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Alteration in Eosinophil's Granular Content in Stained Film as an Index for Estimating the Shelf-lives of Modified Leishman Stains: Implications on Disease Diagnosis

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Authors' contributions

This work was carried out in collaboration between both authors. Author KAF designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author ODA managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Modified Leishman stain is a newly discovered stain which is composed of Leishman powder, absolute methanol and phenol. It is not ususual sometimes in diagnostic haematology laboratories to have improperly stained thin blood films, without any clear-cut reason, leading to misdiagnosis of disease. Notably due to lack of published data on the shelf-lives of modified Leishman stain, we hypothesize that misidentification of cells may be due to expiration of the stain. Four modified Leishman staining solutions were prepared using phenol crystals and liquefied phenols as the sole modifying component ingredient. Thin blood smears were made in multiples of four from each of the patients requiring peripheral blood film review especially those with high eosinophil counts. The

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smears were stained with the prepared dyes for a total of 75.0 seconds or 4.0 minutes and examined for cellular elements of the blood by the researchers. At expiration, while other cellular elements of the blood stained normally, eosinophil granular content altered from orange-red to orange/reddish-brown and shades of brown and that was used to estimate modified Leishman stains' shelf-lives. Estimated shelf-lives of the modified Leishman stain were 25 days (21-29 days), ≤ 28 days, up to 52 days and ≤ 18 days when the first, second, third and fourth methods of preparation respectively were used. Modified stain prepared by initial dissolution of phenol in absolute methanol before using resultant mixture to dissolve Leishman powder has the longest shelf-life- up to 52 days. It was ≤28 days when liquid phenol was used in similar pattern to conventional technique.When phenol crystals were used, the average shelf-life were 25 days (21-29 days) and ≤ 18 days respectively. Conclusively, modified Leishman stain should be prepared and used with the consciousness of the expiry dates. This mystery unfolded prevents misclassification of eosinophils during differential leukocyte counts, thus enhancing disease diagnosis.

Keywords: Eosinophil; haematology; phenol; leukocyte; granules; diagnosis; shelf-life.

1. INTRODUCTION

Accurate haematologic diagnosis is a *sine qua non* condition to right treatment of associated diseases. An indisputable fact that has span centuries especially in developing countries is that manual differential leukocyte count remain the gold standard technique often used to cross-check the results of automated analyzers in diagnostic haematology laboratory [1-2]. This is often the case whenever misclassification of immature leukocytes, extreme leukocytosis or leukopenia and thrombocytosis or thrombocytopenia are suspected. In patient's care, diagnostic formulations rest on a tripod of clinical history, physical examination and laboratory investigations. In fact, it has been established that nearly 70% of clinical diagnoses and decisions in patients' care are based on results of laboratory investigations such as peripheral blood film review including differential leukocyte count [3]. Optimal staining of peripheral thin smears is required to enhance the quality of laboratory results. Conventional Leishman staining solutions and techniques have been used for decades without any major scientific touch. Cellular elements of the blood such as the leukocytes, erythrocytes and thrombocytes may be misclassified sometimes due to improper staining thus leading to erroneous results, wrong diagnosis and wrong treatment especially in under-developed or developing countries where facilities may not be available to do other evaluations to establish or disprove diagnosis.

One of the recent advances in the field of haematology is the development of modified Leishman staining solutions. The modified Leishman stain used accentuating agent phenol as the modifier. Accentuating agents are chemical substances that are used to improve the quality of staining of tissue (fluid tissue inclusive). Without them staining can take place but it will be of low quality. They act by changing the pH of the staining solution with overall effect of increasing the rate of staining uptake by the tissue [4]. Previous study findings showed there were four methods of preparation [4]. The phenol: Leishman powder ratio used per 100 ml of modified Leishman staining solution prepared irrespective of technique was 1:3 or 1:5.

Eosinophil granules contain several basic proteins including a major basic protein known as the eosinophil cationic protein which constitutes the characteristic crystalline core and more than 50% of the granule [5]. This and other basic structures give the eosinophil granule its net negative charge and acidophilic feature [6]. Normal eosinophil measures 12 – 16 microns in diameter. Its nucleus stains purple and it is usually band-shaped or segmented into only two lobes or occasionally more. It is formed in the bone marrow from a committed precursor cell which is morphologically indistinguishable from the myeloblast. Once released into the peripheral blood after spending several days in the bone marrow during maturation, eosinophil circulate with a half-life of 18 hours before migrating to tissues. Of the body's total number of eosinophils, only approximately 1% is found in blood [7-8]. Eosinophilia has been noted in several pathologic conditions including allergic disorders such as asthma, hay fever [9]; skin disorders (e.g. eczema) and hypersensitivity states; parasitic infections (e.g. malaria, hookworm, schistosomiasis), maligancies of

myeloid cells (e.g. chronic myeloid leukemia), drug reactions (sulphonamides, penicillin), and tropical eosinophilia [10]. Identification of eosinophil's morphology and alteration of its cytoplasmic granular colouration is a sure guide the estimation of modified Leishman stain.

2. MATERIALS AND METHODS

2.1 Research Location

This research was performed at the haematology laboratory, Federal Teaching Hospital, Ido Ekiti, Ekiti state. The institution is located at the headquarters of Ido/Osi local government in Ekiti state. Ekiti state established on a total land mass area of 6,353 km^2 has an estimated population of 2, 737, 186. The institution is the only Federal and tertiary health institution, and currently the serving Teaching Hospital for Afe Babalola University for the training of healthcare professionals including Medical, Nursing and Medical Laboratory Science students.

2.2 Ethical Consideration

The informed consent of research volunteers were obtained and analysis was carried out at Federal Teaching Hospital (FTH) following the ethical guidelines of Federal Teaching Hospital, Ido Ekiti.

2.3 Sampling and Peripheral Blood Film Preparation

Up to 4 - 5 ml of K3EDTA anticoagulated samples were collected from routine haematology patients requiring peripheral blood film review (PBF) and those whose PBF demonstrated relative eosinophil counts> 10% were selected for experimental research. Four thin blood films were made from selected patients each day by coverslip method as described by Bain and Lewis [11] and stained with modified Leishman staining solutions prepared by four different methods.

2.4 Modified Leishman Stain Preparation Methods

The first method described required weighing measured amount of both phenol crystals (50 mg or 30 mg) and Leishman powder (150 mg) in a mortar and dissolved them in 100 ml of absolute methanol. The second technique prepared conventional Leishman staining solution [11] and added 50 mg or 30 mg of phenol crytals after the preparation. The third method described involves dissolution of measured amount of liquid phenol (50 µl or 30 µl) in 100 ml of absolute methanol and using the resultant mixture to dissolve the Leishman powder. The fourth method involved adding liquefied phenol (50 µl or 30 µl) after Leishman staining solution has been prepared by conventional approach as in the second method. The phenol: Leishman powder ratio/ 100 mL of Modified Leishman stain optimal for each method of preparation described was 1:3 or 1:5.

2.5 Quality Control Measures

Commercially prepared Leishman powder (BDH Chemicals Ltd, England or Plasmatek Products Ltd, UK) and absolute methanol purified by high performance liquid chromatography which have been used for conventional Leishman staining technique were used for the study. Phenols crystals or liquid phenols (BDH Chemicals Ltd, Pooled, England) used for experimental research were kept at room temperature (18-25°C) in an unoxidized state.The crystals appeared glistering white while in liquid form it is colourless. Pinkish colouration of the phenol was an evidence of oxidation and deterioration of reagent and as such was not used for research. Fresh K3EDTA anticoagulated samples were also used for procedure. Sörrenson's buffer or tap water of pH 6.8 was used for staining process.

2.6 Modified Leishman Staining Techniques

Air-dried PBF were stained using the overall 75.0 seconds or 4 minutes staining procedure as described in the previous study [4].

2.7 Estimation of the Shelf-lives of Modified Leishman Staining Solutions

Four peripheral blood films stained with modified Leishman staining solutions based on four preparation methods were examined each day to observe for alteration in eosinophil's granular appearance from orange-red to a shade of orange/reddish-brown to shades of brown depending on the degree of expiration. Shelf-life of the stain was estimated at the first appearance of alteration in eosinophils' acidophilic granules which showed the stain has begun to lose stability and is said to have expired.The average shelf-life of each staining solution based on the method of preparation was recorded. Similarly five other peripheral blood films were made to demonstrate the effect of modified Leishman

staining on the morphologic appearances of other cellular elements of the blood.

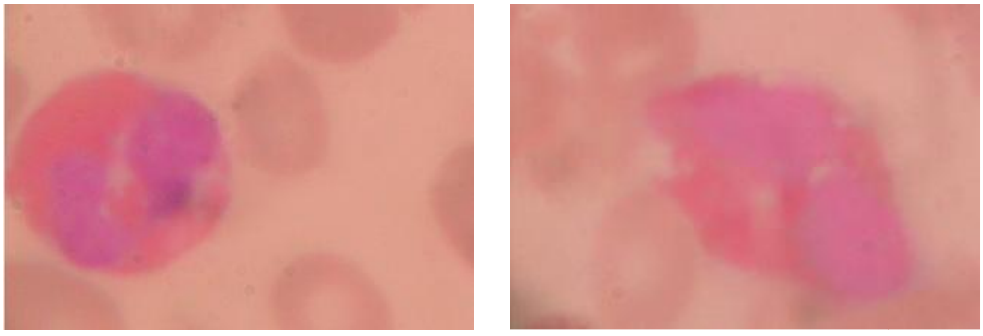
3. RESULTS

3.1 Estimated Modified Staining Solutions' Shelf-lives

With the first method used to prepare modified Leishman staining solution which involved crystallized phenol,the shelf-life was 21-29 days (mean 25 days) at room temperature. The stain lost its stability more rapidly if kept at temperature higher than room temperature (average shelf-life at > 25°C was 18 days). The second method had optimal shelf-life of ≤18 days. When liquefied phenol was added to methanol before using the resultant mixture to dissolve the Leishman powder (i.e. the 3rd method), shelf-life was up to 52 days. Fourth method prepared modified Leishman stain by conventional method followed by subsequent addition of phenol and that resulted in shelf-life of 28 days or less (i.e.≤ 28 days). Slides A and B showed the photomicrographs of morphology of eosinophils prior to stain expiration (observe the orange-red granules). As observed in slide B, conventional technique of preparation showed a less distinctly stained nucleus and granules due to moderate staining uptake by the cell components. Photomicrography similarly had slight effect on the clarity of the outlines of the morphologies of both eosinonophils but the distinct orange-red granular pictures necessary for shelf-life estimation was clear.

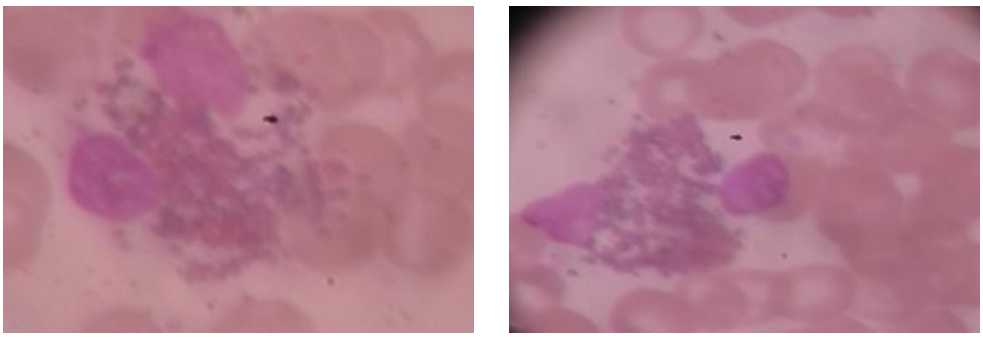
3.2 Effects of Expiration of Modified Leishman Stain on Eosinophil Granules

Expiration of modified Leishman stain had visible effects on the granular content of eosinophils. Slide C and D showed the orange/reddish-brown granules of eosinophils (at first observation of loss of stain's stability, that is, expiration) and brownish eosinophilic granules at protracted expiration.



(A) **(B)**

Fig. 3A-B. A: Demonstration of normal eosinophil morphology in modified leishman stained thin blood film, magnification: X1000); B: Demonstration of normal eosinophil morphology in modified leishman stained thin blood film by conventional method; magnification: X1000



(C) **(D)**

Fig. 3C-D. Demonstration of granular alteration of reactive eosinophil from orange-red to shades of brown (C. Reddish-brown: Slide C; Brownish granules: Slide D) (In expired modified Leishman stain, X1000 magnification)

3.3 Effects of Expiration of Modified Leishman Stain on Other Cellular Elements of the Blood

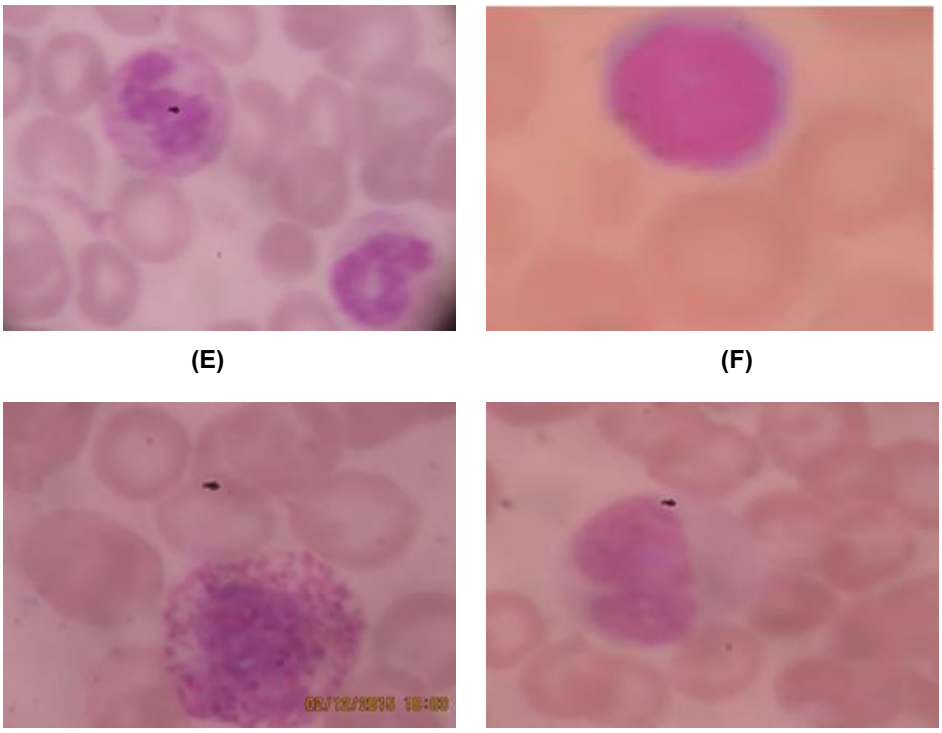
As photomicrographs of slides 3E-H revealed, expiration of modified Leishman staining solution did not have any impact on the morphologic pictures of other cellular elements of the blood. Other leukocytes such as neutrophils, basophils, lymphocytes and monocytes as well as thrombocytes maintained their morphologic appearances following stain expiration. Hence, misclassification of these formed elements of the blood during differential leukocyte counts is uncommon irrespective of clinical diagnosis.

4. DISCUSSION

The active component ingredient of modified Leishman stain is an accentuating agent phenol which is acidic (pKa = 9.95), and that slightly altered the net acidity of the Modified Leishman staining solution. Provided the staining of peripheral blood films with modified Leishman staining technique was performed according to the standard operating procedure, the formed elements of the blood (i.e. red blood cells, leukocytes and thrombocytes) showed their normal characteristic morphologic pictures similar to those observable in conventional Leishman staining technique. As observed from the study findings, modified Leishman staining solutions have different shelf-lives based on their methods of preparation. The reasons behind this remained unclear but some mechanisms were postulated as contributing which were assumed to stem from the modifier. The toxicity of the modifying agent (i.e. phenol) based on some underlying reaction with components of Leishman powder, length of exposure of phenol to Leishman powder, the nature of phenol used (whether crystals or liquid) and the point at which it came into contact with the main solvent of the dye (i.e. absolute methanol). Phenol and absolute methanol have the same terminal hydroxyl functional groups which aid their reactivity and the electron-withdrawing group function of the benzene ring of the modifying agent has been established and that contributed to the slight acidity of the modified Leishman stain when compared with the conventional counterpart [12-13].

Moreover, the fact that the expiration of the modified Leishman staining solution did not have effects on other cellular elements of the blood (as observed in Fig. 3E-I) apart from the eosinophil granular content (as observed in Fig. 3C-D) could only be explained from the perspective that only eosinophil has a major basic protein known as the eosinophil cationic protein which constitutes the characteristic crystalline core and more than 50% of the granule [5]. This and other basic structures give the eosinophil granule its net negative charge and acidophilic feature [6]. The phenol, by impacting on the net pH of modified Leishman stain increased the permeability of the stain by the eosinophilic granules which have net negative charge. There was probably a gradual alteration in pH of the staining solutions as the dyes aged and concomitant change in the morphologic appearance of eosinophil's granular content. The neutrophil granules have net neutral charge [14]. Staining results showed it remained unaffected by the age or expiration of the dye though the mechanism is unclear. The granules of basophils and RNA molecules of the cytoplasm of white blood cells were stained by the basic components of the dye [15], and were unaffected by the phenol component's activity. Morphologies of red blood cells, non-granulated leukocytes (lymphocytes and monocytes) and thrombocytes were also unaffected by the age of the dye or expiration as observed in Fig. 3E-I.

Accurate eosinophil counts during differential leukocyte count analysis have significant impact on the diagnosis of diseases. Most clinicians depend on peripheral blood film review to establish provisional diagnosis and follow up of patients on treatments [1]. Misclassification of relative eosinophil counts during differential leukocyte count procedure due to expiration of modified Leishman staining solution can result in misdiagnosis, lack of confidence in diagnostic results by clinicians, initiation of unnecessary treatment regimens and difficulty in follow-up of hospitalized patients being monitored for either eosinophilia or eosinopenia. Both eosinophilia (eosinophil count > 0.6×10^9/L) and eosinopenia (eosinophil count < 0.04×10^9/L) are associated with certain clinical conditions. Parasitic infections, allergy and reactive causes have been implicated as resulting in eosinophilia [16-18]. Besides, myeloproliferative disorders such as chronic myeloid leukemia have been documented as associated with eosinophilia [19]. Drug use such as penicillin and sulphonamides can similarly result in eosinophilia [10]. Primary immunodeficiency disorders such as Wiskott-Aldrich syndrome, polyendocrinopathy, auto-immune lymphoproliferative disorders, Omenn syndrome are among known differentials of eosinophilia [20]. More recently, Navabi and Upton documented more primary



(E) **(F)**

(G) **(H)**

Fig. 3E-H. E: Thin blood film of a patient with sepsis showing reactive neutrophils with mild left shift and normal RBCs. F: Lymphocyte and normocytic normochromic RBCs in normal subject. G: Basophil and hypochromic RBCs. H: Monocyte and normal RBCs (stained with expired modified Leishman stain, magnification: x 1000)

immunodeficiency disorders with eosinophilia which include but not limited to MHC Class II deficiency, ataxia-telangiectasia, TCRα deficiency, Kostmann disease and neonatal onset multisystem inflammatory disease [21]. They were categorized based on degree of eosinophilia.

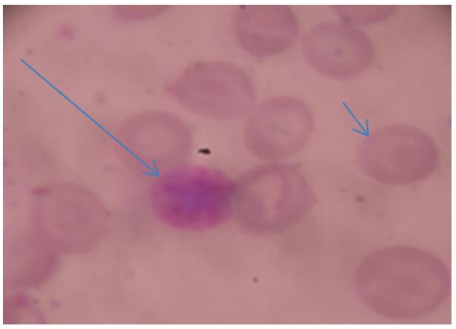


Fig. 3I. Thin blood film showing giant platelet (long arrow) and target cell (short arrow) in an anaemic patient stained with expired modified Leishman stain, magnification: X1000)

Eosinopenia (eosinophil count < 0.04 x 10^9/L) is very rare and has been traditionally associated with enteric fever [22-24]. While some authors have showed that eosinopenia is a biomarker of infections in children [25-26] and adults [27-28], others demonstrated that it is rather a biomarker of mortality in critically ill children [29]. Eosinopenia has been observed as a consequence of cancer drugs [30] prognostic marker of inflammatory-syndrome-associated infectious diseases such as peritonitis [31]. Sometimes, autoimmunity, asthma, urticarial and allergy may be associated with eosinopenia.

5. CONCLUSION

The estimation of modified Leishman staining solutions' shelf-lives and proper understanding of the index used in the estimation enhance the optimal use of the novel dye in haematological diagnosis. Third method of preparation with longer shelf-life is the most preferable technique of preparation by reason of its longer shelf-life.To optimize clinical diagnosis and prevent wrong classification of eosinophils during differential

leukocyte counts, biomedical scientists must note the dates of preparation of modified Leishman staining solutions, method(s) of preparation and storage conditions. Understanding the clinical diagnosis and correlation of such with either eosinophilia or eosinopenia will also improve the quality of laboratory results and minimize errors due to expiration of the dye.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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