ORIGINAL RESEARCH

Comparison of Efficacy and Reliability of different Histochemical stains in Oral Exfoliative Cytology: A Qualitative Analysis

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ABSTRACT

Introduction: Oral cancer constitutes a major health problem representing the leading cause of death The study was performed to develop an economical, quick alternative to the conventional Papanicolaou stain and Haematoxylin & Eosin stain and also to explore newer staining techniques in oral exfoliative cytology like May- Grunwald Giemsa stain, Leishman- Giemsa Cocktail stain**. Material and Methods:** Slightly moistened cytobrush was used to take four smears in 50 patients with OSCC. Each smear was taken from the most representative area and same site (till bleeding spots are established). Scrapings were smeared on four slides; two smears were fixed and stained with H&E and PAP and two were air dried and stained by MGG and LG- cocktail. **Results:** Qualitative analyses of the cytosmears obtained in study cases showed nuclear details were better appreciated with PAP. For cytoplasmic staining the MGG gave comparatively better results. Clear background was seen in PAP stain in the study cases. To make a definite diagnosis in OSCC both PAP and MGG seems to be equally good. **Conclusion:** It can be concluded from the present study that the staining characteristics of PAP proved to be better out of the four stains that are, H&E, MGG, and LG- Cocktail.

KEYWORDS: Oral exfoliative cytology, Papanicolaou stain, May- Grunwald Giemsa stain

INTRODUCTION

Oral cancer is categorised as the sixth most frequent malignancy worldwide and is being detected at an estimated rate of 263,900 new cases and 128,000 deaths in a single year.[1,2] Despite many advances in cancer therapies, the five-year survival rate for oral squamous cell carcinoma (OSCC) has remained at approximately 50% over the past three decades.[1, 3] This is primarily due to delayed diagnosis, with approximately half of all oral cancers diagnosed at stages III or IV.[4]

Histological examinations of biopsied tissues remain the gold standard for diagnosis and identification of oral lesions.[5] But it is an invasive technique with surgical implications, technically a limitation for some professionals and psychologically for some patients [6] Cytology is a non-aggressive, non-invasive process and a relatively pain-free procedure that is well accepted by the patients reluctant to go for a biopsy.[5]

Stains like Haematoxylin & eosin (H & E) and Papanicolaou (PAP) are the routine and universal stain for the cytological procedure and are easy and quick to perform for screening programs.[7] May-Grunwald Giemsa (MGG) stain is easy to prepare, less time consuming, reduces the effects of poor techniques and increases cell yield. The LG cocktail (Leishman Giemsa- cocktail) is an easy, cost – effective one-step technique. It needs less time and less infrastructural support.[7] These newer stains like MGG, LG – cocktail are not been utilized in oral exfoliative cytology. Hence, the present study was designed to carry out the qualitative evaluation of cytologic smears of patients with malignant lesions using four different stains that is H&E, PAP, MGG, LG cocktail. Also to ascertain the efficiency and reliability of these stains in evaluating cellular and nuclear atypia and correlating the cytopathological and histopathological grades.

MATERIALS AND METHODS

The study was conducted in the Department of Oral and Maxillofacial Pathology & Microbiology of our institute. The study was approved by the Ethics Committee. Smears and biopsy samples were obtained from 50 patients clinically showing of OSCC and smears from 10 subjects with clinically normal oral mucosa (considered as control) which were confirmed histopathologically. Written informed consent was obtained from each patient before taking the smear and biopsy samples. Subjects were asked to rinse their mouth thoroughly with water before taking the smear. Slightly moistened cytobrush was used to take four smears. Each smear was taken from

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the most representative area and same site (till bleeding spots are established). Scrapings were smeared on four slides; two smears were fixed and stained with H&E and PAP and two were air dried and stained by MGG and LG- cocktail.

The stained cytosmears were viewed under the compound light microscope and cytopathologically graded based on the criteria given by *von Hamn*.[8] The biopsy was carried out from the lesion and histopathologically graded based on WHO classification for oral squamous cell carcinoma (WHO, 2005).[9] The slides were analyzed for four parameters. (Table 1)

Parameters	Score
Cytoplasmic details:	
Not preserved	0
Non-transparent masking of nuclear details	1
Non-transparent with intact cell membrane	2
Transparent, intact cell membrane without masking of nuclear details	3
Nuclear details:	
Poor preservation	0
Smudgy	1
Fair preservation but chromatin granularity not appreciable	2
Excellent preservation with crisp chromation	3
Background staining:	
Intensely stained obscuring cellular details	0
Moderately stained with better cellular details	1
Less intense staining with crisp cellular details	2
Ability to make a definite diagnosis:	
Ability to make a definite diagnosis as benign	0
Ability to make a less definitive diagnosis as atypical or malignant	1
Ability to make a less definitive diagnosis as intermediate	2
Ability to make a definite diagnosis as malignant lesion	3

Table 1: Qualitative Parameters

Statistics: The resulting data were analyzed by using SPSS software version 19. Data has been expressed as the mean & standard deviation. Differences between variables were analyzed using ANOVA test and Post Hoc test followed by Bonferroni test. Descriptive statistics including the mean values, standard deviations, ranges were calculated for each variable. The qualitative data were compared by linear coefficient depicting the trend in various stains and parameters under variable time period. Inter observer reliability was calculated by Cohen's Unweighted Kappa.

Quality index for four stains was assessed by considering all the qualitative parameters. [QI = actual score obtained/maximum score possible].

Comparison of cytopathological scores with histopathological scores was performed by Mann-Whitney U test.

The true and false positives and negatives were based on :
True positives: Samples which were positive histologically and cytology.
True negative: Samples which were negative histologically and cytology.
False positive: Samples those were negative histologically and positive cytology.
False negative: Samples those were positive histologically and negative cytology.

To check and quantify the reliability of the stains, sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy were assessed.

The Receiver operating characteristic (ROC) curve plotted with sensitivity along Y-axis and 1-specificity along X-axis for each stain.

RESULTS

Qualitative analyses of the cytosmears obtained in study cases showed nuclear details were better appreciated with PAP. For cytoplasmic staining the MGG gave comparatively better results. Clear background was seen in PAP stain in the study cases. To make a definite diagnosis in OSCC both PAP and MGG seems to be equally good with the score (2.88±0.33) and (2.56±0.71) respectively which were superior to the other two stains (Table 2).

Parameters / Stains	Cases	Cytoplasmic details	Nuclear details	Background	Ability to make a definite diagnosis
Haematoxylin- Eosin Stain	OPMD	2.88±0.33	2.76±0.44	1.88±0.33	2.64±0.49*
	OSCC	2.28±0.46	1.92±0.49	1.84±0.47	1.92±0.81*
Papanicolaou Stain	OPMD	2.84±0.37	2.92±0.28	1.92±0.28	2.88±0.33*
	OSCC	2.32±0.80	2.24±0.44	1.84±0.47	2.56±0.51*
May-Grunwald Giemsa Stain	OPMD	2.92±0.28	2.68±0.63	1.8±0.41	2.88±0.33*
	OSCC	2.56±0.51	1.92±1.04	1.64±0.49	2.56±0.71*
Leishman Giemsa Cocktail Stain	OPMD	2.8±0.41	2.72±0.54	1.84±0.37	2.60.58*
	OSCC	2.32±0.63	1.88±0.60	1.68±0.48	1.84±1.07*

Table 2: Qualitative analysis of cytological parameters in oral potentially malignant disorder cases and oral squamous cell carcinoma. * Correlation is significant at the P< 0.05 level.

The Cohen's unweighted Kappa statistics was applied to observe the interobserver agreement between three observers in four different stains that is, Haematoxylin – Eosin stain, Papanicolaou stain, May-Grunwald Giemsa stain and Leishman Giemsa Cocktail stain and in four different staining parameters, that is cytoplasmic details, nuclear details, background and ability to make a definite diagnosis. The Kappa statistics showed slight agreement in recording cytoplasmic details between all observers. A statistically significant result was obtained in cytoplasmic details between Observer 1 vs 3 and Observer 2 vs 3 and statistically non- significant result was obtained between Observer 1 vs 2. For nuclear details, fair agreement was seen between Observer 1 vs 2, ($p<0.05$). And slight agreement was found between Observer 1 vs 3 as well as observer 2 vs3, ($p<0.05$). For the parameter where background staining was evaluated, slight agreement was seen between Observer 1 vs 2 and Observer 2 vs 3 and

poor agreement was found between Observer 1 vs3, ($p>0.05$). A fair agreement was seen in the parameter where ability to make a definite diagnosis between observer 1 vs 2, ($p<0.05$). Whereas, a slight agreement was found between Observer 1 vs 3 and Observer 2 vs 3, ($p<0.05$) in the parameter ability to make a definite diagnosis (Table 3).

Parameters / Observers	Cytoplasmic details	Nuclear details	Background	Ability to make a definite diagnosis
Observer 1 vs 2	0.10	0.25 *	0.09	0.33 *
Observer 1 vs 3	0.13 *	0.13 *	-0.04	0.17 *
Observer 2 vs 3	0.17 *	0.14 *	0.01	0.15 *

Table 3: Kappa analysis for qualitative assessment showing the extent of agreement between three observers. * Correlation is significant at the P< 0.05 level.

The quality index of each of the four stains showed PAP to be a better stain statistically (<0.05) with the highest quality index score of 0.813. MGG stain scored 0.800, which was comparable to the quality index of the PAP. H&E stain scored 0.775. The scores for all the four parameters of LG Cocktail stain were lower than the three other stains that are 0.756 (Table 4).

Parameters (max score)/ Stains (actual score)	CD 180	ND 180	B 120	AD 180	QI 660
H&E	156	143	111	116	0.775
PAP	149	157	112	138	0.813
MGG	164	139	100	138	0.800
LG- Cocktail	146	140	104	113	0.756

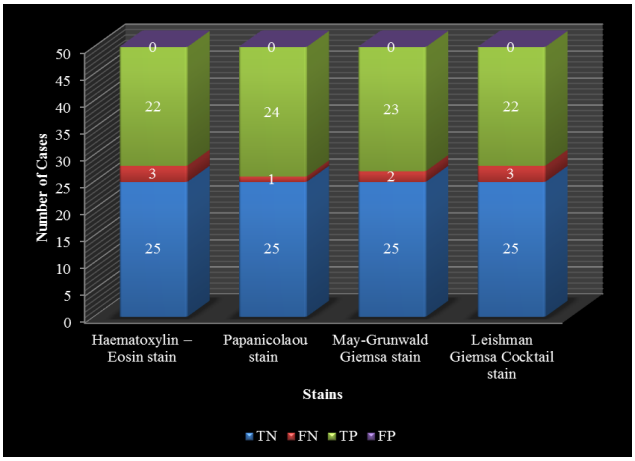
Table 4: Quality index of different stains

On correlation of cytopathological diagnosis with histopathological diagnosis, it was observed that PAP stain was best to detect true negative and LG cocktail was found to have maximum false negative result. Whereas in detecting benign and malignant lesions, i.e, true positive, four stains were found to be equally good. And all benign lesions were identified correctey in all the four stains. Thus suggesting that PAP had highest diagnostic accuracy (Table 5, Graph 1).

Stains	Cytological diagnosis	No. of cases	Histopathologic diagnosis		P-value
			TN (%)	FN (%)	
Haematoxylin – Eosin stain	Benign	n=28	25 (89.28%)	3(10.7%)	< 0.01
	Atypical	n=22	22 (100%)	0 (0%)	
Papanicolaou Stain	Benign	n=26	25 (96.18%)	1 (3.84%)	< 0.01
	Atypical	n=24	24 (100%)	0 (0%)	
May-Grunwald Giemsa Stain	Benign	n=27	25 (92.59%)	2 (7.40%)	< 0.01
	Atypical	n=23	23 (100%)	0 (0%)	
Leishman Giemsa Cocktail Stain	Benign	n=28	25 (89.28%)	3 (10.7%)	< 0.01
	Atypical	n=22	22 (100%)	0 (0%)	

Table 5- Correlation of cytopathological with histopathological diagnosis.

The reliability of the stains were checked and quantified. PAP stain showed better value in relation to sensitivity, specificity, positive predictive value and diagnostic



Graph 1: Correlation of cytopathological with histopathological diagnosis in study cases.

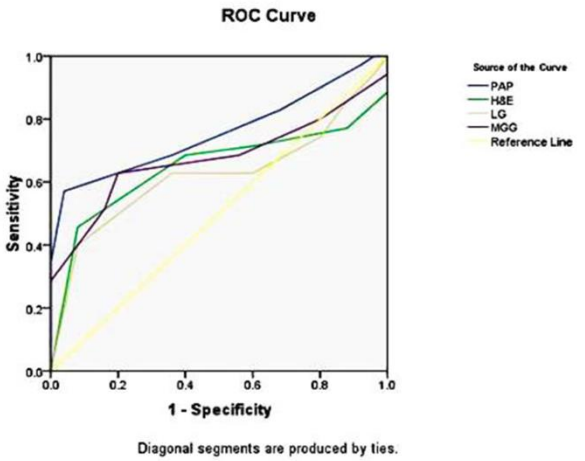
accuracy than the three other stains. In evaluating the negative predictive value both the PAP and MGG were found to be equally good with equal scores. In evaluating the diagnostic accuracy, PAP showed to have 80.0% diagnostic accuracy with MGG stain with the score of 78.3% comparable with PAP. Thus, PAP showed to have the maximum comparable result to classify between the presence or absence of a disease (Table 6).

Stains	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy
H&E	48.6%	88.0%	85.0%	55.0%	65.0%
PAP	71.4%	95.8%	96.2%	69.7%	80.0%
MGG	68.6%	92.0%	88.9%	69.7%	78.3%
LG-Cocktail	47.1%	80.8%	76.2%	53.8%	61.7%

Table 6: Sensitivity, specificity, positive predictive value, negative predictive value and accuracy of all stains.

Receiver operating characteristic (ROC) curve: The present study was first of its kind where ROC curve was also plotted for the four stains. The ROC curve plotted showed PAP has the maximum area under the curve that is (0.757). The result was found to be statistically significant ($p<0.05$) thus indicating that PAP has the best sensitivity and specificity values when all the qualitative parameters were considered for producing best results in evaluating cytological smear from oral epithelial cells in oral potentially malignant disorders and oral squamous cell carcinoma. Area under the curve of MGG scored 0.676 which was found to be higher than H & E with the area under the curve (0.649). The result showed MGG to be a better than H&E ($p<0.05$). While LG Cocktail stain showed the least area under the curve (0.623) and the result were found to be statistically non-significant ($p>0.05$) indicating that LG Cocktail stain has the least diagnostic value as compared to the other stains that is H& Eosin, PAP and MGG in observing cellular and nuclear parameters in oral cytological smears (Graph 2).

ROC curve has shown to be an excellent statistical test in the present study to evaluate the overall accuracy of a diagnostic test i.e, different stains. According to the results obtained PAP stain shows fair accuracy



Stain	Area under the curve	P- value
PAP	0.757	0.001
MGG	0.676	0.02
H&E	0.649	0.05
LG- Cocktail	0.623	0.10

Graph 2: ROC curve in four different stains

comparable with MGG, but H&E and LG Cocktail showed poor accuracy.

DISCUSSION

Oral exfoliative cytology is a relatively pain-free procedure and can provide intact cells from different layers of the epithelium. In the past, this technique had been of limited usage due to poor sensitivity and specificity but has re-emerged as a diagnostic and predictive method due to the improved technique.[10] It is now being promoted as an adjunct to biopsy and a screening method useful for the diagnosis of oral cancer.[6,11,12] It is gaining importance as a rapid method for various screening procedures and many stains has been employed to study the exfoliated cells.[13] The major value of cytology is the rapid, simple and relatively pain-free procedure which can provide the intact cells from different layers of the epithelium.[18] It is mostly well accepted by the patient and is, therefore, an attractive option for the early diagnosis of oral cancer, including epithelial atypia and squamous cell carcinoma.[13]

The increasing popularity of Oral exfoliative cytology, as a primary diagnostic procedure has demonstrated the utility and adaptability of stains such as H&E and Romanowsky stains.

In the present study for cytoplasmic staining MGG (2.56±0.51) and in evaluating nuclear details PAP (2.80±.422) gave better results, as it stains nuclear chromatin well, gives good differential cytoplasmic counterstaining and produces good cytoplasmic transparency.[14,15,16] Due to vivid metachromatic staining of certain cytoplasmic products, stromal, and background elements, many cytologists prefer Romanowsky stains over PAP or H&E for FNA specimens[17] and also the air-dried smears are used, which increases cell yield.[18] PAP and H & E stain showed better background and MGG stain with high background staining obscure the background material and also the cellular details. Therefore, MGG needs preparation of fresh solution every day.[5] PAP stain uses two dyes that differ in their affinity, and there is no chemical interaction between nuclear and cytoplasmic solutions as they bind electrostatically, resulting in salt formation. PAP has the benefit of staining cells from various layers differentially.[6,19] According to the study conducted by Belgaumi and Shetty, PAP stain results were found to be a better stain than LG and MGG, but results of LG were comparable to PAP.[5] Similar study conducted by Sujathan et al. suggested MGG stain to be a better stain than PAP stain.[16]

The qualitative assessment of four stains was done by scoring four different parameters for each stain the first study of its kind. Kappa statistics were applied to measure the agreement between three observers over the qualitative parameters. Thus the maximum agreement between the three observers was found in the parameters nuclear details and ability to make a definite diagnosis, which was also found to be statistically significant at the (<0.05 level), showing the extent to which the data collected in the study are the correct representation of the variables measured. Hence, these parameters can be used in future.

The QI were also assessed and scored to compare the diagnostic efficacy. PAP seems to be a better stain statistically (<0.05). This is in accordance to the study conducted by Idris and Hussain where they concluded PAP to have the maximum quality index of (0.87). [14]

On correlating the cytologic diagnosis with the histopathology reports, PAP stain showed statistically significant value (<0.05) and comparable results with MGG stain in the study cases which is in accordance with the study by Mitra et al. and Gabriel et al. [6,19]

The sensitivity of PAP stain scored (71.4%) which is the highest among the other three stains. This shows PAP has the highest probability of interpreting correctly that someone who has the target disease. The specificity of PAP stain scored (95.8%), depicting PAP has the highest probability to show that someone who does not have a disease. PAP also showed to have the maximum positive predictive value that is (96.2%) showing its efficiency to correctly tell the positive test result actually has the disease or not. While in evaluating the negative predictive value both the PAP and MGG were found to score equal (69.7%) depicting both are equally good with equal scores, and they have the ability to tell correctly the probability that a person with negative test results does not have the disease. Thus suggesting that both PAP and MGG can be used to diagnose accurately weather the person is truly free from disease or not. Finally, the diagnostic accuracy was obtained in which PAP and MGG stain was found to be of a comparable score. Suggesting that, both PAP and MGG stains can be used as a routine chair side stains and also as a screening test

to reduce the morbidity or mortality by detecting the disease in their early stages when the treatment will have a good prognosis. Also in seeing the accuracy, the results of MGG are found to be comparable with PAP and better than H&E and LG- Cocktail. H&E scored inferior in sensitivity, specificity, positive predictive value, negative predictive value and accuracy than PAP and MGG, but the results were superior to LG- Cocktail. Among all the four stains LG- Cocktail scored least in sensitivity, specificity, positive predictive value, negative predictive value, and accuracy. There is no chemical interaction between the nuclear and the cytoplasmic solutions (different to Romanowsky stains) as they bind electrostatically resulting in salt formation. PAP has the benefit of staining cells from various layers differentially.[6,19]

The Receiver operating characteristic (ROC) curve was also plotted for the four stains with sensitivity along Y-axis and specificity along X-axis, which is the first study of this kind. ROC curve provides a way to measure the accuracy of a diagnostic test by measuring the Area under the curve (AUC), that is, larger the area, the more accurate the stain. PAP stain was a better stain with the highest area under the curve 0.757 in ROC curve, indicating that PAP stain has the highest diagnostic value as compared to the other stains in observing cellular and nuclear parameters in cytological smears. Also, the curve for PAP stain was steepest, in the beginning, indicating a maximum number of true negatives and fair accuracy in diagnosis. As this curve gives a clear picture and appropriate comparison, it should be used more often for such comparative studies.

CONCLUSION

It can be concluded from the present study that the staining characteristics of PAP proved to be better out of the four stains that are, H&E, MGG, and LG- Cocktail. The MGG was found to give results comparable to the PAP. Hence, keeping in mind the advantages of MGG as a single step, cost-effective procedure the study also supports the idea of utilizing this stain in oral exfoliative cytology for PMDs and OSCC.

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