ORIGINAL ARTICLE

Analysis of effectiveness of Leishman-Giemsa twin stain preparation in cytopathology: A novel step in laboratory quality assurance

Anandraj Vaithy K[1], E. S. Keerthika Sri[2], Prima Suchita Lakra[1], Harishkumar Rameshkumar Bohra[1], Sanjeet Kumar Singh[1]

[1]Department of Pathology/Lab Medicine, All India Institute of Medical Sciences, Deoghar- 814152 (Jharkhand), India, [2]Department of Pathology, Mahatma Gandhi Medical College & Research Institute, Sri Balaji Vidyapeeth (Deemed to be University), Puducherry- 607403, India

Abstract

Background: Cytopathology plays dynamic role with respect to diagnostic laboratories in terms of diagnosing broad disease categories and is the primary screening procedure for any superficial swelling of the body. While being daycare procedure with known advantages and limitations, optimal cytochemical stains and its quality forms vital component of any cytology laboratory. Studies have been conducted to analyze standalone and combined cytological stains and to evaluate their efficacy based on standard quality parameters. *Aim and Objectives*: To undertake a novel analysis of two standard air-dried smears, namely standalone Giemsa stain and the Leishman –Giemsa (LG) cocktail, and to evaluate their impact on the effective diagnosis of cytological smears. *Material and Methods:* This prospective study was designed as a comparative observational study, with standard inclusion and exclusion criteria established. The LG working solution was prepared according to standard protocols and existing literature, and its efficacy was compared with that of the routine standalone Giemsa stain based on smear quality parameters. The collected data were tabulated for analysis and assessment of the Quality Index (QI). *Results:* The study evaluated 80 cases comprising body fluids and cytology aspirates (cystic fluids). Among the fluid samples, pleural fluid accounted for the highest number of cases (24), followed by Bronchoalveolar Lavage (BAL) samples (18) and ascitic fluid (13). The specimens were obtained from a wide age range, spanning from pediatric to adult patients, with the highest incidence observed in the fifth and sixth decades of life. The male-to-female ratio was 3:1. The QI of LG cocktail was 0.95 and that of standalone Giemsa stain was 0.50. *Conclusion:* Effective combination of the LG cocktail proved to be more effective than standalone Giemsa stain air-dried cytology smears, especially for cellular differentiation, background clarity and optimal staining quality parameters.

Keywords: Air-dried cytosmears, Leishman-Giemsa cocktail, Giemsa stain, staining quality, cellular differentiation

Introduction

Cytopathology plays a vital role in effective analysis of body fluids, peripheral blood smears and exfoliative cytology providing early and prompt diagnosis [1, 2]. Being a minimally invasive test, the efficacy in terms of sensitivity and specificity varies based on several attrition factors such as nature of fluid sample, fixation time, technical skills, underlying pathology with confounders, warranting further exploration [2].

Although cytochemical stains remain a cornerstone for the accurate evaluation of cytosmears, they require periodic quality assessment [3]. Several studies have been undertaken to evaluate the efficacy of standalone cytological stains as well as

combination stains in parallel [3]. Among the several staining strategies, Leishman-Giemsa (LG) cocktail and May-Grünwald-Giemsa (MGG) are outstanding techniques routinely utilized for peripheral blood film and cytology smears examination [4, 5]. These stains feature essential equipment for visualizing cell components, diagnosing malignancy, parasitic infections and several other conditions [6, 7].

In the Indian context, the LG cocktail is specifically utilized in identification of parasites on blood films and its role in cytosmears are still sub-optimal [8]. MGG stain is widely used for cytological assessment followed by categorization and blood cellular differentiation [8, 9]. Although each stain plays a crucial role in diagnostic evaluation, there is a need to assess their comparative effectiveness, particularly when applied to air-dried cytology smears [10]. The standalone air-dried smear approach is regularly used for its ease of staining, but it could pose challenges for stain absorption and cellular visualization [11, 12]. This broadens the scope for evaluating the combined cytostain, namely the LG stain, and comparing its performance with the MGG stain in such smears, particularly in terms of lesion identification, cellular morphology, background characteristics, and turnaround time [13, 14]. This study aimed to analyze the nuances of the combined staining strategies and their effectiveness in air-dried cytology smears, offering a deeper understanding of which stain gives better diagnostic effects in this precise putting.

Material and Methods

Study design

This prospective study was conducted for a period of six months after receiving institutional ethical clearance. The effectiveness of cytochemical stain was evaluated primarily based on numerous parameters such as staining quality, differentiation of cells, cytoplasmic and nuclear clarity, and background nature. Standard inclusion and exclusion criteria were fixed wherein fluid sample less than 2 ml and crucial samples were excluded from the study. All the body fluids received were centrifuged and processed with routine standard operating procedure of the laboratory and the reports were dispatched to the respective end users. The patients' age, nature of sample, quantity, and site of aspiration were documented in a structured proforma.

Sampling technique and sample size

The study design being paired comparative observational study, a convenience sample technique was used. Following the application of predetermined inclusion and exclusion criteria, a total of 80 consecutive cytological samples received at the department of pathology during the study period were included. Sixty-three body fluid samples and 17 Fine-Needle Aspiration Cytology (FNAC) samples made up the study material. As part of standard diagnostic procedures, all samples were processed. In order to reduce patient-related bias, the residual sample material that would have been thrown away after diagnostic requirements were met was used for the study. The same specimen was used to create matched air-dried smears for each pair. MGG stain was used to stain one smear and the LG cocktail was used to stain the corresponding paired smear. By using each specimen as its own control, this paired design narrowed the inter-sample variability by enabling direct intra-sample comparison. The analysis did not include samples with poor cellularity, or substandard smear quality. The sample size of 80 cases was determined based on feasibility, specimen availability, and consistency with previously published cytopathology studies evaluating the efficacy of the LG cocktail

staining technique. For instance, Kavya *et al*. (2021) [8] analyzed approximately 70 paired cytological samples, which was taken into consideration while finalizing the sample size. A formal power-based sample size calculation was overlooked as the current study was a laboratory-based method-comparison study that focused on qualitative staining factors rather than outcome estimation or prevalence.

Preparation of working stock solution

The LG cocktail working solution was prepared according to standard protocols and previous literature. As described by Kavya *et al*. (2021) [8], filtered Leishman stain was mixed with an equal volume of Giemsa stain and diluted with distilled water in a 1:1 proportion. The additional air dried slides were prepared and LG cocktail was added and kept for one to two minutes and standard buffer was added and stained for further six minutes and washed and dried for further analysis.

Evaluation parameter and Quality Index (QI)

For calculation of QI, parameters such as overall staining pattern and clarity, cell morphology based on degree of differentiation of cytoplasmic as per prior work done by Kavya *et al.* [8].

For the individual scoring index, each parameter was classified into three levels of quality—satisfactory, good, and excellent—scored 1 to 3, respectively, along with the number of cases in each category. The quality parameters of the cytosmears were evaluated by experienced cytopathologists with reference to standard textbooks. The actual score under each parameter was calculated by adding scores obtained with respect to observed grade with number of cases in reference to prior studies [8] The final QI was calculated by comparing the actual scores obtained for each parameter against the maximum possible score. The data were tabulated for analysis, and the final QI of the MGG stain was compared with that of the LG cocktail.

Results

This prospective study evaluated 80 cases, including body fluids and cytology aspirates (cystic fluids). The comparative analysis of the LG cocktail stain versus the standalone MGG stain was conducted following the methodology outlined by Kavya *et al*. (2021) [8]. Among the body fluid samples, pleural effusion was the most common (24 cases), followed by Bronchoalveolar Lavage (BAL) samples (18) and ascitic fluid (13). Other fluids, including synovial fluid, urine, and sputum, each accounted for one case, as summarized in Table 1. The cystic fluid from thyroid aspirate was commoner followed by degenerative fluid from breast lesions (6) cases. The samples were received from wide range of age group from pediatric to adult with highest range noted in the 5th and 6th decade with male to female ratio of 3:1 as shown in Table 2. Of the 80 cases, 15 lacked sufficient diagnostic material and could not be included in the analytic scoring. The remaining 65 cases, which had adequate material, were evaluated and scored accordingly. The overall staining, cytoplasmic staining and nuclear morphology staining was good with LG cocktail when compared to MGG stain (Table 3). The QI of LG cocktail was 0.95 and that of standalone MGG was 0.51. The differentiation between myoepithelial and ductal epithelial cells in the breast were well made out in the LG stain.

Table 1: Nature of specimens

Type of specimens	Number of cases n (%)
Fluids (n=63)	
Pleural fluid	24 (29.62)
Bronchioalveolar lavage	18 (22.22)
Ascitic fluid	13 (16.04)
Peritoneal fluid	1 (1.23)
Cystic fluid	2 (2.46)
Pericardial effusion	2 (2.46)
Others*	4 (4.93)
FNAC (n=17)	
Thyroid	8 (9.87)
Lymph node	1 (1.23)
Breast	6 (7.40)
Oropharyngeal growth	1 (1.23)
Sub pleural nodule	1 (1.23)
Total	**81 (100)**

**Sputum, synovial fluid & urine each constituted one case*

Table 2: Age and gender wise distribution of study samples

Age group (Year)	Male n (%)	Female n (%)
11-20	1 (1.58)	2 (11.76)
21-30	3 (3.70)	3 (17.64)
31-40	6 (9.52)	4 (23.52)
41-50	10 (15.87)	1 (5.88)
51-60	17 (26.98)	1 (5.88)
61-70	18 (28.57)	3 (17.64)
>70	8 (12.69)	3 (17.64)
Total	**63 (100)**	**17 (100)**

Table 3: Comparison of quality parameters scores and index between standalone MGG and LG cocktail

Parameters	MGG (no. of cases × score)	LG Cocktail (no. of cases × score)
Overall staining		
Satisfactory (1)	2×1=2	0×1=0
Good(2)	61×2=122	64×2=128
Excellent(3)	2×3=6	1×3=3
Score	120	151
Clarity of staining		
Satisfactory (1)	1×1=1	0×1=0
Good(2)	56×2=112	57×2=114

Continued...

Excellent(3)	8×3=24	8×3=24
Score	117	178
Cytoplasmic staining		
Satisfactory (1)	10×1=10	2×1=2
Good(2)	48×2=96	58×2=116
Excellent(3)	7×3=21	5×3=15
Score	105	173
Nuclear staining		
Satisfactory (1)	17×1=17	1×1=1
Good(2)	45×2=90	58×2=116
Excellent(3)	3×3=9	6×3=18
Score	102	155
Background material staining		
Satisfactory (1)	0×1=0	1×1=1
Good(2)	55×2=110	52×2=104
Excellent(3)	10×3=30	12×3=36
Score	115	181
Actual score obtained (Z)	560	880
Maximum possible score (N)	975	975
Quality index (Z/N)	**0.51**	**0.95**

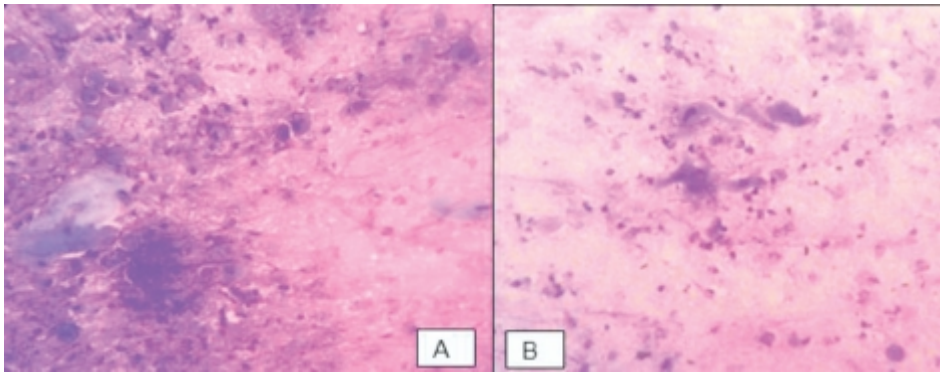

Figure 1A:Giemsa standalone with poor cytoplasmic & nuclear clarity, MGG at 10×

Figure 1B:LG cocktail showing clear background and squamous cell carcinoma pleural fluid, LG stain at 10×

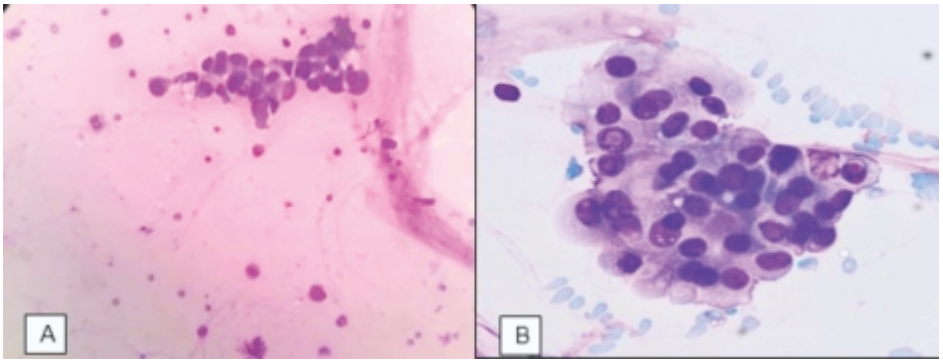

Figure 2A: Giemsa standalone with low clarity of inclusion, MGG at 10×

Figure 2B: LG cocktail showing clear inclusions with distinct cytoplasm of thyroid aspirate, LG stain at 40×

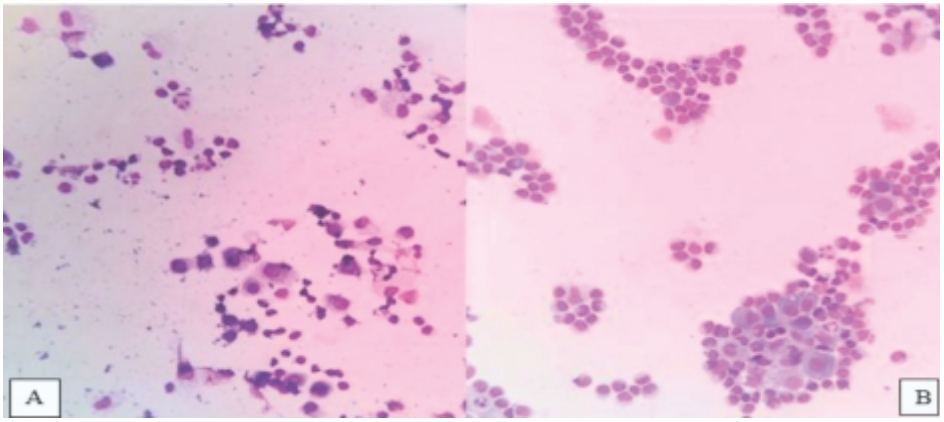

Figure 3A: Giemsa standalone with low clarity on cell morphology, MGG at 10×

Figure 3B: LG cocktail showing clear cells with distinct cytoplasm, LG stain at 40×

Discussion

Cytopathology is a specialized domain in pathology and it is an often preferred technique in sampling owing to its less invasiveness and easy daycare procedure. As a widely used screening technique, it is influenced by several attrition factors, with the quality and efficacy of cytochemical stains playing a key role in achieving an accurate and reliable report [1, 2]. Romanowsky stains encompassing a spectrum of cytochemical stains possess diversified nature of staining characterization when used either as a standalone or with combination of other air-dried dyes[2, 3]. The perennial lacunae with readily available stains is their volatility and variable efficacy which was superseded by conventional stains such as Giemsa, Leishman, and MGM stains [4, 5]. In the Indian context, previous studies assessing the quality and efficacy of cytological stains have employed experimental designs for sampling. In the present study, a convenience sampling technique was used, in line with the approach of Sidhu *et al.* (2018) [6]. As recommended by the National Accreditation Board for

Laboratories (NABL), in any quality assurance analysis during the pre-analytical phase, both the sampling method and the quality of staining are critical factors in determining the outcome and overall efficiency [7-9].

While cytopathologist's expertise plays a vital role in determining the sampling methodology, grading and scoring the QI and its parameters depends on the staining composition and techniques [10-12]. While Leishman stain is a cost-effective stain, it has its own limitation when it comes to cytosmears owing to its nuclear binding and background deposits [13-15]. This limitation was overcome by Giemsa stain wherein the combination of LG cocktail is being used widely in recent times. Ensuring optimal Turnaround Time (TAT) by minimal staining time, LG cocktail enhances QI in hematology as well as in cytosmears as observed by Jani & Satav (2012) [16]. While LG cocktail application on cytosmears is still underrated compared to Wright-Giemsa combination across the laboratories, studies have been conducted to assess its utilities [17-20]. High background intensity obstructing with visualization was another drawback with standalone Giemsa stain which was effectively overcome by LG cocktail as observed in prior studies as well [18-20]. The higher QI observed for the LG cocktail is attributed to its ability to effectively stain cells at different stages of maturation, including polymorphic and mixed reactive cells, as noted by Shilpa *et al*. (2017) [21]. Studies by Kavya *et al*. (2021) [8] and Sahu *et al*. (2024) [25] assessed the QI of the LG cocktail in 90 cases, consistent with our study analysis. Similarly, Joshi *et al*. (2014) [17] evaluated 200 body fluid samples over a five-year period and reported that the LG cocktail was particularly effective in highlighting background material, distinguishing cell types in salivary gland lesions, visualizing granules, and demonstrating metachromasia. Comparable findings were observed in the present study, where the LG cocktail showed a high QI in pleural and ascitic fluids by effectively demonstrating three-dimensional clusters, cellular patterns, and nuclear and chromatin architecture. Thus, LG cocktail was beneficial in assessing primary versus metastatic effusion on cytosmears. Although the Giemsa stain demonstrated substantial scores for cytoplasmic staining, particularly in salivary gland and BAL samples, factors such as background deposits, enlarged nuclear features, and drying artifacts limited its performance, often complicating the diagnosis and classification of malignant effusions.

The major component of analysis in our study was QI which was calculated by the ratio of actual score obtained on the test score stain (LG) with maximum score possible in reference to prior literature [22-24]. The cytology slides were scanned and the scores were awarded based on quality parameters as per literature and studies done by Sahu *et al*. (2024) [25]. Although several authors have reported varying QI scores—typically nuclear features scoring higher than cytoplasmic and background characteristics—with both standalone Giemsa and LG cocktail stains, the present study demonstrated a QI of 0.95 for the LG cocktail compared to 0.5 for the standalone Giemsa stain, indicating nearly a twofold increase in QI. Thus the rationale use of LG cocktail on cytosmears could be implemented across laboratories concurring with the findings of previous studies [26-30].

Another additional observation in the study was enhanced specificity on the International System for Reporting Serous Fluid Cytopathology (ISRSFC) wherein the proportion of Atypia of Undetermined Significance (AUS) in category III was high in

standalone Giemsa stain whereas LG cocktail clearly delineated malignancy versus benign effusions thereby providing a clear management guideline to clinicians. The higher quality index is attributed to the optimal visualization of nuclear parameters and their contents, which facilitates clear delineation of cytomorphology and aids in distinguishing benign from malignant lesions. As category III often warrants repeat sampling or invasive procedures to ascertain the diagnosis, LG cocktail with its enhanced QI had substantially aided in categorizing the fluids as observed in previous studies as well

Limitations

The present study evaluated the LG stain in comparison with Giemsa on air-dried smears and primarily focused on body fluids rather than cystic fluids. Future research may expand the scope to include other air-dried staining techniques, such as Wright's stain, and incorporate a more stratified and larger sample size for comprehensive analysis.

Conclusion

LG cocktail stain is beneficial when compared to standalone Giemsa stain in terms of better QI and parameters aiding enhanced quality assurance for cytology laboratories. The proportion of skeptical diagnostic entities in serous fluids can be minimized by effective staining of LG cocktail. Given its cost-effectiveness, optimal staining efficiency, and reduced staining time, the LG stain can be recommended for use in under-resourced laboratory settings.

References

1. Garbyal RS, Agarwal N, Kumar P. Leishman-Giemsa cocktail: an effective Romanowsky stain for air-dried cytologic smears. *Acta Cytol* 2006; 50(4):403-406.
2. Doddagowda SM, Shashidhar HA, Prasad CSBR. Leishman-Giemsa cocktail – is it an effective stain for air dried cytology smears. *J Clin Diagn Res* 2017; 11(3):EC16-EC18.
3. Gajendra S, Jha B, Goel S, Sahni T, Sharma R, Shariq M, Jaiswal S, Sachdev R. Leishman and Giemsa stain: a new reliable staining technique for blood/bone marrow smears. *Int J Lab Hematol* 2015; 37(6):774-782.
4. Gupta J, Gupta K, Agarwal R. Comparison of different stains in exfoliated oral mucosal cell micronucleus of potentially malignant disorders of oral cavity. *J Cancer Res Ther* 2019; 15(3):615-619.
5. Metgud R, Naik S, Patel S. Spritzer: For diagnostic cytopathology. *J Cancer Res Ther* 2017; 13(6): 964-967.
6. Sidhu SK, Ramalingam K, Goyal S, Poonia M, Rajawat GS, Sharma N. Comparing the efficacy of Leishman-Giemsa cocktail stain, Giemsa stain, and Papanicolaou stain in potentially malignant oral lesions: A study on 540 cytological samples. *J Cytol* 2018; 35(2):105-109.
7. Akhlaghi A, Ahmadi-Hamedani M. Introducing a combined Leishman-Giemsa stain as a new staining technique for avian blood smears. *Iran J Vet Res* 2019; 20(2):147-150.
8. Kavya PR, Abraham S, Padmakumar V, Raveendran Pillai KP. Cellular composition and cytomorphological characteristics of periodontal pockets of patients with periodontitis using Leishman-Giemsa cocktail staining. *Diagn Cytopathol* 2021; 49(4):518-527.
9. Belgaumi U, Shetty P. Leishman Giemsa cocktail as a new, potentially useful cytological technique comparable to Papanicolaou staining for oral cancer diagnosis. *J Cytol* 2013; 30(1):18-22.
10. Nivedhita G, Brundha M. Analysis of Papanicolaou stain on peripheral smear compared to Leishman's stain: A prospective study. *Int J Clinicopathol Correl* 2020; 4(2):40-45.
11. Srilatha T, Singh J, Raja D, Jain T, Chandra A, Agrawal R. Comparative analysis of staining efficacy of Leishman's stain with hematoxylin and eosin stain in the assessment of keratin pearl formation and mitotic figures in different grades of oral squamous cell carcinoma. *SRM J Res Dent Sci* 2022; 13(2):49-53.
12. Sareen R, Kapil M, Gupta GN. Incubation and its effect on Leishman stain. *J Lab Physicians* 2018; 10(3): 357-361.
13. Barcia JJ. The Giemsa stain: its history and applications. *Int J Surg Pathol* 2007; 15(3):292-296.

14. Vaithy KA, KS, Anandhani G, Kotasthane DS. Analysis of quality assurance: a novel step in fixing the turn-around time of surgical pathology reports as a part of quality management system. *IP J Diagn Pathol Oncol* 2017; 2(4):100-103.
15. Dhotre PS, Dhotre SV, Shaikh AKAR. A comparative study of pre-analytical errors in central clinical laboratory in a tertiary care hospital in Maharashtra. *J Krishna Inst Med Sci Univ* 2020; 9(2): 67-72.
16. Jani P, Satav V. Revolutionizing traditional pathology practice in today's computer age. *J Krishna Inst Med Sci Univ* 2012; 1(2):163-167.
17. Joshi A, Mahajan N, Karmarkar PJ, Mahore SD. Diagnostic utility of various techniques used in body fluid cytology. *IOSR J Dent Med Sci* 2014; 13(1):13-18.
18. Ramana PV, Padma S, Kante R, Kumar OS. Shravan Kumar. A comparative study of staining characteristics of Leishman-Geimsacocktail and Papanicolaou stain in cervical cytology. *Asian Pac J Health Sci* 2018; 5(3):233 -236.
19. David A, Kana S, Basu D. Standardization of Leishman-Giemsa cocktail stain and its comparison with conventional Leishman and Giemsa stains in peripheral blood and bone marrow aspirate smears. *Int J Lab Hematol* 2022; 44(4): e181-e184.
20. Liyanage TG. A quick and a cost-effective stain for cytology; 12 years of experience in using Leishman stain. *Galle Med J* 2023; 27(3):105-108.
21. Shilpa M, Hemalatha S, Sridhar C. Leishman-Giemsa cocktail - Is it an effective stain for air dried cytology smears. *J Clin Diagn Res* 2017; 11(3): 16-18.
22. Mishra TR, Mishra P, Dash M, Panigrahi KR. Leishman Giemsa cocktail, a cytological staining comparable to papanicolaou stain for oral cancer diagnosis - A prospective observational study. *Student J Health Res Afr* 2025; 6(6): 5-9.
23. Agrawal A, Kumar K, Singh HC, Agrawal KC. Comparative analysis study of efficacy of Papanicolaou smear & Leishman-Giemsa cytological technique in the diagnosis of oral neoplastic lesions. *Int J Sci Res* 2019; 8(2):1601-1604.
24. Sathpathi S, Mohanty AK, Satpathi P, Mishra SK, Behera PK, Patel G, *et al*. Comparing Leishman and Giemsa staining for the assessment of peripheral blood smear preparations in a malaria-endemic region in India. *Malar J* 2022;13:51
25. Sahu A, Gharei R, Mishra TR, Panda S, Panda C. Leishman-giemsa cocktail, effective stain in air dried smear- An institutional study. *Panacea J Med Sci* 2024; 14(1):134-138.
26. Iqbal W, Tariq U, Javed S, Channa SA, Ahmed A, Surwaich A. Comparing the efficacy of Leishman –Giemsa cocktail stain, Giemsa stain, and Papanicolaou stain in potentially malignant disorders: A comparative study. *Prof Med J* 2024; 31(03):480-485.
27. Bose S, Mukherjee G, Roy A, Rai V. Evaluating an alternative cost effective protocol to screen and detect oral pre-cancerous and cancerous lesions. *Int J Appl Dent Sci* 2017; 3(4):178-184.
28. Jovanovic M, Zivkovic N, Gligorijevic N, Igic M, Petrovic M, Bojovic M, *et al*. Cytomorphometric and clinical changes in gingival tissue after subgingival tooth preparation-A pilot study. *Healthcare (Basel)* 2023; 11(3):414.
29. Mitra S, Bose S, Mukherjee G. Comparative studies on the Leishman Giemsa stains and Papanicolau stains for cytological diagnosis of oral lesion. *Sci Cult* 2011; 77(3-4):139-140.
30. Dey S, Misra V, Singh PA, Mishra S, Sharma N. Role of intraoperative imprint cytology in diagnosis of suspected ovarian neoplasms. *Asian Pac J Cancer Prev* 2020; 11(5):1389-1391.

***Author for Correspondence:**

Dr. Anandraj Vaithy K, Department of Pathology/Lab Medicine, All India Institute of Medical Sciences (AIIMS) Deoghar -814152, Jharkhand India
Email: kanandrv@gmail.com

How to cite this article:

Anandraj VK, Keerthika ESS, Lakra PS, Bohra HR, Singh SK. Analysis of effectiveness of Leishman-Giemsa twin stain preparation in cytopathology: A novel step in laboratory quality assurance. *J Krishna Inst Med Sci Univ* 2025; 14(4): 57-65

***Submitted:** 19-June-2025 **Accepted:** 26-Aug-2025 **Published:** 01-Oct-2025*