

ORIGINAL RESEARCH

Prevalence of Candida and Efficacy of Gomori Methenamine Silver and Calcofluor-White Staining In Oral Submucous Fibrosis: A Cyto and Histopathologic Study

Arti Singh Rajput¹, Rajkumar N. Parwani², Sangeeta Panjab Wanjari³, Apurva Pathak⁴, Satpal Yadav⁵, Praveen Singh Rajput⁶, Babita Singh Rajput⁷

ABSTRACT

Background: Various precancerous lesions and cancers have been associated with candida to the extent of it being causative agent. A need for prevalence of candida and efficacy of Gomori methenamine silver (GMS) and Calcofluor-white staining (CFW) in Oral submucous fibrosis (OSMF) in Cyto and Histopathologic sections, this study was conducted.

Materials and Methods: The study group consisted of patients with OSMF (n=30) and control group (n=30). Presence of Candida was confirmed by culture inoculation along with a germ tube and carbohydrate fermentation test. The cytopathological smears were analyzed by CFW, PAS and Gram staining, whereas tissue sections were stained by PAS, GMS and CFW staining.

Results: In 30 cases of study group, in Cytological smears CFW stain showed highest sensitivity (94.7%) than GMS and Gram's stains (89.5% each) whereas Gram's stain showed highest specificity (82.9%) than GMS (75.6%) and CFW (73.2%) stains. In Histopathological sections high sensitivity was observed with CFW stain (50.0%) than PAS and GMS stains (42.9% each) while specificity of CFW stain was lower (56.3%) than PAS and GMS stains (68.8% each).

Conclusion: The ease, versatility and rapidity of CFW stain could be advantages to evaluate Candida in at least suspected OSMF cases. Controversy of betel quid chewing habit to inhibit or promote adherence and invasion of Candida remains wide open.

Keywords: Candida, OSMF, CFW, GMS, PAS

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¹Dental S.R., Baba Saheb Ambedkar hospital, Delhi, ²Professor, ³Professor and HOD, Department of Oral Pathology, ⁴Professor, Department Of Pathology and Microbiology, Modern Dental college and Research centre, Indore, M.P, ⁵Senior Pediatric consultant, Jaipur Golden hospital and Gupta hospital, Delhi, ^{6,7}Medical officer, GRMC, Gwalior, M.P. India.

Corresponding author: Dr. Arti Singh Rajput, Dental S.R., Baba Saheb Ambedkar hospital, Delhi, India.

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INTRODUCTION

Oral submucous fibrosis was first reported in India in 1953.¹ The term is given in 1952 by Schwartz.² The hallmark of the disease is submucosal fibrosis that affects the oral cavity and progressively involves the pharynx and the upper esophagus.³ Worldwide, estimates of OSMF show a confinement to Indians and Southeast Asians, with overall prevalence rate in India to be about 0.2% to 0.5 % and prevalence by gender varying from 0.2-2.3% in males and 1.2-4.57% in females. It has been suggested that ingestion of chillies, genetic susceptibility, nutritional deficiencies, altered salivary constituents, autoimmunity and collagen disorders may be involved in the pathogenesis of this condition.⁴ Mini et al. reported 7.6% of malignant transformation rate of OSMF.⁵

C. albicans is the predominant species isolated in premalignancy and carcinoma. Candidal infection can induce epithelial atypia and lead to malignant

transformation through the release of chemical carcinogens like nitrosamine compounds.⁶ The association of Candida and premalignant states, such as leukoplakia and lichen planus, has been extensively studied. However, literature reveals very few studies linking the association of betel quid chewing habit and related lesions, such as OSMF with Candida.⁷ The present study was aimed to determine Prevalence of Candida and efficacy of Gomori Methenamine silver and Calcofluor-White staining in Oral submucous fibrosis in Cytology & Histopathology.

MATERIALS AND METHODS

The present study was conducted in the Department of Oral and Maxillofacial Pathology of Modern Dental College and Research Centre, Indore, India. Study sample comprised of 30 cases of OSMF clinically graded according to Haider et al.⁶ and age and sex matched equal number (30) of control subjects. The oral rinse technique was used to collect samples. For identification, we used Gram's staining, a germ tube test, carbohydrate fermentation test. Smears were prepared by scrape cytology and prepared three smears. Gram's stain was done with one smear and observed under a light microscope. Remaining two smears were wet-fixed by dipping the slide in 95% ethyl alcohol for one hour and subsequently stained with GMS and CFW staining.

For confirmation of provisional diagnosis of OSMF patients were subjected to punch biopsy or scalpel biopsy. Control group were not subjected to biopsy for ethical concern. Tissue was further processed to obtained the paraffin embedded sections for PAS, GMS and CFW staining.

STATISTICAL ANALYSIS

Group analyses were performed with the chi-square test and the differences between two independent samples were analyzed with the unpaired *t*-test. A *P*-value<0.05 was considered statistically significant.

RESULTS

Study sample comprised of 60 subjects, 30 each of study group and 30 of control group. Out of 30

cases, 16 had habit of quid with tobacco and 14 cases had habit of betel quid without tobacco. Association of smoking habit was seen in 12 cases (40%) and burning sensation was present in 26 (86.7%) of the 30 cases of study group. Subjects were clinically graded into 3 groups of 10 cases each based on Haider et al.⁶ classification. Evaluation of epithelial dysplasia was done in histopathological sections. Moderate dysplasia in 3 cases (10.0%), Mild dysplasia in 6 cases (20.0%), and no dysplasia in 21 cases (70.0%) was observed. Culture was performed in all 60 cases (30 of study group & 30 of control cases), as it is considered as "Gold standard/Reference method" diagnosis of an infection. Out of 60 samples (30 OSMF and 30 control group) which were cultured, 14 samples (46.7%) in study group showed candidal colonies, fulfilling all the diagnostic criterias whereas only 5 samples (16.7%) from control group were positive for candidal growth. With positive culture report of the samples, quantitation of the Candida was done.

The mean colony count in OSMF and control groups was 55.53 ± 205.81 and 10 ± 43.90 CFU/ml respectively. The SD was extremely high owing to the extreme values in the observation.

In study group 14 out of 30 cases, showed candidal colonies, candidal carriage was observed in 6, 5 and 3 samples of grade I, II and III cases respectively. Chi-square test was performed to evaluate the association between different cytopathological & histopathological stains and grading of OSMF. There was no significant association between Gram s stain, CFW s and clinical grading ($p = 0.659$ and 0.109 respectively). However, there was significant association between GMS s and clinical grading ($p = 0.024$). All the individuals with clinical grading I were positive for GMS s stain while only half of them were positive in grade II and III. There was no significant association of CFW p, GMS p PAS p with clinical grading ($p = 0.175$, 0.563 , 0.155 respectively). It is imperial to state that the sample size per grade was small.

Culture isolates were further subjected to mycological tests; Germ tube test, Carbohydrate fermentation test to identify various species of candida. Out of 14 culture positive samples of OSMF, 10 cases showed positive germ tube test

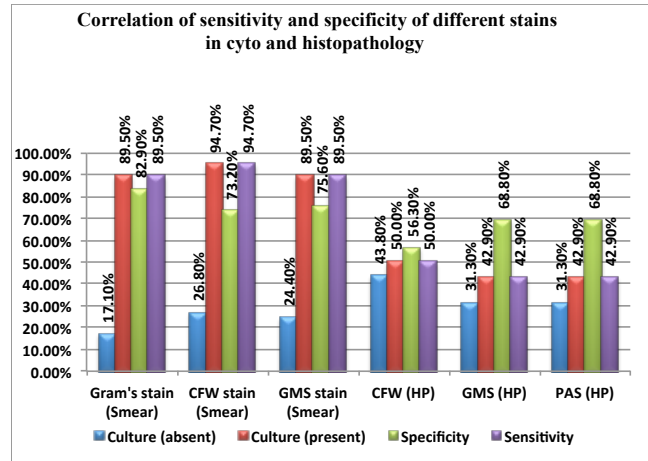
and out of 5 culture positive samples of control group, 4 cases showed the same. For the identification of species in combination with morphological observations, all the 19 (14 of study group and 5 of control group) culture positive samples were subjected to carbohydrate fermentation tests. There was significant association of Gram s, CFW s, GMS s with Culture ($p= 0.007, 0.024, 0.038$ respectively). Majority of the individuals who showed positive culture were also positive in the staining methods of Gram s, CFW s, and GMS s (89.5%, 94.7%, 89.5% respectively), similarly those who had negative culture were also negative in staining methods of Gram s, CFW s and GMS s (82.9%, 73.2%, 75.6% respectively).

In cytopathological smear, CFW showed highest presence of Candida (73.3%) followed by GMS (66.7%) and Gram's stain (60%) whereas in control group CFW and GMS stains had equivalent presence (23.3% each) followed by Gram's stain (20%).

Correlation analyses of various staining procedures were done by using two ways Pearson Correlation. Staining ability of CFW was found to show strong positive correlation with GMS and Gram's stain in cytopathological smear. There was significant association of Gram s, CFW s, GMS s with Culture ($p = 0.007, 0.024, 0.038$ respectively). Majority of the individuals who showed positive culture were also positive in the staining methods of Gram s, CFW s, and GMS s (89.5%, 94.7%, 89.5% respectively), similarly those who had negative culture were also negative in staining methods Gram s, CFW s and GMS s (82.9%, 73.2%, 75.6% respectively). In histopathological sections, CFW stain showed highest presence (46.7%) followed by GMS and PAS (36.7% each) (showed in Graph 1). Stained slides were observed under light microscope for presence of candida where candida can be seen in the superficial layer of epithelium and hyphal fragments penetrating the epithelium both vertically and horizontally (showed by figures-1,2,3).

DISCUSSION

Candida is an opportunistic fungal pathogen that may colonize, invade and induce lesions in any



Graph-1: Correlation of sensitivity and specificity of different stains in cyto and histopathology

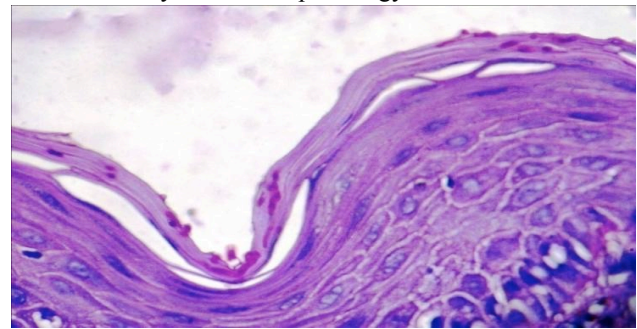


Figure-1: Candidal hyphae (Magenta red coloured) stained by PAS stain in histopathological section (Light microscope, 40X with zoom)

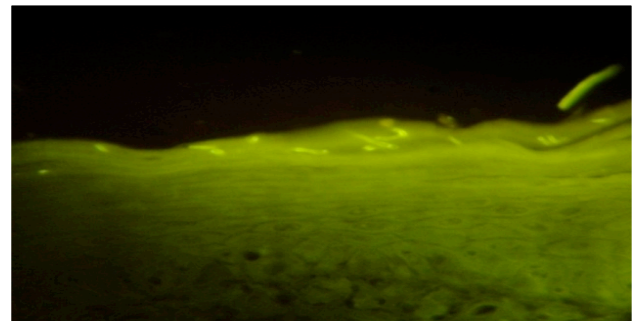


Figure-2: Candidal hyphae (Apple green coloured) stained by CFW stain in histopathological section (Fluorescence microscope, 40X with zoom)

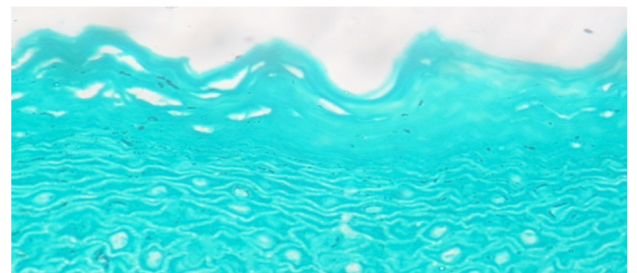


Figure-3: Candidal hyphae (Black coloured) stained by GMS stain in histopathological section (Light microscope, 40X with zoom)

part of the oral cavity in both immunocompetent and immunocompromised individuals. The presence of *Candida* in the mouth together with epithelial changes may predispose individuals to candidal infection.⁶

Similarity of our finding of high candida carriage has been reported by various authors such as Ariyawardana et al.⁸ Rashmi Santosh Kumar et al.,⁹ K Anila et al.⁶ and Mamta et al.⁷ However Reichart et al.¹⁰ observed almost equivalent *Candida* carriage in betel quid chewers (46%) as against non-chewers (44%). In study of Ariyawardana et al.⁸ *Candida* was isolated in 63.6% of OSMF patients and 50% of the control group. But, K Anila et al.⁶ revealed in their study a higher candidal prevalence in OSMF patients (40%) when compared to a control group (15%), and mean scores of candidal growth were also higher in OSMF patients than controls. Rashmi Santosh Kumar et al.⁹ recorded seven of 24 (29.16%) cases of OSMF cases were culture positive for *Candida* growth and Mamta et al.⁷ observed eleven (36.67%) cases in the study group (OSMF), and two (10%) cases in the control group, yielded *Candida* on culture.

However in all previous studies, there was no statistically significant difference between the two groups, a finding similar to our study ($p=0.24$). This finding of increased *Candida* carriage in some OSMF patients implies that alterations in the overlying epithelium in OSMF would breach the physiological barrier offered in healthy status, thereby favoring a conducive microenvironment that eventually increases the colonization of *Candida*.⁷

In our study, we revealed non significant association ($p=0.314$) between habit of betel nut quid with and without tobacco and *Candida* carriage in OSMF, though a significantly higher proportion of *Candida* carriage in individuals who had smoking habit than those who didn't had the habit of smoking ($p<0.001$). Similarly David N. Crockett et al.¹¹ and Daftary and his colleagues¹² in their separate studies found *Candida* invasion almost entirely among tobacco users while Arendorf et al.¹³ D. E. Oliver and E. J. Sillitoe¹⁴ found in their studies, a significantly increased candida carriage rate in cigarette smokers than non smokers. On the other hand, Gergely and Hilman and Kissin found no qualitative change of the oral mycotic

flora in smokers as compared with non-smokers.¹²

Out of 30 cases of study group, *Candida* was detected in 14 cases (46.7%) whereas in control group, *Candida* was detected in only 5 cases (14.6%). In study group, we observed *Candida albicans* in 9 cases (30%), *Candida tropicalis* in 2 cases (6.7%), *Candida glabrata*, *Candida krusei* and mixed species in 1 case each (3.3%) whereas in the control group, *Candida albicans* in 2 cases (6.7%), *Candida tropicalis* in 1 cases (3.3%) and mixed species in 2 cases (6.7%). *Candida tropicalis* was the most common species isolated in the study group which is similar to the studies by Ariyawardana et al.⁸ and Rashmi Santosh Kumar et al.⁹ These were the most dominant species, even in healthy individuals. Isolation of mixed species of *Candida* was similar to study of K Anila et al.⁶ Reichart et al.¹⁰ Ariyawardana et al.,⁸ in their studies also reported *C. dubliniensis* for the first time in both groups whereas in our study we didn't find this species.

In our study Gram stain was used as a basic stain. Amongst study group of 30 cases of OSMF, 18 cases (60%) were positive and 12 cases (40%) showed negative detection for *Candida* by Gram's stain whereas Rashmi Kumar et al.⁹ in her study showed 33.33% (8 out of 24 cases) positivity in OSMF patients.

Monheit introduced the addition of CFW to PAP stain, without altering or destroying the diagnostic cytopathological features, while still allowing the fungi to be identified.¹⁵ In this study, we used CFW stain in cytopathological smear to demonstrate fungal elements in OSMF under fluorescence microscope and compared with Gram's stain and Gomori Methenamine silver (GMS) stain. Higher percentage of detection of *Candida* in 73.3% (22 out of 30 cases) was observed with CFW stain when compared with that of Gram's staining (60%) and GMS (66.7%) in OSMF. Similarly, Rashmi Kumar et al.⁹ observed higher percentage detection of *Candida* with CFW stain (54.16 %) as compared with that of Gram's stain (33.33%) in OSMF. Staining ability of CFW was found to show strong positivity with GMS and Gram's stain in cytopathological smear by 'Two Way Pearson correlation'.

CFW stain showed highest sensitivity (94.7%) than GMS and Gram's stain (89.5% with each

stain) in cytopathological smears; similarly Rashmi Santosh Kumar et al.⁹ observed in their study, higher sensitivity with CFW stain (85.10%) as compared to Gram's stain (48.93%). In this study we used the most commonly used PAS stain for tissues. In 30 cases of study group, it showed 11 cases (36.7%) of OSMF to be positive for candidal presence in histopathological sections which were similar to results of Rashmi Kumar et al.'s study.⁹ She showed in her study 33.33% (8 out of 24 cases) positivity of Candida in histopathological sections of OSMF patients. Jacqueline E., Monheit et al. Anna R. Graham et al.⁹ Denis P. Lynch et al.¹⁶ in their study used GMS and CFW stain and observed similar positivity in both staining.¹⁶ In our study, we also used GMS stain to demonstrate candidal infection in 30 cases of OSMF and observed positivity in 11 cases (36.7%) which was similar to PAS staining positivity.

CONCLUSION

The observations of this study suggest that OSMF favours the colonization of Candida. Thus mucosal/epithelial alterations due to underlying disease process, coupled with other factors might lead to Candida colonization, even in the absence of clinically relevant mycotic manifestations. Controversy of betel quid chewing habit to inhibit or promote adherence and invasion of Candida remains wide open.

Thus, further authenticated studies are definitely required to explore association of Candida along with species characterization in OSMF patients, factors favouring or discouraging its association as well as role of betel quid habit.

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