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Research Protocol

Pathology Section

A Cross-sectional Analysis of Conventional Leishman's Stain versus Modified Leishman's Stain: A Study Protocol

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ABSTRACT

Introduction: The conventional Leishman's stain and modified Leishman's stain are widely used in medical laboratories for the microscopic examination of blood smears. The conventional stain provides good visualisation of cellular morphology, aiding in the identification of various blood cell types. The modified Leishman's stain, on the other hand, incorporates alterations to enhance specific cellular structures, improving diagnostic accuracy.

Need of the study: In the present study, the authors will observe the differences between the conventional staining method and the modified Leishman's stain method, as well as the advantages of the modified Leishman's staining over the conventional staining method.

Aim: To compare Conventional Leishman's Stain with Modified Leishman's Stain.

Materials and Methods: The cross-sectional study will be conducted from October 2022 to October 2024 at the Department of Pathology, Jawaharlal Nehru Medical College (JNMC), Sawangi (Meghe), Wardha, Maharashtra, India. Ethylenediamine Tetraacetic Acid (EDTA) blood samples will be used for staining. Conventional Leishman's Stain employs the traditional Leishman's staining method, while Modified Leishman's Stain incorporates specific modifications aimed at improving staining quality and enhancing the detection of eosinophils and leukocytes. Several quality control parameters will be assessed, including staining quality, eosinophil and leukocyte visibility, and overall diagnostic accuracy for both peripheral blood smear samples. A panel of experienced haematologists, blinded to the staining technique, independently examines the slides to minimise potential bias. The numbers and morphological characteristics of eosinophils and leukocytes are meticulously recorded, and statistical analysis will be conducted to compare the two staining methods.

Keywords: Acidic, Basic, Leucocyte analysis, Microscopic analysis, Modified stain, Phenol, Staining efficiency

INTRODUCTION

The staining procedure has been named after the Russian Surgeon Dmitri Leonidovich Romanowsky (1861-1921). The nearest popular stain applied globally for staining blood films is the Romanowsky stain, which combines an acidic stain and a basic [1]. The initial Romanowsky method was adapted by William Boog Leishman's, a British pathologist, and is referred to as Leishman's stain. A variety of adjustments have been introduced to Romanowsky dyes, with Leishman's stain being central to several of the available modifications [2].

The two components, eosin Y (tetrabromofluorescein) and Azure B (trimethyl thionine), play a crucial role in the distinctive ability of Romanowsky stains to differentiate stains within all cellular granules [3]. Azure B is the favoured oxidative product derived from methylene blue and surpasses other azure dyes, making it the predominant component in Romanowsky stains. The initial Romanowsky mixture included a blend of polychrome methylene blue and eosin. The International Committee for Standardisation in Haematology recommended combining Azure B and eosin Y [4].

The advantage of Leishman's stain over Giemsa staining is that it can be easily prepared, is cost-effective, and the staining process is complete within 10-15 minutes. Few modifications in the preparation of Leishman's stain have been employed that can help reduce the staining duration of blood films [5]. Commercial production of Leishman's Stain involves the oxidation of methylene blue. However, even when meticulously controlled through spectrophotometric methods, it may not consistently yield reproducible outcomes. Inadequate oxidation can lead to irregular staining responses, presenting challenges in the morphological assessment of cells [6].

Traditional blood stains, such as the Giemsa stain, are part of the Romanowsky stain group and are valuable for routinely staining peripheral blood smears to study blood cell morphology, differential leukocytes, and aid in the diagnosis of various blood parasite infections. The conventional Giemsa stain blood smear examination under light microscopy continues to be the preferred method for diagnosing blood parasites in endemic regions. This approach offers informative, sensitive, reasonably affordable, permanent records, and can complement other disease control initiatives [7].

The method of fixing and staining preparations is similar to that employed in Leishman's process. First, immerse the preparations in the undiluted solution for one minute, then continue staining for an additional three minutes after adding between two to four volumes of distilled water. After this step, thoroughly rinse the films for about 20 seconds using distilled water, achieved by employing a wash-bottle. It is not necessary to let the water stand for a minute, as recommended by Leishman's, if the rinsing process is performed well. With only a modest amount of experience, you can generate clean and uniformly stained preparations [8].

For staining the blood smear, Leishman's stain is frequently utilised. The key stages in the process of staining a blood smear include applying the stain for fixing the smear, introducing fluid onto the stain for the staining process, and subsequently rinsing the smear [9].

The present study will mainly focus on the properties of Leishman's stain and how over time the staining techniques have been modified to give better results for examining patients' samples. Leishman's stain has been proven to be a landmark in staining history for cytological samples, which gives detailed knowledge of their internal structures. The modified Leishman's staining method offers advantages over the conventional Leishman's staining method as it

is cheap, easily available, shows rapid results, and is reliable. With time, authors have seen a modifications in the staining properties of Leishman's stain. In the present study, authors will observe the differences between the conventional staining method and the modified Leishman's stain method, as well as the advantages of the modified Leishman's staining over the conventional staining method.

The objectives of the present study was to compare Conventional Leishman's Stain with Modified Leishman's Stain and to compare the quality of staining between the two stains and to assess and compare the time needed for fixation and staining of Conventional and Modified Leishman's stains.

1. To compare the quality of staining between Conventional Leishman's stain and Modified Leishman's stain.
2. To assess and compare the time needed for fixation and staining of Conventional and Modified Leishman's stains.

REVIEW OF LITERATURE

Mathi A et al., conducted a study by adding phenol to traditional Leishman's stain, particularly at a concentration of 1:5. This simple modification allows for the creation of a modified Leishman's stain that can be used to stain peripheral blood smears faster [5].

Manmadhan AA et al., conducted a study motivated by the subpar quality of some standard stain brands. The updated approach offers three benefits over the traditional method: easy access to chemicals within the laboratory, cheaper ingredients, and quicker staining. Villanueva had poorer chromatin patterns and neutrophil granular staining than Leishman's. While Leishman's staining provided an overall better result, the current study's use of the modified stain allowed for the development of an alternative staining technique using laboratory-available reagents. The researchers believe that studying alternative staining techniques is important, particularly during crises like Coronavirus Disease-2019 (COVID-19), which can impact global chemical manufacturing and transportation. A major concern is ensuring an ongoing supply of high-quality stains [6].

Hye RA et al., conducted a study where the preferred stain for peripheral blood films was the Leishman's stain. The modified stain uses phenol's emphasising property to optimise staining differently. Phenol modifies the pH of the adapted Leishman's stain, enhancing its penetrability and halving the staining duration. Phenol and methanol, being polar organic compounds with terminal hydroxyl functional groups, exhibit greater reactivity than non polar organic substances. In this research, the outcomes of peripheral blood samples stained using both the Modified and Conventional Leishman's staining methods were compared. Based on the characteristics of the background pattern, platelets, neutrophil and eosinophil granules, the nucleus, and the Red Blood Cell (RBC) pattern, it was determined that smears stained with the modified stain yielded superior outcomes compared to the standard preparation. Furthermore, the modified approach is rapid, cost-effective, and reliable. Due to the simplicity and efficiency of the modified Leishman's stain procedure, it is expected to have a substantial impact on diagnostic haematology practice, reducing issues related to delayed peripheral smear reports for very ill patients [10].

Fasakin KA et al., conducted research on the use of modified Leishman's stain and its potential impact on diagnostic hematology practices. The conclusion is drawn based on the ease of revenue creation compared to the cost-effectiveness of the modified Leishman's stain, and the simplicity and practicality of the staining processes. Its usage in diagnostic haematology laboratories is supported by its stability, knowledge of how this unique dye functions, and its ability to stain large quantities of thin blood films. In busy private and public laboratories, batches of thin blood films can now be stained in between 75 and four minutes thanks to automated slide stainers. The implementation of these new rapid procedures is expected to significantly reduce the morbidity and mortality caused by late haematological findings, especially in hospitalised patients with serious illnesses [11].

Essgir PK and Anantharamaiah H, conducted a study to determine that in this investigation, the efficacy of altered Leishman's stains on days 1, 5, and 10 after stain preparation. Smears stained on day 10 displayed enhanced staining qualities, exhibiting a Quality Index (QI) of 0.89, in contrast to the values of 0.71 on day 1 and 0.73 on day 5, and were comparable to traditionally stained peripheral Leishman's smears. Thin peripheral blood smears can be stained in four minutes using a modified Leishman's stain [12].

Raghuveer CV et al., determined in their research that Peripheral Blood Smear (PBS) was outperformed by Medicare Current Beneficiary Survey (MCBS) and Quantitative Buffy Coat (QBC). The novel method, as sensitive as QBC and as focused as PBS, is capable of eliminating QBC's false positives. MCBS is also simple to use and reasonably priced. However, further research is necessary before MCBS can be confidently considered the new gold standard for diagnosing malaria [13].

Akhlaghi A and Ahmadi-Hamedani M, reported in their research the findings of the current investigation, which demonstrated for the first time that avian blood cells labelled with a novel L&G combination are more appealing. When used alone, it provides a greater nuclear and cytoplasmic differential staining than the standard Giemsa and Leishman's stains [14].

Sidhu SK et al., suggested in their study that the LG stain, together with the Rapid Papanicolaou (PAP) stain, could be employed as a crucial method in oral exfoliative cytology and be advised for the early detection and follow-up of patients with either a potentially malignant condition or oral malignancy [15].

Ahmed H et al., conducted a study demonstrating that Leishman's stain can be removed from clinical laboratory wastewater using natural clay from the Qulapalk region as an adsorbent. The adsorption process was discovered to follow a pseudo-second-order kinetic model, with intraparticle diffusion controlling the adsorption. The activation energy of 37.942 kJ/mol indicates an activated chemisorption process. The temperature dependence of the pseudo-second-order rate constants further supports the idea of an activated chemisorption mechanism. The adsorption was discovered to be spontaneous and endothermic, with values of 34.25 kJ/mol for enthalpy (H) and 145.98 J/mol for entropy (S). The experimental data were well-fitted by both the Freundlich and Langmuir models. The Langmuir isotherm was used to calculate the maximum adsorption capacity, which was found to be 455.37 mg/g at 25°C. Therefore, before introducing the slide washings from clinical labs to sewage, the Leishman's stain can be removed using natural clay from the Qulapalk area. More research on continuous systems (column systems) is necessary [16].

Srilatha T et al., showed the creation of keratin pearls and mitotic figures. Both H&E and Leishman's stains showed good overall staining intensity and specificity patterns. The keratin pearls and mitotic figures were more noticeably and intensely stained by Leishman's stain. However, H&E showed higher specificity [17].

MATERIALS AND METHODS

The cross-sectional study will be conducted from October 2022 to October 2024 at Department of Pathology, Jawaharlal Nehru Medical College (JNMC), Sawangi (Meghe), Wardha, Maharashtra, India. The study group will consist of 60 EDTA blood samples received in the Department of Pathology. These criteria ensure that the blood samples included in the study are relevant to the investigation of the morphology of red blood cells and meet quality standards for accurate and reliable results in the comparative analysis of Conventional Leishman's Stain and Modified Leishman's Stain in microscopic diagnostics. The study is approved by the

Institutional Ethics Committee of Datta Meghe Institute of Medical Sciences, Wardha, Approval no.- DMIMS(DU)/IEC/2022/104, Dated: 20/07/2022.

Inclusion criteria:

- Patients willing to participate in the study;
- Patients of age group 18 to 50 years with disease prevalence, diagnostic relevance, and consistent health status;
- Patients of both sexes will be included.

Exclusion criteria: Cases still in therapy, patients in emergencies, patients from ethnic minority groups, homeless persons, nomads, refugees, and minors were excluded from the study.

Sample size calculation: The sample size formula for the difference between two proportions [10] is as below:

$N=(Z\alpha/2+Z\beta)^2*(p1(1-p1)+p2(1-p2))/(p1-p2)^2$,

Where,

$Z\alpha/2$ is the critical value for the normal distribution at $\alpha/2$ (e.g., for a confidence level of 95%, α is 0.05 and the critical value is 1.96)=1.96.

$Z\beta$ is the critical value for the normal distribution at β (e.g., for a power of 80%, β is 0.2 and the critical value is 0.84)=0.84.

p1 and p2 are the sample proportions of the two groups.

p1=Proportion of acceptable neutrophil granules by the conventional method=85.9%=0.859.

p2=Proportion of acceptable neutrophil granules by the modified method=99%=0.99.

$N=(1.96+0.84)^2*(0.859(1-0.859)+0.99(1-0.99))/(0.859-0.99)^2=59.85=60$ participants needed in the study.

Preparation of peripheral blood smears: Two distinct smears will be prepared from each patient for contaminated or improperly collected specimens. To prepare a peripheral blood smear, place a drop of blood on a glass slide, spread it thinly, and air dry before staining for microscopic examination. One smear will be used for the application of the Conventional Leishman's staining technique, while another smear will be employed for the implementation of the Modified Leishman's staining procedure.

- **For conventional method:** The smear is covered with undiluted stain, and this is allowed to sit for 1-2 minutes. Staining is a common technique in microbiology and histology used to enhance the visibility of cellular structures or microorganisms. After the initial staining period, twice the volume of buffered water (with a pH of 6.8) is added to the smear to dilute the stain. Buffered water helps maintain a stable pH environment during staining. The stain is mixed with the buffered water by gently blowing air into the mixture using a plastic bulb pipette or a straw. This step ensures the uniform staining of the smear. The smear is then allowed to stain for a longer duration, typically 10-12 minutes. After the staining period, excess stain is washed off the smear using tap water. This step helps remove unbound or excess stain from the slide. The smear is immersed in buffered water (pH 6.8) for two minutes. This rinse step likely helps to further remove any residual stain and ensures the smear is at the appropriate pH level for subsequent analysis. Final rinse and drying: The smear is rinsed with regular water, and the back of the slide is wiped clean. The slide is then left to air dry, which is a crucial step to ensure the sample is properly fixed and ready for microscopic examination.
- **For modified method:** The smear is initially covered with undiluted Modified Leishman's stain for a specific duration, which is 25 seconds in this case. Staining with Leishman's stain is commonly used in haematology for the visualisation of blood cell morphology. After the initial staining period, twice the volume of buffered water (pH 6.8) is added to the smear to dilute the stain. This step is important to ensure that the stain doesn't over-accumulate and interfere with the visualisation of cellular features. The smear is allowed to stain for a longer period, specifically 50 seconds. This extended staining time may help in achieving better contrast and more detailed staining of cellular components. After the staining period, excess stain is washed off with water. This step is crucial to remove any unbound or excess stain that could obscure the morphological features of the blood cells. The smear is flooded with buffered water with a pH of 6.8 for two minutes. This rinse step helps to further remove any residual stain and ensures that the smear is at the appropriate pH level for microscopic examination. After rinsing with regular water and wiping the back of the slide, the slide is left to air dry. Drying is important to fix the stained cells and prepare them for examination under oil immersion microscopy at a high magnification of 100x. The evaluation of staining quality involved examining and scoring various aspects such as the pattern of red blood cells, nuclear pattern, neutrophil granules, eosinophil granules, platelets, and background staining [10]. The conventional method, which requires a total of 10 to 12 minutes to complete the staining process, modified Leishman's staining techniques require only 75.0 seconds and 4.0 minutes.

Primary outcome:

Staining time: This refers to the time taken for each staining method to complete the staining process. It could be measured in minutes or hours.

Quality of staining: This involves assessing the clarity, contrast, and overall quality of the stained cells under a microscope. It could be subjective, based on the judgement of experienced observers, or quantified using specific criteria.

Secondary outcome:

Fixation efficiency: This refers to the effectiveness of the fixation method in preserving cell morphology and structure. It could be evaluated by comparing the appearance of fixed cells before and after staining, considering factors like cell shrinkage, distortion, or loss of detail.

Cost-effectiveness: This involves comparing the costs associated with conventional and modified Leishman's staining techniques, including the cost of reagents, equipment, and labour. It could help determine which method offers better value for money without compromising quality.

Bias: All required calculations to analyse bias at all quantities will be taken. In the present observational study, specimens are received daily in the Pathology Department. Cytology is considered for comparing Conventional and Modified Leishman's stain. A standard supply of stains and reagents, with components sourced from various companies, ensures the production of appropriate and consistent alterations, which are an essential element of the staining process. Alterations in the treatment approach affect the outcome of cell staining, thereby influencing the interpretation of diagnostic samples.

STATISTICAL ANALYSIS

In the present study, authors will use Statistical Package for Social Sciences (SPSS) version 23.0 for our statistical analyses. Significance will be assessed at a threshold of $p \leq 0.05$. As part of the initial data exploration, authors will focus on characterising the central tendency of inhibition zones through the computation of mean values. For the subsequent inferential analyses, authors will use Analysis of Variance (ANOVA) to investigate both patient-specific data and repeated measures. This choice of statistical tool is particularly suitable for comparing means across multiple groups. It is imperative to acknowledge that, in the context of our research, a p-value of 0.05 or lower will be considered indicative of statistical significance.

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