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Comparative studies on efficacy of the Leishman-Giemsa stains, Hematoxylin-Eosin & Papanicolaou stains for cytological diagnosis of oral lesions

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ABSTRACT

Oral potentially malignant disorders are the precursors of oral cancers in India. Severealcoholism, use of tobacco like cigarettes, smokeless tobacco, betel nut chewing and humanpapilloma virus (HPV) are the most common risk factors for oral potentially disorder andmay also occur due to poor dental care and poor diet. The incidence of oral cancer is highest inIndia, South and Southeast Asian countries.Papanicolaou (PAP) stain is the routine and universal stain for the cytological procedure and iseasy and quick to perform for screening programs. The Leishman-Giemsa (LG) is an easy, cost–effective one-step technique. It needs less time and less infrastructural support. TheHematoxylin-Eosin stains are used routinely in Histopathological procedures. These newerstains like, LG are not much been utilized in oral exfoliative cytology. Hence, the present studywas designed to carry out the qualitative evaluation of cytological smears of patients with pre-malignant lesions using three different stains that are H&E, PAP, and LG. This is to ascertain theefficiency and reliability of these stains in evaluating cellular and nuclear atypia and correlatingthe cytopathological and histopathological grades.

Abbreviation
H&E Stain- Hematoxylin-eosinstain, Pap stain- Papanicolaou stain, LGstain- Leishman -Giemsastain, OPMD- oral potentially malignant disorders.

INTRODUCTION

Oral potentially malignant disorders are the precursors of oral cancers in India. Severe
alcoholism, use of tobacco like cigarettes, smokeless tobacco, betel nut chewing and human
papilloma virus(HPV) are the most common risk factors for oral potentially disorder [1] and
may also occur due to poor dental care and poor diet. The incidence of oral cancer is highest in India, south and Southeast Asian countries. In India, 90 -95% of the oral cancers is squamouscell carcinoma. The international agency for research on cancer has predicted that India's
incidence of cancer will increase from 1 million in 2012 to more than 1.7 million in 2035 due tohigh risk of oral potentially malignant disorders. [2]In oral potentially malignant disorder development, tobacco plays an etiologicrole. It is believed that tobacco carcinogens induce generation of free radicals and reactiveoxygen species responsible for the high rate of oxidation/peroxidation of polyunsaturated fattyacids which affects the cell membrane and also cause DNA damage thus involved incarcinogenesis. Oral cancer is categorized as the sixth most frequent malignancy worldwide andis being detected at an

estimated rate of 263,900 new cases and 128,000 deaths in a singleyear [3]. This is primarily due to delayed diagnosis, with approximately half of all oral cancersdiagnosed at stages III or IV [4]. Cancers in the oral cavity, such as, cancers of the lip, tongue andmouth (WHO, ICD 10 classification: C 01–06) account for 48% of all head and neck cancers.Out of all these 90% are epithelial cell carcinomas. [5] The other oral sites of cancer include cheeklinings, gingiva (gums), and palate (roof of the mouth).

Papanicolaou (PAP) stain is the routine and universal stain for the cytological procedure and is easy and quick to perform for screening programs. The Leishman-Giemsa (LG) is an easy, cost – effective one-step technique. It needs less time and less infrastructural support. The

Hematoxylin-Eosin stains are used routinely in Histopathological procedures. These newer stains like, LG are not much been utilized in oral exfoliative cytology. Hence, the present study was designed to carry out the qualitative evaluation of cytological smears of patients with pre-malignant lesions using three different stains that are H&E, PAP, and LG. This is to ascertain the efficiency and reliability of these stains in evaluating cellular and nuclear atypia and correlating the cytopathological and histopathological grades.

Review of literature

World Health Organizations (WHO) in 1972 subdivided precancerinto lesions and conditions;

with their definitions. In 2005 the working group of WHO is not recommended such

subdivision and they use of the term oral potentially malignant disorder (OPMD) [6].It has beenrenamed potentially premalignant oral epithelial lesions (PPOELs) by its important to differentiate among PPOELs, which is a broad term to define a wide variety of clinical lesions, and oral epithelial dysplasia, which should be reserved specifically for lesions with biopsy-proven foci of dysplasia.[7]Chen et al. (2007) used methylene blue in fifty-eight patients with suspicious oral cavity lesions.They reported that the overall sensitivity of methylene blue uptake in cases with suspectedlesions was 90%, specificity 69%, and accuracy 79%. They also reported that the positivepredictive value was 74% and the negative predictive value 87% [8].It

was observed that the cytoplasmic staining was betterinL-G stain when compared to

PAP stain and nuclear stain was better inPAP stain,Gabryalet al. [9]PAP stain is widely used but it is more expensive and more time consuming and itrequires infrastructural sensitivity on other hand L-G stains in routine cytological procedure which is very cost effective,less time consuming and less infrastructural support give equal result.[10]Liu et al. (2016) state that the non-invasive detection techniques evaluated are divided into fourcategories: vital staining with a solution that can be used as a mouth rinse or applied onto asuspected area of the mouth, light-based detection systems, optical diagnostic technologies thatemploy returned optical signals to reflect structural and morphological changes within tissuesand salivary biomarkers.[11] Recent clinical diagnostic tools for early detection of oral cancer include tolonium chlorideor Toluidine blue dye, Oral CDx brush biopsy kits, salivary diagnostics and lastly opticalimaging systems.Among them oral exfoliative cytology is noninvasive most useful tools.[12,13]Histological examinations of biopsied tissues remain the gold standard for diagnosis andidentification of oral lesions. But it is an invasive technique with surgical implications,technically a limitation for some professionals and psychologically for some patients. Cytologyis a non-aggressive, non-invasive process and a relatively pain-free procedure that is wellaccepted by the patients reluctant to go for a biopsy. Stains like Hematoxylin; Eosin (H &E)and Papanicolaou (PAP) are the routine and universal stain for the cytological procedure to perform for screening programs.

The newer stains like, LG – cocktail is not been utilized in oral exfoliative cytology [13].Vezhavedhan N et al. (2011) has shown that oral exfoliative cytology has a Sensitivity of 88%

and a Specificity of 87% [14].Belagaumi et al. (2013) reported that the results from the histologically confirmed cases ofsquamous cell carcinoma and the number of cases diagnosed by PAP and LG cocktail werealmost identical and both were superior to MGG. The P value obtained for the confirmed casesof squamous cell carcinoma in comparison for Pap vs MGG was 0.001, MGG vs LG cocktail was0.001 and LG cocktail vs Pap was 0.157. Hence, no statistically significant difference wasobserved between the diagnostic ability of Pap and LG cocktail stains [15]Sidhu et al. (2018) explored that in the normal group, staining of LG was highly significant among potentially malignant lesions, LG was observed to be highly significant when compared with G and was not significant when compared with PAP. Inthe malignant group, LG was highly significant. LG was superior with the highestaverage staining than PAP and G. they concluded that LG cocktail is a betterstain with excellent cytoplasmic and nuclear staining intensity compared to PAP and G stains [16].

Materials & method

1. Subject selection

The study was conducted on the Patients visiting the Outpatient Department of Oral Medicine andRadiology of Awadh Dental College & Hospital, Jamshedpur. The study was approved by theEthical Committee. One smears each three such were obtained from 50 different potentiallymalignant disorders and 30 cases of control group respectively. Informed consent was obtainedfrom each patient before taking the smear. Subjects were asked to rinse their mouth thoroughly withwater before taking the smear.

1.2 Methodology

Prior to the collection of samples from the subjects, detailed case history was recorded. Both menand women in the age group between 18 to 85 years were subjected to clinical examination andfollowing readings were recorded in. The characteristics of different lesions were evaluated in detail

1.3 Inclusion criteria

Red and white Oral lesions including various forms of tobacco habits.

1.4 Exclusion criteria

Patients with other systemic, endocrinal abnormalities excluded from this study.

1.5 Collection of Sample

On the day of collection, participating subjects thoroughly wash his/her

mouth properly before the cytopathological examination. Scrapings will be taken from the oral

cavity with a wooden spatula from suspicious areas. Material obtained was smeared on grease freeglass slides, and immediately fixed in 95% ethyl alcohol and air dried.

1.6 Leishman-Giemsa staining Procedure

The smears were stained in the case of Leishman- Giemsa staining; the amount of stain sufficient tocover the smear was added. After about 2 minutes, double the amount of buffered water was addedand mixed with the stain present. A proper time is elapsed according to the stock used (about 8-10minutes) and the slide was washed off.

1.7 Hematoxylin-Eosin staining Procedure

In case of H&E staining, after about 10-15 minutes of fixing with isopropyl alcohol, the slide wasstained first with Hematoxylin for 15-20 minutes and then washed. Then, 1% acid alcohol was addedfor differentiation and the slide washed in running tap water. The slide was then placed in water for5 minutes for bluing. Finally, the slide was dipped in Eosin for 3-4 dips for counterstaining andwashed off. Hence, we can compare the staining time of both the procedures.It is about 10 to 12 minutes for LG stain while it is about 30 to 40 minutes for Hematoxylin andEosin stain.

1.8 PAP staining Procedure

In case of PAP the slides were fixed in 95% ethanol for 15 min followed by immediate dipping in50% ethanol for 2 min. After that, the slides were washed in tap water for 10 s. After the water hadbeen removed from the slides using tissue papers, the slides were kept in Harris hematoxylin stainfor 1 min. Then the slides were washed in tap water until clear. 0.5% acid alcohol was used for thedifferentiation of 2–3 quick dips. The nuclear stain was checked under the light microscope to ensurethe clarity of the nuclei. The slides were washed in water for ten dips followed by ten dips in twochanges of 95% ethanol. Immediately, the slides were placed in O-G-6 for 3 min. The

slides weredipped in two changes of 95% ethanol for ten dips each. After that, the slides were placed in EA-50for 4 min. The slides were dipped in three changes of 95% ethanol for ten dips each. Then, the slideswere dipped in three changes of absolute ethanol for ten dips each. The slides were dipped in threechanges of xylene for 15 dips each. Finally, the slides were mounted in DPX.

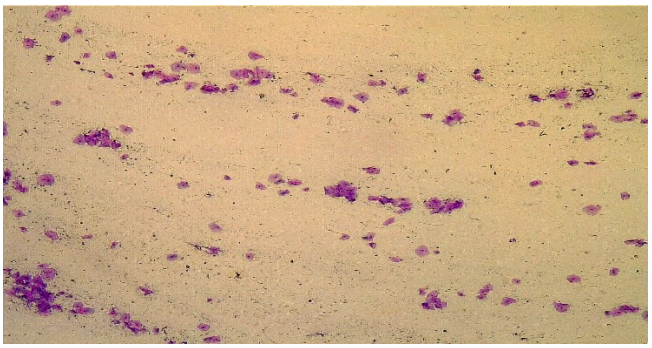


Figure :1 photomicrograph showing of H&E stained slides of Patient having oral potentially disorders (4x resolution)

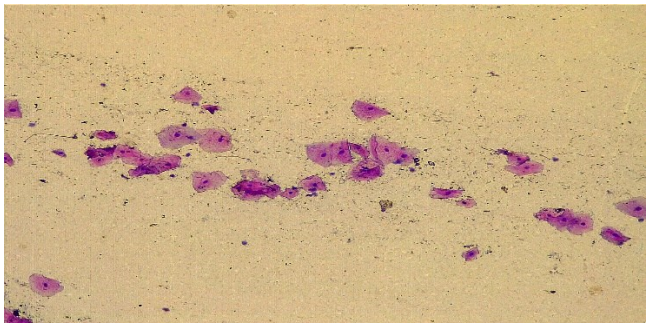


Figure :2photomicrograph showing of H&E stained slides of Patient having oral potentially disorders (10x resolution)

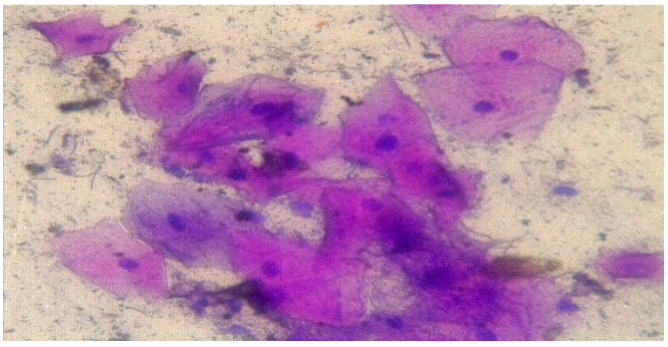


Figure :3photomicrograph showing of H&E stainedslides of Patient having oral potentially disorders (40x resolution)

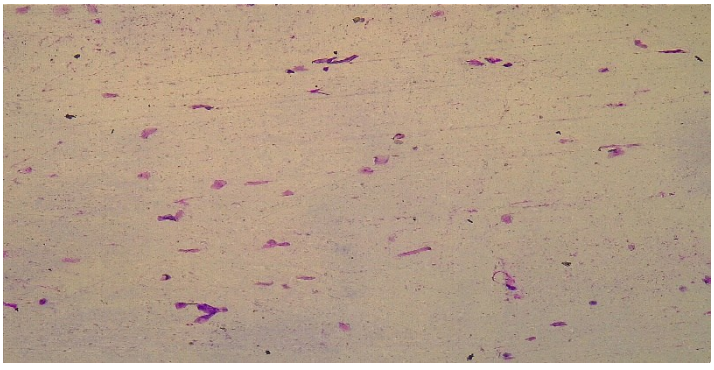


Figure :4photomicrograph showing ofLeishman-Giemsastainedslides of

Patient having oral potentially disorders (4x resolution)

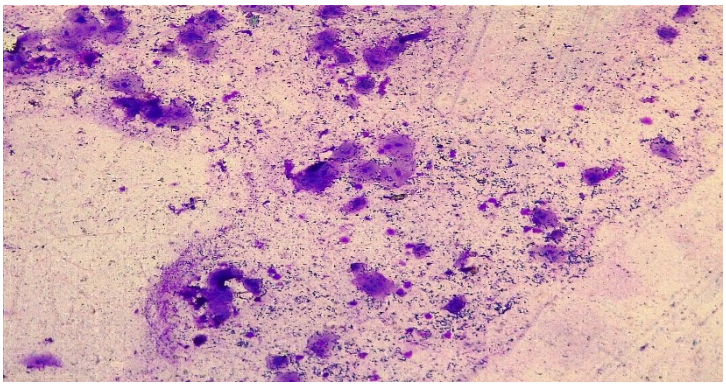


Figure :5photomicrograph showing ofLeishman-Giemsa -stained slides of Patient having oral potentially disorders (10x resolution)

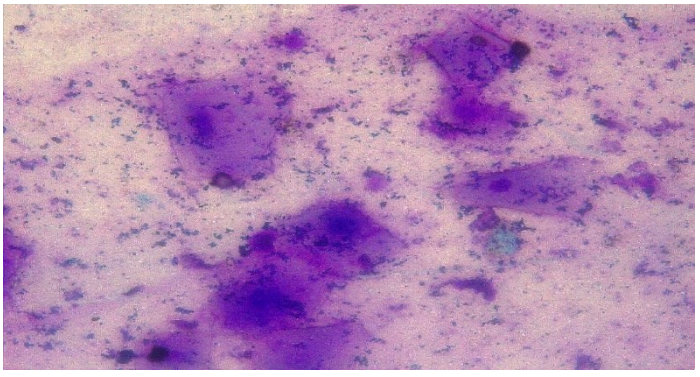


Figure :6photomicrograph showing of Leishman-Giemsa-stained slides of Patient having oral potentially disorders (40x resolution)

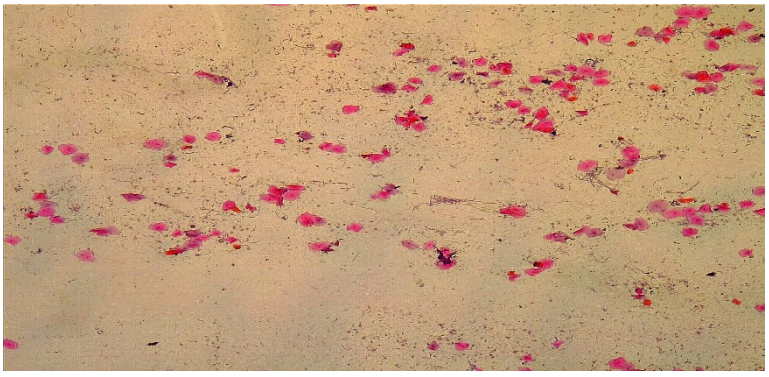


Figure :7photomicrograph showing of PAP -stained slides of Patient having oral potentially disorders (4x resolution)

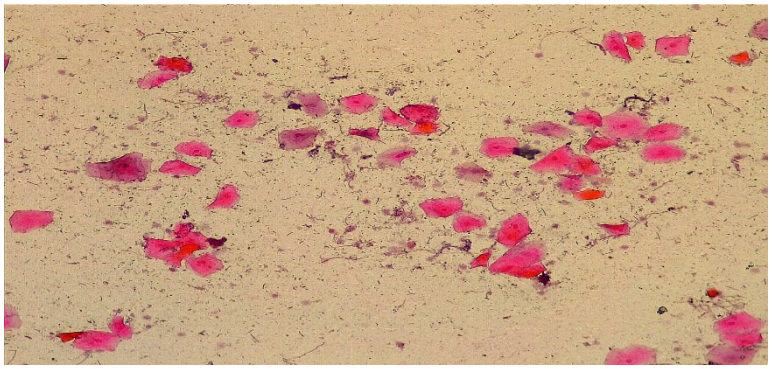


Figure :8photomicrograph showing of PAP-stained slides of
Patient having oral potentially disorders (10x resolution)

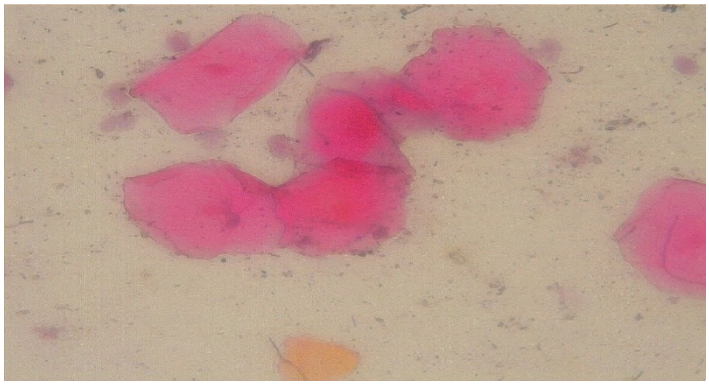


Figure :9 photomicrograph showing of PAP -stained slides of
Patient having oral potentially disorders (40x resolution)

Table 1:

Semi quantitative analysis parameters

PARAMETERS	**SCORE**
Cytoplasmic details:	
Not preserved	0
Non-transparent masking of nuclear details	1
Non-transparent with intact cell membrane 2	2
Transparent, intact cell membrane without masking of nuclear details	3
Nuclear details:	
Poor preservation	0
Smudgy	1
Fair preservation but chromatin granularity	2
Excellent preservation with crisp chromatin	3
Background staining:	
Intensely stained obscuring cellular details Moderately stained with better cellular details	0 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

2. Statistical analysis

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. Inter observer reliability was calculated by Cohen's Kappa. To check and quantify the reliabilityof the stains, sensitivity, specificity value and diagnostic accuracy were assessed. The Receiveroperating characteristic (ROC) curve plotted with sensitivity along Y-axis and 1-specificityalong X-axis for each stain.

3. Observations &result

The analyses of thecytosmears obtained inthe present studyshowed comparatively significant results withPAP which was goldstandard for cytoplasmicstaining and nuclear
details. Clearbackground was seen inPAP stain in the studygraph but thebackground of both LGand H & E seems to beconsistent with the score(1.80 ± 0.76) and (1.66± 0.75) (Table 2).Tomake a definitivediagnosis in OPMD bothLG and H &E showedstatistically significant (P≤ 0.001) equally good with the score (1.13 ± 0.73 *) and (1.06 ± 0.58*)respectively in comparison to PAP.

Table 2:

Semi- quantitative analysis of cytological parameters in oral potentially malignant disorder cases and control case

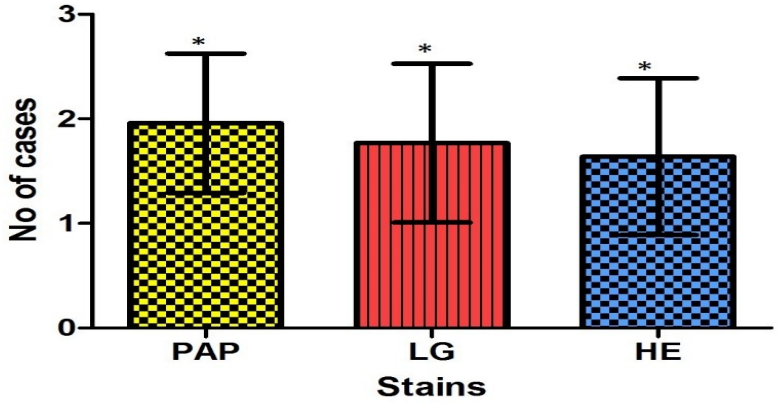
Parameter / Stain	Cases	Cytoplasmic details (Mean ± SD)	Nuclear detail (Mean ± SD)	Background (Mean ± SD)
Papanicolaou Stain (PAP)	OPMD	2.16 ± 0.698	2.10 ± 0.80	2.50 ± 0.68
	CONTROL	2.36 ± 0.614	2.26 ± 0.691	1.86 ± 0.62
Leishman-Giemsa Stain (L-G)	OPMD	1.63 ± 1.098	1.23 ± 0.85	1.80 ± 0.76
	CONTROL	1.73 ± 1.014	1.90 ± 0.71	1.96 ± 0.55
Hematoxylin-Eosin Stain (H &E)	OPMD	1.56 ± 0.89	2.1 ± 0.66	1.66 ± 0.75
	CONTROL	1.53 ± 0.81	1.36 ± 0.96	1.86 ±0.57

Table: 3

Correlation of cyto-pathological diagnosis

Parameter / Stain	Cases	Ability to make a definite diagnosis (Mean ± SD)	P- Value
Papanicolaou Stain (PAP)	OPMD	0.85 ±2.23 *	< 0.001
	CONTROL	0.81 ±1.60 *	
Leishman-Giemsa Stain (L-G)	OPMD	1.56 ±0.77 *	<0.001
	CONTROL	2.20 ±0.76 *	
Hematoxylin-Eosin Stain (H & E)	OPMD	1.86 ±0.77 *	<0.001
	CONTROL	2.20±0.88 *	

Graph 1



Correlation of cyto-pathological diagnosis

Receiver operating characteristic (roc) curve: the roc curve plotted showed pap has the
Maximum area under the curve that is (0.703). 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

In oral potentially malignant lesions. Area under the curve of lg scored 0.702 which was found
To be higher than h & e with the area under the curve (0.618). The result showed lg to be a
Better than H&E (p>0.05). Whereas lg and h &e stain showed the comparatively same areaunder the curve with respect to pap and the result were found to be statistically significant(p<0.05) indicating that both the stains have the same diagnostic value as compared to the othergold standard stains in observing cellular and nuclear parameters in oral cytological smears(graph 2, table 4). Roc curve has shown to be an excellent statistical test in the present study toevaluate the overall accuracy of a diagnostic test i.e., different stains.

Graph 2:

Roc curve for diagnostic efficacy of the studied stain.

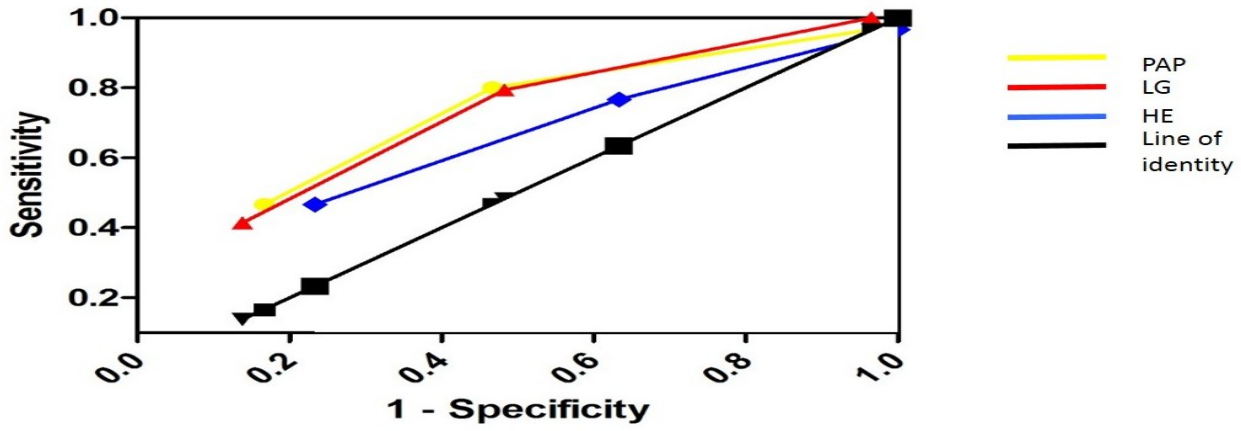


Table 4:

Diagnostic efficacy of the studied stain

Stain	AUC	Sensitivity	Specificity	Cut-off	P value
Papanicolaou Stain (PAP)	0.703	46%	86%	> 2.500	0.005
Leishman-Giemsa Stain (L-G)	0.702	41%	83%	> 2.500	0.005
Hematoxylin-Eosin Stain (H & E)	0.618	46%	76%	> 2.500	0.005

The reliability of the stains was checked and quantified. PAP stain showed better value in
relation to sensitivity, specificity, and diagnostic accuracy than other two other stains. In
evaluating the diagnostic accuracy, PAP showed to have 86% diagnostic accuracy with LG stain
between the presence or absence of a disease (Table 5).
with the score of 83% comparable with PAP and H&E score is 76% which little less
comparable with PAP. Thus, PAP showed to have the maximum comparable result to classify

Table 5:

Kappa analysis for showing the extent of agreement between two observers. Correlation is significant at the p < 0.005 level

Parameter / Observers	Ability to make a definite diagnosis	P value
Observer 1 vs 2 (PAP)	0.94	.001
Observer 1 vs 2 (LG)	0.96	.001
Observer 1 vs 2 (H&E)	0.88	.004

Value of Kappa	Level of Agreement	% of Data that are Reliable
0-.20	None	0-4%
.21-.39	Minimal	4-15%
.40-.59	Weak	15-35%
.60-.79	Moderate	35-63%
.80-.90	Strong	64-81%
Above .90	Almost Perfect	82-100%

DISCUSSION

In our study we found that staining specificity of PAP was 86% in compare to LG83% and H&E 76% regarding cytoplasmic details, nuclear details and background staining. Also, in seeing the accuracy, the results of H &E and LG are found to be comparable with PAP. The Receiver operating characteristic (ROC) curve was

also plotted for the three 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 0.703 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 positivity or fair accuracy in diagnosis. As this curve gives a clear picture and appropriate comparison, it should be used more often for such comparative studies. But LG stain (AUC 0.702 and H &E stain (AUC 0.618) also had ROC value comparable with PAP which can be interpreted that they also have the accurate diagnostic value in comparison to PAP. It can be concluded from the present study that the staining characteristics of PAP proved to be better out of the two stains i.e. H&E and LG respectively. But they were found to give results comparable to the PAP. Hence, keeping in mind the advantages of stains as a single step, cost-effective procedure the study also supports the idea of utilizing which stain in oral exfoliative cytology for PMDs and OSCC.On the cytological diagnosis with 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.[10] and Gabyal et al.[9]. In our study we found LG is more comparable to PAP.In the study of Maumita B et al. [17] PAP showed to have the maximum positive predictivevalue that is (96.2%) showing its efficiency to correctly tell the positive test result actually hasthe 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, In ourspresent study for cytoplasmic staining PAP (2.16 ± 0.698), LG (1.63± 1.098) and H&E (1.56± 0.89) and in evaluating nuclear details PAP (2.10±0.80), LG (1.23± 0.85) and H&E (2.1± 0.66) gave better results in comparison with PAP, as it stains nuclear chromatin well, gives good differential cytoplasmic counterstaining and produces good cytoplasmic transparency. LG (1.80± 0.76) and H &E (1.66 ± 0.75) stain also showed better background staining obscure the background material and also the cellular details in comparison to PAP (2.50 ± 0.68). The
qualitative assessment of three stains was done by scoring on parameters of ability make a definite diagnosis 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 two observers was found in the parameter on 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. In our study Kappa analysis PAP stain showed better value in relation to sensitivity, specificity, and diagnostic accuracy than other two other stains. In evaluating the diagnostic accuracy, PAP showed to have 86% diagnostic accuracy with LG stain with the score of 83% comparable with PAP and H & E.

Summary

As we summarized our data among the 50 subjects 19(38%) were males and 31(62%) were females. The minimum age of the study subjects was 18 years and the maximum age was 85 years.

- Among the 50 subjects 20(40%) had the habit of smoking, 28(56%) had the habit ofchewing, 7(9.3%) had the habit of chewing and smoking, 3(4%) had the habit of smoking and alcohol consumption and 4(5.3%) had all three habits together with a p-value of which is statistically significant. Thus, a positive correlation between smoking, chewing, alcohol consumption and development of precancer and cancer has been established.
- The most common sites for leukoplakia were in the retro-commissure area with 5 (45.5%) subjects followed by buccal mucosa 5(45.5%) and one in floor of the mouth.
- The present study showed all cases of OSMF in the buccal mucosa 6(42.8%) in Grade III and 4 each in Grades I and IV and none in Grade II.
- Among the total of 30 (60%) subjects, 19(38%) had lesions in the buccal mucosa, 11(22%) had in the tongue, 4(8%) had in the palate, 5(10%) had in gingivo-buccal sulcus of mouth and 5(10%) in the lip and 6(12%) in retro-molar area. OPMD was mostly seenin the buccal mucosa, followed by tongue, lip, gingivo-buccal mucosa and palate.

CONSLUSION

Theseresearch projecton comparative study on the Leishman-Giemsa stains, Hematoxylin-Eosin; Papanicolaou stains for Cytological Diagnosis of Oral Lesions was conducted in the Department of Oral Maxillo-facial pathology, Awadh Dental College and Hospital, Jamshedpur, Jharkhand to compare and correlate the efficacy of LG, H&E and PAP stains on cytopathological diagnosis of various potentially malignant lesions in comparison to healthy individuals and to determine the efficacy of stains compare to each other in a very cost-effective way and less infrastructural supports.

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CONSENT FORM (for the subject/patient)

I, ...hereby voluntarily give my free and whole hearted consent for myself/my ward,....., to participate in the Clinical Study on *"Comparative Study on the Leishman-Giemsa Stains, Hematoxylin-Eosin & Papanicolaou Stains for Cytological Diagnosis of Oral Lesions"*. The aims and objectives and details of the methodology of the said study have been adequately explained to me by the investigator to the fullest of my satisfactory and understanding. I have the high regards for professional capacity and ethical values to the investigator.

I am at liberty to withdraw myself/ my ward from the study at any moment when I wish.

Signature of the Patient/Guardian

Relation:

Date:

सहमति फार्म (विषय / रोगी के लिए)

मैं,

..... फिर से स्वेच्छा से अपने और अपने वार्ड के लिए अपनी स्वतंत्र और पूरी दिल से सहमति... .....।, "लीशमैन-गिमेसा के दाग पर तुलनात्मक अध्ययन, हेमेटोक्सिलिन-एओसिन और पापेनिकोलाउ दाग पर मौखिक घावों के रोग निदान के लिए तुलनात्मक अध्ययन" पर भाग लेने के लिए। उक्त अध्ययन की कार्यपद्धति के उद्देश्य और उद्देश्य और विवरण मुझे मेरे संतोषजनक और समझ के अनुसार अन्वेषक द्वारा पर्याप्त रूप से समझाया गया है। मेरे पास पेशेवर क्षमता और अन्वेषक के लिए नैतिक मूल्यों के लिए उच्च संबंध हैं।

मैं अपनी इच्छा से किसी भी क्षण अध्ययन से खुद को / मेरे वार्ड को वापस लेने के लिए स्वतंत्र हूं।

रोगी / अभिभावक का हस्ताक्षर

रिश्ता:

दिनांक: