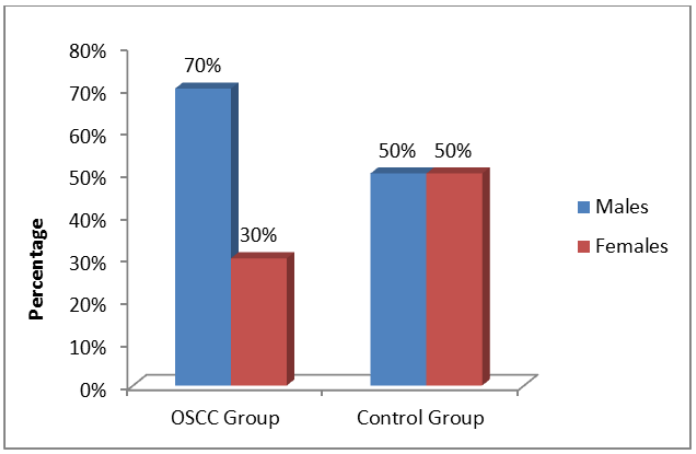
All the 30 cases of OSCC and 10 cases of normal buccal mucosa were available for high quality immunohistochemical staining. Immunohistochemical staining was performed on 4µm thick sections. All the procedures were performed at room temperature. The sections were deparaffinised through a series of xylene baths and rehydrated in graded concentrations of alcohol. Tissue sections were treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Antigen retrieval was carried out by microwave with 0.01 M sodium citrate buffer solution for tree cycles of 800 W for 5 min twice and 200W for 14 min and later was subjected to two washes of tris buffer solution for 5 min each. Sections were then incubated with ready to use primary antibody (rabbit polyclonal to H.pylori, DAKO, Bengaluru, India). After washing with tris buffer solution, the sections were then incubated for 30 min with anti mouse secondary antibody and visualized using 3, 3'-diaminobenzidine (DAB) chromogen. Section then visualized under microscope (4X, 10X and 40X) magnification and were assessed for the cytotoxic associated gene expression on the outer membrane of H.pylori.

**Statistical analysis**

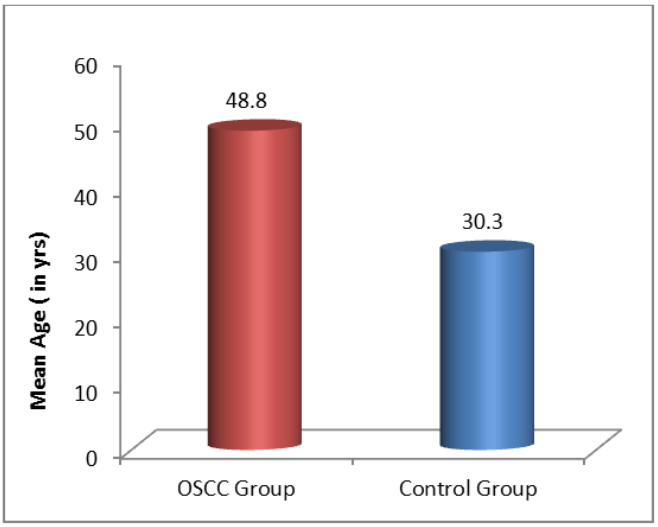
Helicobacter pylori presence was looked both in the epithelium as well as the connective tissue and any correlation was assessed and they were analysed as present or absent. The data obtained were tabulated. Statistical values were analyzed using Chi-square test and Mann Whitney U test. A p-value of 0.05 or less was considered statistically significant.

**RESULTS**

In our study, the age of the OSCC patients ranged from 35 to 68 years and patients of normal buccal mucosa ranged from 23 to 42 years. The mean age of the OSCC patients was 48.8 years and of normal buccal mucosa patients 30.3 years. In the gender distribution 70% were males and 30% were females in OSCC group and 50 % male and 50% female in the normal buccal mucosa patients (Graph-1). The presence or absence of H.pylori was assessed. . In 22 (73.3%) cases of OSCC H.pylori has been detected while complete absence (0%) is seen in cases of normal buccal mucosa. H.pylori was detected in the epithelial layer (23.3%), lamina propria (46.7%), blood vessel, salivary gland (26.7%), muscular component (10%) of OSCC tissue. Here, the p value found to be stastically significant between the OSCC and the control group (p<00.1).



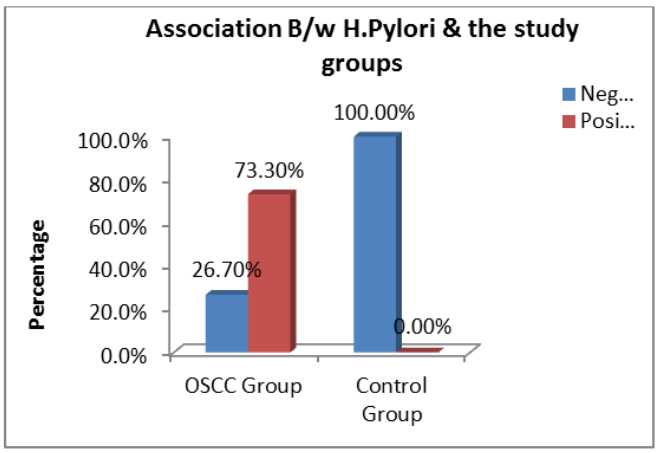

**Graph-1: Shows genderwise Distribution of Study Participants among 02 groups**



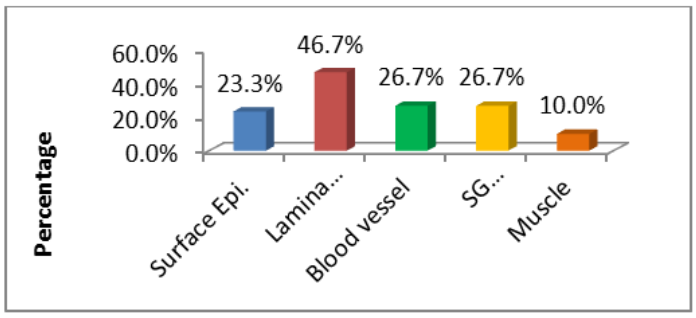

**Graph-2: Agewise distribution of study Participants among 02 groups**

**Table-1: Distribution of Lesion & Habit Chacrteristics with *H. pylori* Diagnosis in OSCC group**

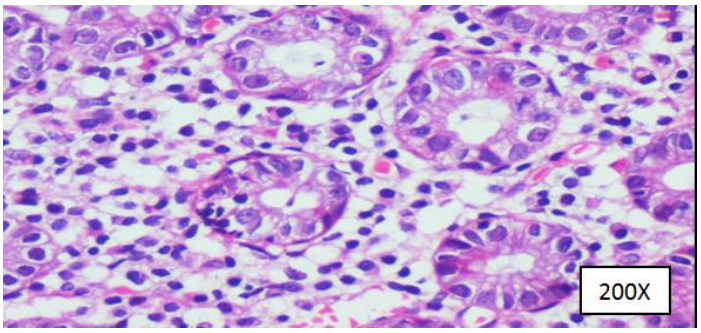
| Variables | Categories | n | % |
|---|---|---|---|
| Site of Lesion | Buccal Mucosa | 30 | 75.0% |
| | Gingiva | 4 | 10.0% |
| | Palate | 1 | 2.5% |
| | Retro Molar Area | 2 | 5.0% |
| | Tongue | 3 | 7.5% |
| Habit History | Betel Quid | 3 | 10.0% |
| | Tob. Chewing | 13 | 43.3% |
| | Tob. Smoking | 3 | 10.0% |
| | Tob. Chewing + Smoking | 1 | 3.3% |
| | Info NA | 10 | 33.3% |
| HP Diagnosis | Well Diff. OSCC | 16 | 53.3% |
| | Mod. Diff. OSCC | 11 | 36.7% |
| | Poorly. Diff. OSCC | 3 | 10.0% |



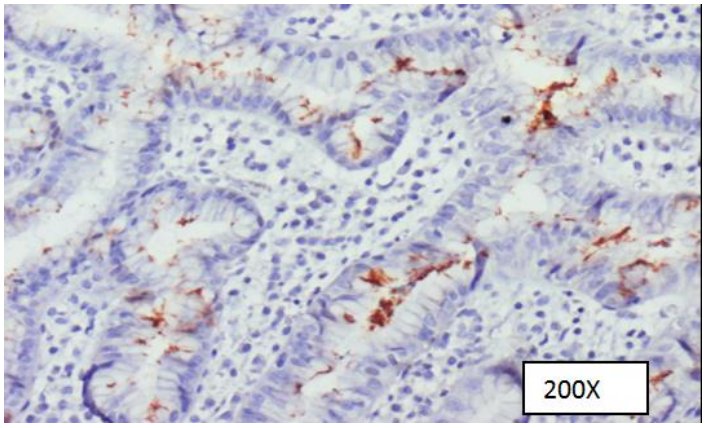

**Graph-3: Showing association B/w H.Pylori & the study groups**



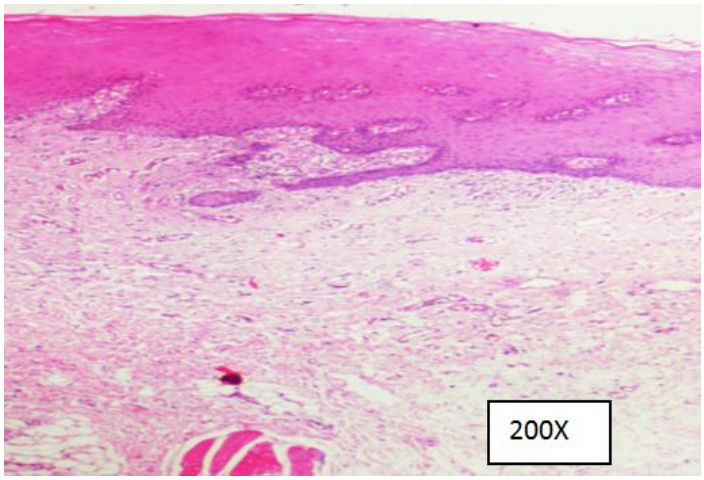

**Graph-4: Showing the presence of H.Pylori bacteria in different areas of OSCC Tumor Region**



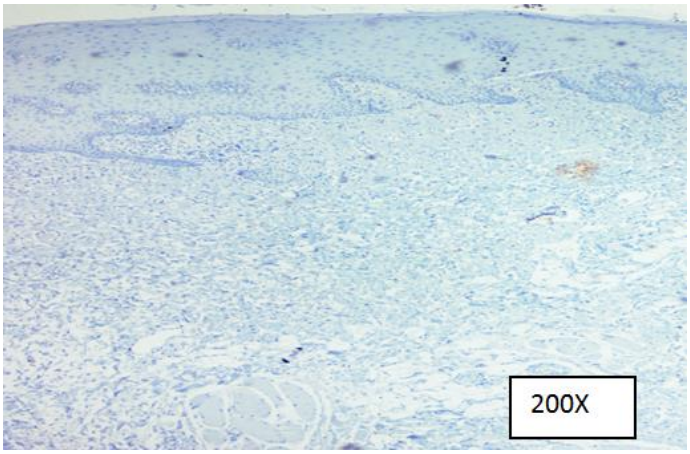

**Photograph-1: Photomicrograph showing spiral shaped H.pylori seen in the lumen of gastric mucosa (H&E)**



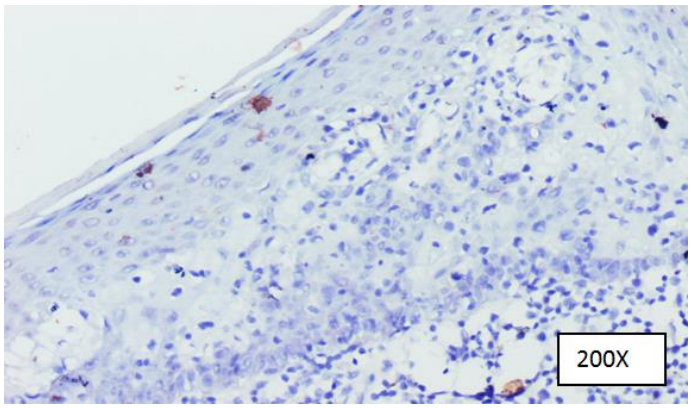

**Photograph-2: Photomicrograph showing H.pylori in both spiral and coccoid form in the lumen of Gastric mucosa (IHC stain)**



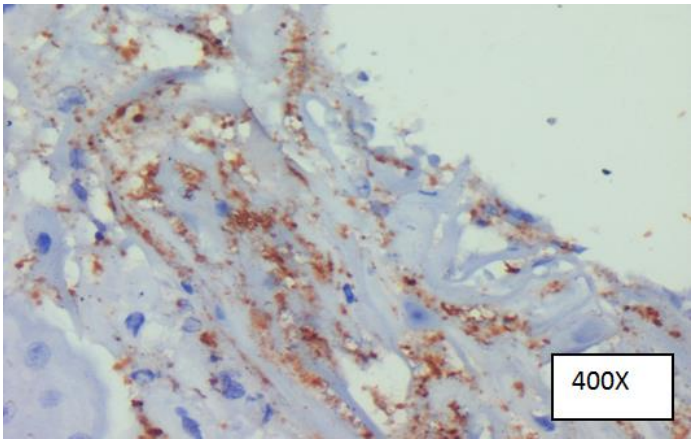

**Photograph-3: Photomicrograph of normal buccal mucosa (H&E)**



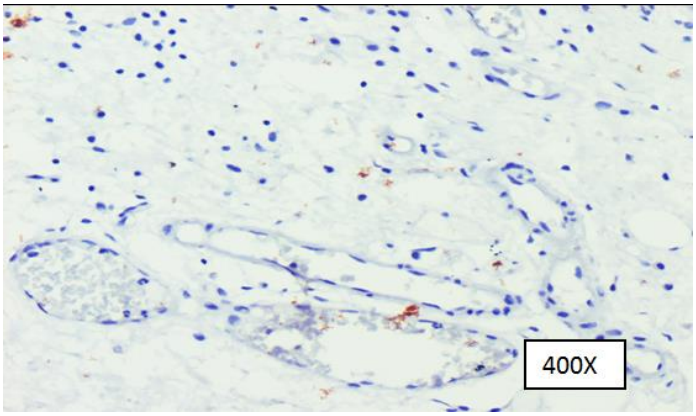

**Photograph-4: Absence of H.pylori in Normal buccal mucosa (IHC stain)**



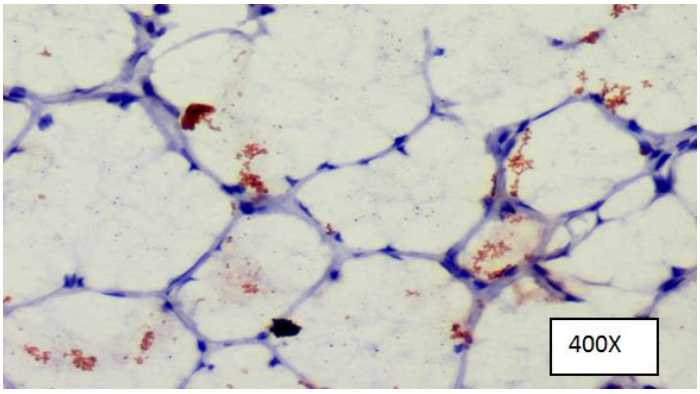

**Photograph-5: Presence of H.pylori in epithelium of OSCC (IHC stain)**



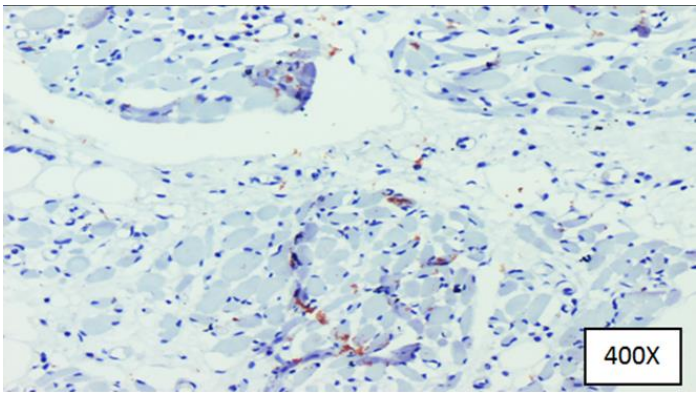

**Photograph-6: Presence of H.pylori in Lamina propria of OSCC (IHC stain)**




**Photograph-7: Photomicrograph showing presence of H.pylori in Blood vessels of OSCC (IHC stain)**




**Photograph-8: Photomicrograph showing presence of H.pylori in Salivary component of OSCC (IHC stain)**




**Photograph-9: Photomicrograph showing presence of H.pylori in Muscular component of OSCC (IHC stain).**

## DISCUSSION

Helicobacter pylori was first discovered in the stomach of patients with gastritis and stomach ulcers in 1984 by Dr. Barry Marshall and Dr. Robin Warren and is considered to be the most common chronic bacterial infection in humans [2]. It is thought to promote tumour growth through inflammation dependent mechanism in epithelial cells leading to mucosal atrophy, inflammation and gastric malignancies [3]. In this H.pylori has gained more attention as soon as it was declared as class I carcinogen by WHO and a definitive causative agent for gastric carcinomas [2]. Since an initial portion or the gate of the gastro-intestinal tract (GIT) is oral cavity; many diseases of oral cavity may affect the integrity of mucous membrane of oral cavity and the remaining portions of the GIT. It is also associated with the changes in oral enviroment, including its microbial colonization and then infection in the oral cavity. Thus oral cavity can serve as a reservoir of micro-organisms and source for the infection of the stomach and gut or alternatively. It may serve as the transmission gate of external germs for further colonization of GIT [4]. As we know OSCC is the most common malignancy and accounts for almost more than 90% of all the oral malignancies and is a multifactorial disease with various etiological factors playing role in its pathogenesis. So we tried to correlate if any kind of association can be seen with OSCC and presence of H.pylori in the oral cavity since

only few studies are mentioned in the literature. But there are different opinions concerning the presence of Helicobacter pylori in the oral cavity. Song Q have suggested that Helicobacter pylori may belong to the normal oral flora of the human oral cavity, maintaining a commensal relation with the host, but present in very low numbers such that the identification is very difficult [5]. There are several methods to detect H.pylori in the oral cavity like urea breadth test, culture and special stains like giemsa but in our study Immunohistochemistry (IHC) was preffered due to to its high specifity and sensitivity (>90%), to show the location of H. pylori inside the tissue (lamina propria) if present and also since it can detect coccoid /post treatment forms of H.pylori. In the present study, 30 cases of OSCC and 10 cases of normal mucosal biopsies were evaluated for H.pylori immunohistochemically. Twenty two number of cases (73.3%) of OSCC were found to be positive for H.pylori while eight cases of OSCC and all the 10 cases of normal buccal mucosa were negative for H.pylori. H. pylori has been detected in various regions of the oral cavity and a high variation is seen in its presence in the oral cavity i.e dental plaque (82.3%), gargles (51.1%) and the dorsum surface of tongue (37.5%) [6]. The two possible mechanisms involved in H. pylori pathogenesis are firstly, H. pylori interacts with surface epithelial cells, developing direct cell damage or producing pro-inflammatory mediators [7-9]. Secondly, H. pylori reaches the underlying connective tissue to stimulate an immune response, leading to the release of various cytokines and oxygen radicals that transform the chronic gastritis into gastroduodenal ulcers and gastric carcinoma. Also H. pylori produces some extracellular products that cause local and systemic immune responses, which can result in tissue damage [10-12]. With the advancement of biochemical techniques, new information about the pathogenicity and virulence factors of H.pylori has emerged, indicating that infection by H.pylori requires a complex interaction of both bacterial and host factors. Bacterial proteins like urease are necessary for colonization of gastric mucosa by H.pylori. The urease produced by the bacteria alters the microenvironment of the organism to facilitate colonization. The bacteria adhers to the surface of gastric mucosa due to its mobility by using its flagella and attaches via adhesions to glycolipid receptor on the apical membrane of surface epithelial cells. H.pylori produces cecropins to inhibit the growth of competing organisms. Once attached to gastric mucosa, H. pylori causes tissue injury by a complex cascade of events that depends on both the organism and the host. H. pylori, like all gram negative bacteria have in its cell wall lipopolysaccharide, which acts to disrupt mucosal integrity. Furthermore, H.pylori releases several pathogenic proteins that induce cell injury. Once it gets colonized in the gastric mucosa, the immunogenic properties of H.pylori induce an inflammatory reaction with neutrophilic gastritis that ultimately results in the clinical manifestations of the infection [13]. In present study the mean age of occurrence of OSCC was found to be 48.8 or middle age (Graph-2) same as given in the literature, suggestive of either late development of OSCC or due to late detection of OSCC because many of the times it get unnoticed when it is asymptomatic. Male predominance is seen in our study (Graph -1) with almost 70% being male and 30% female and this can be attributed to the more involvement of male towards using various forms of tobacco and also the highest percentage (43.3%) is found in the patients who use chewable form of tobacco as compared to other forms of tobacco. This also suggest that why in our study buccal mucosa (70%) was the most common site involved due to tobacco chewing habit of the individuals in Indian subcontinent . All the three demographic data i.e age of occurrence ,sex and site involved are in accordance with earlier studies [14-16]. Most of the cases reported are well differentiated ,followed by moderately and poorly differentiated OSCC (Table-1). In our present study, 22 (73.3 %) cases of OSCCs showed H. pylori positivity (Graph-3). Fernando *et al*., done a study to show the presence of H.pylori in oral cancer patients who are betel and non-betel chewers and found that as compared to non betel chewers the H.pylori presence was statistically significant in betel chewers [17]. Rubin *et al*., working on 61 samples from head and neck malignant and premalignant conditions, detected H. pylori positivity in 16.3% of oral cavity samples [18]. Grimm *et al*., in 2014 analyzed the prevelance and influence of H.pylori in OSCC immunohistochemically and found that H.pylori prevelance was found to be 21.5% and was associated with the disease free survival [2]. Dayamma *et al*., in found positive result in only 3% of cases of OSCC using culture and PCR technique but the male predominance and age involvement coincides with our study.[21]Okuda et al in 2000 done a study using swab samples of the oral mucosa and cancer lesion surfaces, but no positive PCR results were obtained [19]. Chitsazi *et al*., in 2007 reported that 40% of 39 patients had viable H. pylori in their oral cavities despite H. pylori eradication. Also 56% of those without detectable H. pylori in the mouth before treatment had H. pylori in the oral cavity when re-examined after H. pylori eradication [20]. In the present study, H. pylori oral colonization was seen in both the coccoid and the spiral forms. Wang *et al*., suggested that the coccoid form of H. pylori is viable and maintains the integrity of the nucleic acid contents, involved in active protein synthesis and is able to synthesize DNA [21]. The present study detected the coccoid form of H. pylori, which might be a proof for its long-standing presence in the oral cavity and revealing the role of H. pylori in the pathogenesis of OSCC. Several studies support the hypothesis that the oral cavity is a reservoir for re-infection of the stomach [22]. On the other hand, some other investigations have shown that presence of H. pylori in the oral cavity does not relate to gastric

infection and that H. pylori can also be found in the oral cavity without any gastric infection [23-25]. Song *et al*., shown that H. pylori DNA sequences differed between oral samples and gastric samples within the same individual [5]. Previous studies on the gastric mucosa indicated the presence of H. pylori in the lamina propria, the intercellular space as well as in the gastric lumen [24] coinciding with our study where we have seen presence of H.pylori in the epithelium as well as in the lamina propria. The presence of H. pylori in the stromal cell of the lamina propria, far from the epithelial basement membrane, indicates invasion [25]. As seen in our study 14 cases of OSCC (46.7%) were positive for epithelium and also shows their presence inside the lamina propria (Graph no.4). Various studies have shown H. pylori invasion into the lamina propria of gastric mucosa, which can be an important factor in the induction and development of gastric inflammation [26, 27]. In the present study H. pylori was found in the epithelial layer 23.3% and in the lamina propria in 46.7%, blood vessels and salivary gland (26.7%) and muscle (10%) which can be clear evidence for the invasion of the bacteria. Petersen et al found that H. pylori is able to pass through the endothelial layer [28]. In our study 26.7% of cases ,H. Pylori was detected inside the blood vessels, suggestive of H. pylori bacteremia, resulting in a systemic response.In our study the detection of H.pylori is found highest in lamina propria suggestive of the invasive behaviour of the bacteria especially in ulcerated cases where they get more serum factors.Our results are very much similar to the study done by Soussan irani *et al*., where H.pylori positivity is seen in 26.5% cases of OSCC but here H.pylori presence is maximum in the epithelium followed by lamina propria, blood vessel and salivary gland duct, the reason here can be given as we have taken more number of ulcerated cases, which are devoid of epithelium.

## CONCLUSION

Hence, we can suggest that there is a positive relationship between the presence of H.pylori in the oral cavity and OSCC and it can acts as risk factor for the development of OSCC. Since, it causes direct cell damage by producing various proinflammatory mediators and also by stimulating an immune response, can lead to production of different cytokines and oxygen radicals which might help in further progression of OSCC [29, 30].

However we also assessed for the presence of H.pylori in normal mucosal biopsies. None of the buccal mucosal biopsies in our study showed positivity for H.pylori. So this raises the doubt whether oral cavity acts as a real H.pylori reservoir or whether it is transiently stored in mouth as a result of gastroesophageal reflux or when passing to the stomach [31]. Before concluding, several limitations of our current study might be considered as follows. Firstly we failed to obtain clinical history of the patients including gastrointestinal symptoms or other stomach disorders. Secondly it is always advisable to do two or more diagnostic methods to confirm the presence of H.pylori. Thirdly the presence of H.pylori in oral cavity is low in number and may be suppressed by the complex oral microflora [32]. Thus further large scale research with appropriate diagnostic methods in this field could open doors to understand the true nature of H.pylori in OSCC.

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