

**DIAGNOSTIC ACCURACY OF THE ACRIDINE ORANGE FLUORESCENCE
STAINING AND PAPANICOLAOU STAINING IN DETECTION OF ORAL
CANCER**

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ABSTRACT

Oral cancer is a major health problem in South East Asia including India. It is also estimated to be the sixth most common malignancy attributing majority of them as squamous cell carcinomas. Early diagnosis and prompt treatment of early malignant lesions offer the best hope of improving the prognosis in patients with oral squamous cell carcinoma. Oral exfoliative cytology is a simple and non-invasive diagnostic technique that could be used for early detection of oral premalignant and malignant lesions. The present study aims at determining the reliability of acridine orange stain and Papanicolaou stain in cytodiagnosis.

Keywords: Exfoliative Cytology, Fluorescence, Squamous Cell Carcinoma.

INTRODUCTION

Oral cancer is a major health problem in South East Asian subcontinent including India. The incidence of oral carcinomas is increasing substantially with younger population affected at a larger rate. [1] The oral cancer is estimated to be the sixth most common cause of mortality and highest incidence is seen in Indian population. [2] 94% of oral malignancies are oral squamous cell carcinoma. Because of great numerical dominance, the term *oral squamous cell carcinoma* is synonymously used as *squamous cell carcinoma*. [3] Since oral cancer is a major global threat worldwide and represents approximately 5% of all malignant tumours involving the body, its early detection is desirable so that successful therapy can be carried out. [4] Haematoxylin and eosin stained sections by far means remains the gold standards in regards to diagnosis of these lesions. However, the advent of newer diagnostic approaches has overcome some of the shortcomings of this technique. The early and chairside detection of these lesions by the dentist can imply earlier therapeutic approaches in this area.

The predominant cytochemical property of a malignant cell is the great abundance of nucleic acids. This content of nucleic acid can be demonstrated by exfoliative cytology using acridine orange staining method and Papanicolaou staining. [5] The purpose of the study was to demonstrate malignant cells cytochemically, based on the increase in nucleic acids, and to compare acridine orange fluorescence microscopy technique with the conventional cytological technique using Papanicolaou stain.

Material and methods

This retrospective study was carried out in department of Oral Pathology and Microbiology, Sharad Pawar Dental College, Sawangi (M), Wardha after the approval of institutional ethical committee.

The study group comprises of following cases –

1. Group I consists of 100 histologically diagnosed cases of malignancy of various grades.
2. Group II consists of 25 healthy control subjects.

Inclusion and Exclusion criteria –

1. The study group consisted of 100 individuals which were selected irrespective of their age and sex.
2. The control group comprised of patients which were of age group above 20 years without any clinically observable lesions.
3. For every case in the study group, the most representative areas were selected to obtain the smears.
4. Toluidine blue was not used due to possible interaction of acridine orange.

Exfoliated material was collected from study group and control group with the help of wooden stick and carried on the slide. The smear was prepared and fixed with 90% alcohol. The slides were stained simultaneously both for PAP and Acridine Orange. The apparatus used in our study was Leica Microscope. The smears stained with AO were screened using 10X and 20X objectives. Morphological details of the exfoliated cells were studied with 40X objective and the photographs were obtained.

EVALUATION:

The most significant difference between interpretation of malignancy by the Acridine orange staining and Papanicolaou techniques is in relative importance of the appearance of cytoplasm. In PAP, interpretation mainly depends upon nuclear characteristics & cytoplasmic features are of little importance (utilized chiefly for determination of cell type). On the other hand flaming orange red fluorescence of the cytoplasm of malignant cells is the most striking feature.

CRITERIA FOR EVALUATION:

Acridine Orange Fluorescence Technique

- I- Negative:-Cytoplasm shows green fluorescence & nucleus yellow in colour
- II- Suspicious:- Cytoplasm shows weak orange red fluorescence but morphological criteria of atypia are present
- III- Positive :- Cytoplasm shows bright orange red fluorescence (increased RNA) & nucleus bright yellow fluorescence (increased DNA)

Papanicolaou staining technique

- I-Negative :- No malignant cells are observed in the smear.
- II- Suspicious :- Cells are seen that do not fulfill the malignant criteria adequately to allow a Positive report, yet reveal enough deviation from normal to indicate that follow up studies should be performed.
- III- Positive :- Criteria for malignant cellular change are observed.

Histopathological Evaluation:

- I. Well differentiated squamous cell carcinoma.
- II. Moderately differentiated squamous cell carcinoma.
- III. Poorly differentiated squamous cell carcinoma.

RESULTS AND OBSERVATIONS

The cases were compared according to the demographic characteristics in accordance to age, sex and site of the lesion. The study population was divided into various age groups and it was found in the study that incidence of OSCC is in 5th to 7th decade of life .In our study, it was observed that, male: female ratio was 2:1 affected by OSCC. The probable reasons attributed to this fact could be due to high indulgence of habits at an earlier age in males followed by low socioeconomic status, peer pressure etc.

The most affected site of OSCC as observed in our study was buccal mucosa (51%) followed by gingivobuccal sulcus (49%).The reason for such distribution could be due to habit of quid placement or kharra chewing for longer duration with more frequency of habit.

The results obtained using acridine orange and Papanicolaou staining were evaluated. Using fluorescent acridine orange method, the patients with study group I was evaluated. The total cases of squamous cell carcinoma were 100. Out of which 14 cases were of Well Differentiated Squamous cell carcinoma, 81 cases were of moderately differentiated squamous cell carcinoma, 5 were of poorly differentiated squamous cell carcinoma as diagnosed histopathologically. Out of 14 cases of well differentiated squamous cell carcinoma, 9 (64.28%) cases appeared to be negative, 2 (14.28%) cases appeared to be suspicious for staining with acridine orange, 3(21.42%) cases were positive for staining.

[Table -1]

Out of 81 cases of Moderately differentiated squamous cell carcinoma, 3 (3.70%) cases were negative, 31 (38.27%) cases were suspicious, 47(58.02%) cases were positive for staining

[Table – 1]

Out of 5cases of poorly differentiated squamous cell carcinoma, none of the cases showed negative and suspicious for staining. All the 5 (100%) cases showed positive for malignancy

.[Table-1]

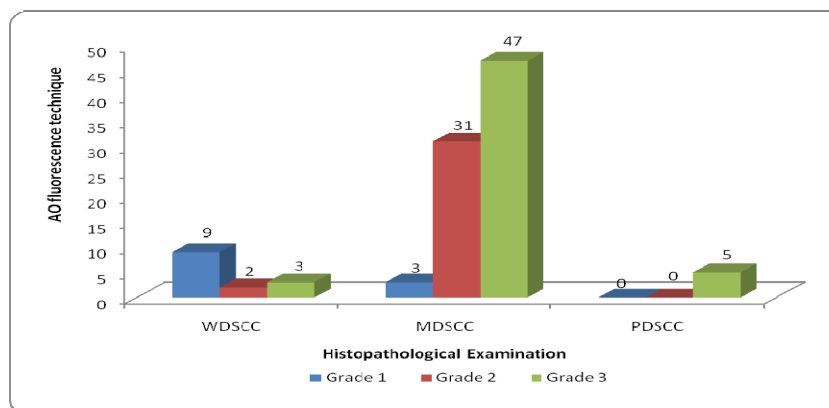
The smears were also stained for Papanicolau staining.Out of 14 cases of WDSCC 12 cases were positive for grade I,2 cases were positive for grade II and none of the cases were positive for grade III.Out of 81 cases of MDSCC, 39 cases were positive for grade I, 42 cases were positive for grade II,none of the cases were positive for grade III. Out of 5 cases of PDSCC, 1 case showed positive for grade I, 4 cases showed positive for grade II and none of the case were positive for grade III.[Table-2] The results obtained were thus statistically significant.(p <0.05).

Table 1: Correlation between AO fluorescence techniques and histopathological examination

		Histopathological Examination			Total
		WDSCC	MDSCC	PDSCC	
AO fluorescence technique	Grade 1	9	3	0	12
	Grade 2	2	31	0	33
	Grade 3	3	47	5	55

		Histopathological Examination			Total
		WDSCC	MDSCC	PDSCC	
AO fluorescence technique	Grade 1	9	3	0	12
	Grade 2	2	31	0	33
	Grade 3	3	47	5	55
Total		14	81	5	100

- Chisquare Value=45.80,p-value=0.000, S,p<0.05

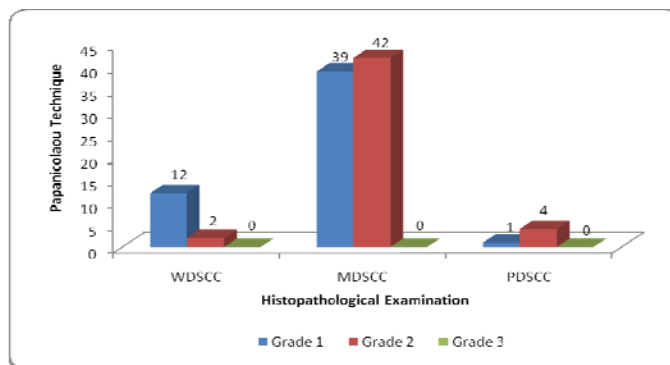


- Interpretation : The correlation of AO fluorescence technique with histopathological examination shows significant association i.e Chisquare Value=45.80,p-value=0.000, S,p<0.05. In AO fluorescence technique Grade I is WDSCC is 9, MDSCC is 3. In Grade II WDSCC is 2, MDSCC is 31 and in Grade III is WDSCC is 3, MDSCC is 47.

Table 2: Correlation between Papanicolaou Technique and histopathological examination

		Histopathological Examination			Total
		WDSCC	MDSCC	PDSCC	
Papanicolaou Technique	Grade 1	12	39	1	52
	Grade 2	2	42	4	48
	Grade 3	0	0	0	
Total		14	81	5	100

- Chisquare Value=8.90,p-value=0.012, S,p<0.05

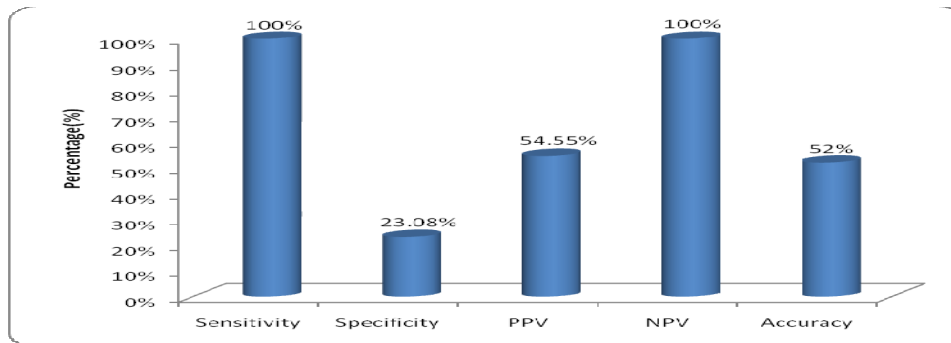


- Interpretation : The correlation of Papanicolaou Technique with histopathological examination shows significant association i.e Chisquare Value=8.90,p-valule=0.012, S,p<0.05 but it was less than AO fluorescence technique .In Papanicolaou Technique Grade I is WDSCC is 12, MDSCC is 39, PDSCC is 1. In Grade II WDSCC is 2, MDSCC is 42, PDSCC is 4 and in Grade III is WDSCC is 0, MDSCC is 0 and PDSCC is 0. The staining of acridine orange and Papanicolau technique compared and the results showed that specificity was 23.08% and the sensitivity was 100%. Furthermore, positive predictive value of the disease was 54.55% which is suggestive of the total no of cases actually affected by the disease. The negative predictive value is 100% which indicates that patient does not have the disease and is indicative of total true negative cases in the study .[table – 3]

Table 3 Sensitivity of AO fluorescence technique and Papanicolaou Technique

		Papanicolaou Technique		Total
		Positive	Negative	
AO Fluorescence technique	Positive	48	40	88
	Negative	0	12	12
Total		48	52	100

- Sensitivity=100%
- Specificity=23.08%
- PPV=54.55%
- NPV=100%
- Accuracy=52%



- Interpretation : For getting Sensitivity of AO fluorescence and Papanicolaou Technique Grade II and III in both technique were consider as positive and Grade I was considered as negative, its sensitivity came to be 100 %, Specificity=23.08%,Positive Predictive Value =54.55%, Negative Predictive Value =100% and Accuracy=52%. Accuracy was 52% because true negative cases are less.

DISCUSSION

Oral cancer is the most life-threatening disease of oral tissues. Most cancers of the oral cavity are oral squamous cell carcinomas (OSCC). Tobacco, alcohol and betel nut use are the main risk factors for these and many potentially malignant lesions (PML). The main high risk groups are older adult males who have habit of tobacco and alcohol consumption. Diagnosing oral cancers at an early stage is critical in improving the survival rate and reducing the morbidity associated with the disease. [6] Exfoliative cytology is a simple and reasonably effective technique for rapid initial evaluation of a suspicious oral lesion. However, since the conventional Papanicolaou technique is demanding in terms of time and skill of the examiner, various method to expedite cytological diagnosis have been proposed. In a similar study done by Bertalanffy, [7] Group I consisted of 32 patients with oral lesions suspicious of malignancy.

Twenty of the 32 (63%) patients were positive for carcinoma, 11 (34%) were negative and only 1 lesion was questionable when stained by fluorescent acridine orange method. Malignant disease was diagnosed by biopsy in 16 (50%) of the lesions. The malignant disorder in each of the cases was squamous cell carcinoma. Four (13%) of the lesions were reported as "false positive" by the fluorescent acridine orange method, when compared with biopsy reports. There were no "false-negative" reports with this technique. Three (18%) of the 16 malignant lesions were reported "false negative" by the conventional Papanicolaou method, when compared with the biopsy findings. No "false positives" were reported by this technique.

Group II consisted of 21 oral lesions, which did not suggest malignancy. The smears obtained were stained by fluorescent acridine orange method and were compared only to the conventional Papanicolaou method. Eleven (53%) of the lesions were reported as positive when stained by fluorescent acridine orange method.

Thus, 11 of the 21 lesions were "false positive" when compared to the conventional Papanicolaou method. However, there were no "false-negative" reports. Two "false-positive" reports were obtained by the conventional Papanicolaou method and both the reports were verified by biopsy reports. There were no "false-negative" reports using the conventional Papanicolaou method.[7]

The application of acridine orange-fluorescence microscopy to exfoliative cytologic screening for cancer cells is based upon the high protein synthesis of cancerous tissue, since the fluorescence of malignant cells appears to be proportional to their DNA and RNA moieties.

Although most malignant cells have more nucleic acids than their normal prototypes some cancer cells have no significant increase in DNA and decreased RNA has been found in less virulent malignant cells. Thus it is not surprising that some exfoliated malignant cells failed to show sufficient fluorescence to permit their rapid recognition by Acridine Orange – Fluorescence Microscopy.(AO-FM) Most of the AO-FM failures in this study were due to lack of fluorescence of well differentiated squamous carcinoma cells.

Since most exfoliated cells have reached their fullest maturation prior to desquamation, it is remarkable that such a high proportion still exhibits such brilliant fluorescence. Our results show that the fluorescent acridine orange method was more reliable in demonstrating malignant cells in oral lesions that were clinically suggestive of cancer than

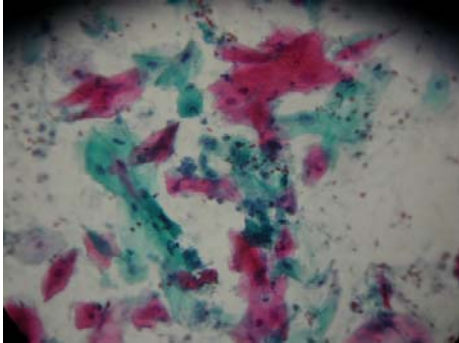


Fig 1 A – Normal exfoliated cells demonstrated by Papanicolaou stain (10×)

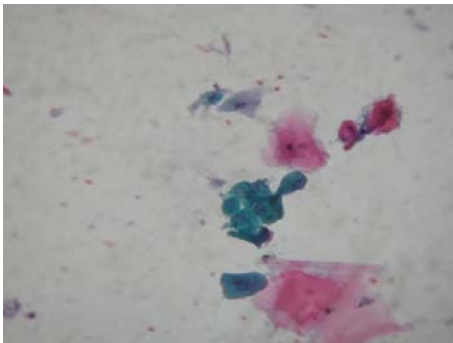


Fig 1 B – Malignant cells demonstrated by Papanicolaou stain (10×)

This was confirmed using the biopsy findings. The acridine orange technique demonstrates malignant cells based on the increase in nucleic acid content. Malignant cells with moderate amounts of RNA show reddish brown cytoplasmic fluorescence and the cytoplasm of cells with large amounts of RNA shows bright orange to flamingo-red fluorescence. [Fig- 2 A,B]

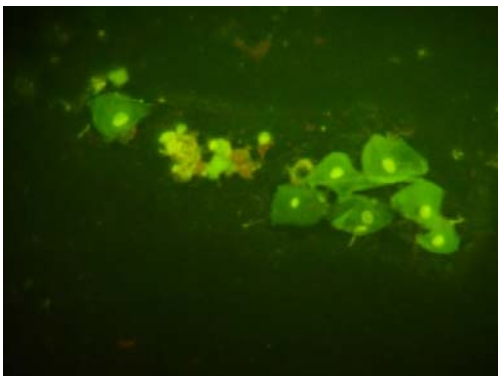


Fig 1 B – Normal cells demonstrated by Papanicolaou stain (10×)

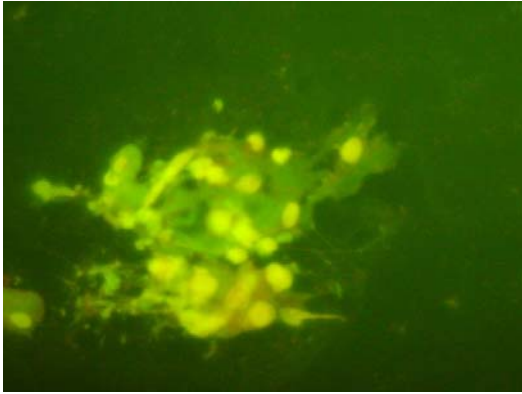


Fig 2 B : Malignant cells demonstrated by fluorescent acridine orange stain (10x)

The diagnosis of malignant cells using conventional Papanicolaou method relies on the morphologic details. However, the results of acridine orange fluorescent stain were not acceptable when applied to a group of oral lesions (traumatic ulcers, herpetic lesions, etc.), which were not clinically suggestive of cancer. Up to 45% of the lesions showed "false-positive" results indicating carcinoma. The accentuated protein synthesis and the resultant increase in the nucleic acid content in these proliferating cells accounts for the discrepancy in diagnosis. The Papanicolaou stain results were superior when applied to this group of lesions.

Conclusion

The fluorescent acridine orange method can be used reliably for the screening of carcinomas and it is especially helpful in the follow-up detection of recurrent carcinoma in previously treated cases. However, it should be cautiously used in oral ulcerative lesions, which may clinically mimic cancer.

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