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Comparative Demonstration of Somatic Cells using Modified Staining Techniques

[1*]Akpulu SP, [2]Egaji GO, [1]Mairiga AA, [2]Agbon AN, [2]Oderinde GP, [3]Okonkwo LO

[1]Department of Medical Laboratory Sciences, Faculty of Health Sciences, Federal University of Lafia, Nasarawa, Nigeria.
[2]Microscopy and Stereology Research Unit, Department of Human Anatomy, Faculty of Basic Medical Sciences, Ahmadu Bello University Zaria, Nigeria.
[3]Department of Medicine, Faculty of Clinical Sciences, Ahmadu Bello University Zaria, Nigeria.

E-mail: petosw2000@yahoo.com; +234 8037914275

ABSTRACT

Demonstration of somatic cells (SC) in the last few years been performed commonly by the method of Prescott and Breed. Other advanced techniques have been developed to demonstrate SC. Most of these methods are rather time-consuming and expensive. Demonstrating SC using a more rapid, less expensive, readily available and accurate method is of great value. This study demonstrated SC using modified staining technique with Methylene Blue (MB), Toluidine Blue (TB) and Hematoxylin (HX) stains. Milk was obtained from healthy cows at Zango Stall Barn, Zaria. Milk (3 ml) from each cow was aseptically collected. Smears were made by using 10 µl of the milk samples and immediately stained in freshly prepared MB, TB and HX for two minutes each. The stained slides were examined using light microscope. Staining characteristics and the general appearance of the somatic cellular structures were observed, and their photomicrographs captured using digital microscope camera. The results revealed features of the SC stained with MB to be preserved and distinctly stained with a clearly differentiated background aiding visualization and identification. Staining with HX revealed no distinct affinity for SC in the smears with poorly demonstrated SC. HX stain mostly reacted with the background features. The TB stained smear presented a slightly homogenous staining background giving it a blurry appearance with less affinity for fat droplets. In conclusion, the modified staining protocol with MB stain for demonstrating SC is satisfactory with well-preserved cellular features. This protocol is recommended for adoption in biomedical research and diagnostics in emergency cases.

Keywords: Fat droplet, Hematoxylin, Methylene blue, Staining affinity, Toluidine blue

INTRODUCTION

Somatic cells are the cells in the body other than sperm and egg cells (which are called germ cells). Somatic cells are white blood cells that occur naturally in milk and comprise of cells that contain nucleus i.e. lymphocytes, macrophages, polymorphonuclear neutrophils and epithelial cells that are produced by the cow's immune system to fight an inflammation or infection (mastitis) in the mammary gland. [1, 29] The somatic cells slough off from the lining of udder during milking or in response to an injury in the mammary gland. [1, 30] Somatic cell count is an important parameter for hygienic quality of milk and for animal health.[1, 30]. An elevated somatic cells count level in milk can serve as an indicator for udder infection (mastitis) of lactating cows and thus may be an indication of an insufficient hygiene practices on farms. Somatic cells count is used for various purposes such as milk payment, checking compliance with regulations, and milk recording for genetic evaluation and farm management.[1, 2 19, 30]

There are several laboratory methods used in the determination of somatic cells count in milk. Electronical counting of firm particles (somatic cells fixed by formaldehyde) during their passage through defined electrical field in capillary was previous procedure.[28] Recently, other procedures such as fluoro-opto-electronic counting and modern flow cytometry, are used preferably. [3, 4, 5, 30] Direct microscopic somatic cell count is the actual number of somatic cells seen in milk and counted by the use of microscopic method. It is a method recognized by the International Diary Federation (IDF). [5, 20]

The first microscopic procedure for examination of milk films was described by Zajác et al. [5] and, Presscott and Breed [18] where in the experiments smears from milk were stained, and the somatic cells observed counted.[5, 21 22] There are several dyes (stains) used to demonstrate somatic cells. Some common dyes include the modified Newman-Lampertm ethylene blue stain solution, methyl green-pyronin Y stain solution, ethidium bromide stain solution and Romanosky stains such as Leishman, Giemsa, etc.[6] However, the methodologies related to the use of these dyes involve laborious processes and have high carcinogenic potentials. [7, 6] Thus, a more rapid, less expensive, readily available and accurate method is of great value. [29, 30]

Methylene blue (MB) is an aromatic compound that comes as a dark green powder; it turns a deep blue when in aqueous solution. Chemical formula of MB is: C16H18N3SCl. It is also used as a pigment used in microbiological settings to examine nucleic acid chains. It can be added to a solution to dye RNA and DNA so that it may be visually analyzed. MB staining is useful in determining cell mortality. [8, 27] Toluidine blue is also an acidophilic metachromatic dye that selectively stains acidic tissue and cells components and has affinity for nucleic acids, it is a member of the thiazine group and partly soluble in both water and alcohol. [9, 28] Toluidine blue stains tissues based on the principle of metachromasia and it is highly selective and only certain tissue structures can stain metachromatically which might be the cause of not being able to stain cow milk somatic cells very well. Hematoxylin is the most used dye in the histopathology and histochemistry laboratories.[10] It can be used as a primary stain and as a counter stain

where it will differentiate acidophilic materials.

This study comparatively demonstrated somatic cells using modified staining technique for Methylene Blue, Toluidine Blue and Hematoxylin stains.

MATERIALS AND METHODS

Staining Dyes: The following stain dyes (powder) were obtained:

Methylene blue (BDH Chemicals LTD product number: 26132, made in England), Toluidine blue (Sigma-Aldrich 89640) and Hematoxylin (Hopkin and Williams LTD, 4516.Chadwell Heat Essex, England) were purchased from a reputable chemical store Cardinal Scientific, Zaria, Kaduna State, Nigeria.

Chemicals and Reagents: Ethylene glycol, Distilled water, Xylene, Potassium Aluminum Sulfate, Sodium Iodate, and Ethanol were obtained from Cardinal Scientific, Zaria.

Other materials used in this study include, but not limited to, the following: Hot Air Oven (Gallenhamp Ovh-010-x, made in Germany), Microscope Glass Slides (22x22), Distyrene plasticizer and xylene (DPX) mountant, Optical Microscope (HM-LUX, Leitz Wetzlar, Germany) and Amscope Digital Camera for microscope (MA 500 Amscope, USA), etc.

Stains Preparation: The staining solutions were prepared according to the manufactures instructions. Briefly, the method is as follows:

i. **Methylene Blue (MB) Stain**: 0.5 gm methylene blue powder was dissolved in 30 ml of water. The Methylene Blue solution was transferred to a clean brown bottle, labelled and stored at room temperature.
ii. **Harris Hematoxylin (HX) Stain**: 5 gm of Hematoxylin powder dye was dissolved in 50 ml of ethylene glycol while slowly heating. 100 g of potassium aluminum sulfate was also dissolved in 950 ml of distilled water by heating. Both solutions were mixed, stirred and gradually heated to boiling. It was the allowed to cool and 370 mg of sodium iodate was added with constantly stirring. It was labelled and stored for use.
iii. **Toluidine Blue (TB) Stain**: 0.1 g of Toluidine Blue powder dye was dissolved in 100 ml of water by stirring. The freshly prepared staining solution was all filtered before use.

Sample Collection and Preparation: Milk was obtained from five apparently healthy cows at Zango Stall Barn, Zaria, Kaduna State, Nigeria. Three (3) ml of milk from each of the cows were aseptically collected into sterile plain (5 ml) sample bottles and immediately transported over ice pack to Histology Research Laboratory Unit, Department of Human Anatomy, Ahmadu Bello University (ABU), Zaria for analysis.

Ten microliter (10 uL) from each of the milk samples was placed at the center of a clean grease free slide and smears were made by spreading the milk circularly and outward from the center point on the slide using a pipette teat. Three duplicate smeared slides were made from each of the three cows. One of each of the duplicates slides was labeled MB for methylene blue stain, and the other two slides were labeled as TB and HX for toluidine blue and hematoxylin staining

respectively. (*See Figure 1 for summary of experimental protocol*).

Staining Protocol: The smeared slides were treated with a modified staining protocol. Specifically, the modification was in the staining duration, reduced to two minutes for each of the stains. This slight modification in the staining procedure from the standard (traditional) method for over a century, is to determine whether there will an enhanced staining quality in the demonstration of somatic cells.

Briefly, the staining protocol is as follows: (a). Fixation of the smears by heat drying in oven at 45°C for ten minutes to bind the specimen to the slide so that it does not wash off during staining. (b). Defatting the smeared slides after the fixation, by cooling the slides for 10 minutes and then dipping in xylene for two minutes to remove fat globules. (c). Hydration of the smears slides through descending grades of ethanol (100%, 90% and 70%) respectively. (d). Staining, the smeared slides immediately after hydration were stained by dipping the slides into 50 ml solutions of the freshly prepared stains; methylene blue, toluidine blue and Harris hematoxylin in a staining jar for two minutes. (e). Removal of excess stain from the smears by rinsing in a running tap water briefly. (f). Air drying of the slides by standing racks at room temperature for 10 minutes. (g). Clearing in xylene briefly and mounting (cover slipped) with mixture of distyrene plasticizer and xylene (DPX) (*See* Table 1).

Histological Examination: The stained slides were examined at ×400 magnification using optical light microscope (Optical Microscope; HM-LUX, Leitz Wetzlar, Germany). Staining characteristics (color intensity and background clarity) and the general appearance of the somatic cellular structures were observed. Photomicrographs were captured using digital microscope camera, (MA 500 Amscope, USA) in the Microscopy and Stereology Laboratory, Department of Human Anatomy, ABU, Zaria.

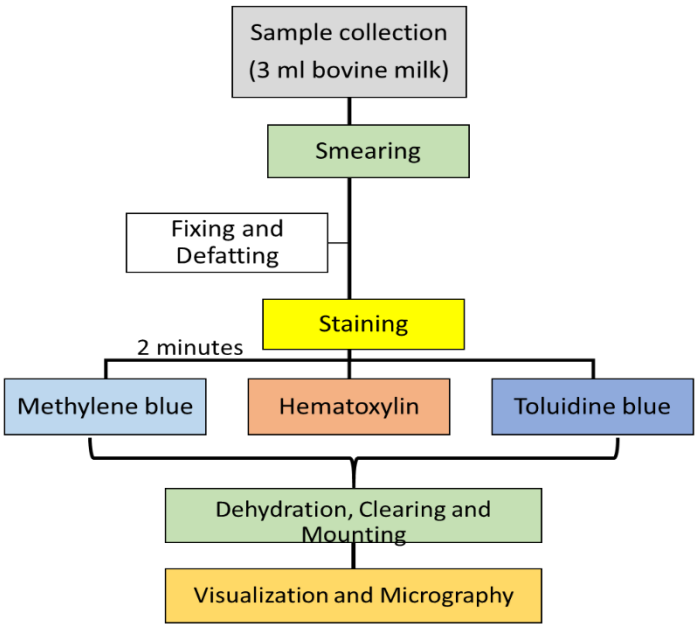

Figure 1: Experimental protocol (Adapted by the authors)

Table 1: Staining outlines

Staining process	Methylene Blue	Hematoxylin	Toluidine Blue	Activity
No of smeared slides	3	3	3	
Fixation by heat	45 ^{0}C	45 ^{0}C	45 ^{0}C	heat drying
Xylene	2 min	2 min	2 min	
Hydration	briefly	briefly	briefly	through graded ethanol
Staining	2 min	2 min	2 min	
Rinse in water	briefly	Briefly	briefly	
Air drying	10 min	10 min	10 min	
Xylene	briefly	Briefly	briefly	clearing before mounting
Mounting	DPX	DPX	DPX	cover slipping

RESULTS

The cellular structures of the somatic cells stained with the MB appeared preserved and distinctly stained (localized). A clearly differentiated background was observed that aided the visualization and identification of the somatic cells. Additionally, MB stain demonstrated numerous fats droplets with vary sizes (Figure 2).

Staining with HX revealed no observable or distinct affinity for somatic cells in the smears. Thus, somatic cells were poorly demonstrated. However, HX stain mostly reacted with the background features including cellular structures in the smears. HX stain demonstrated numerous fats droplets with varying sizes (Figure 3).

The TB stained smear presented a slightly homogenous staining background giving it a smudging (blurry) appearance. A relatively distinct staining of somatic cells was observed; few somatic cells retaining their cellular structural integrity. However, TB stain demonstrated less affinity for fat droplets (Figure 4).

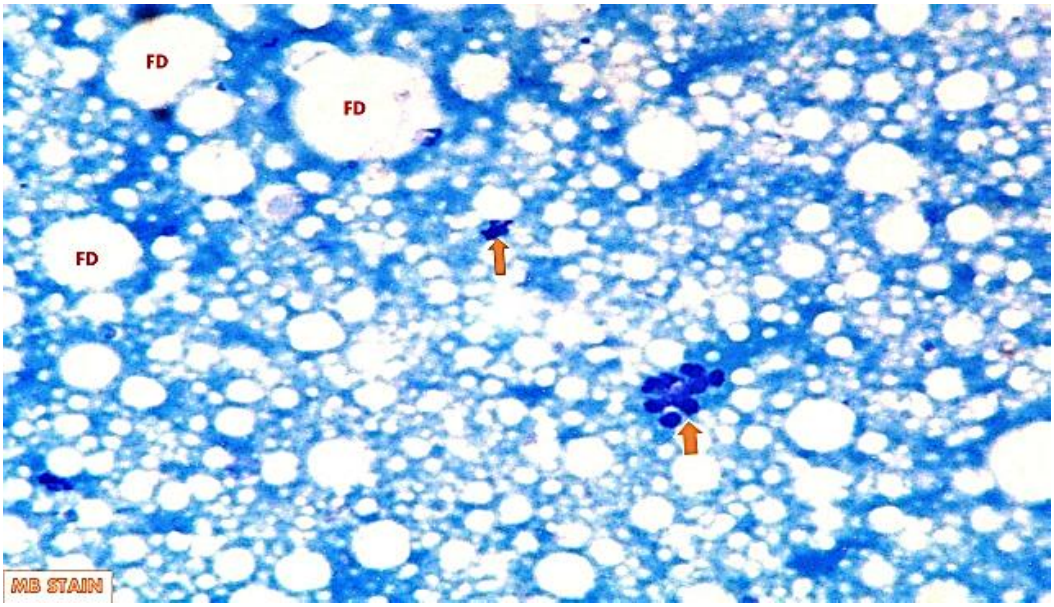

Figure 2: Methylene blue stained cow milk smear demonstrating cytoarchitectural features (MB ×250).

Aggregation of distinctly stained (deep blue) somatic cells (orange arrows) with intact cellular structures; numerous fat droplets (FD), well-preserved light blue background with varying sizes.

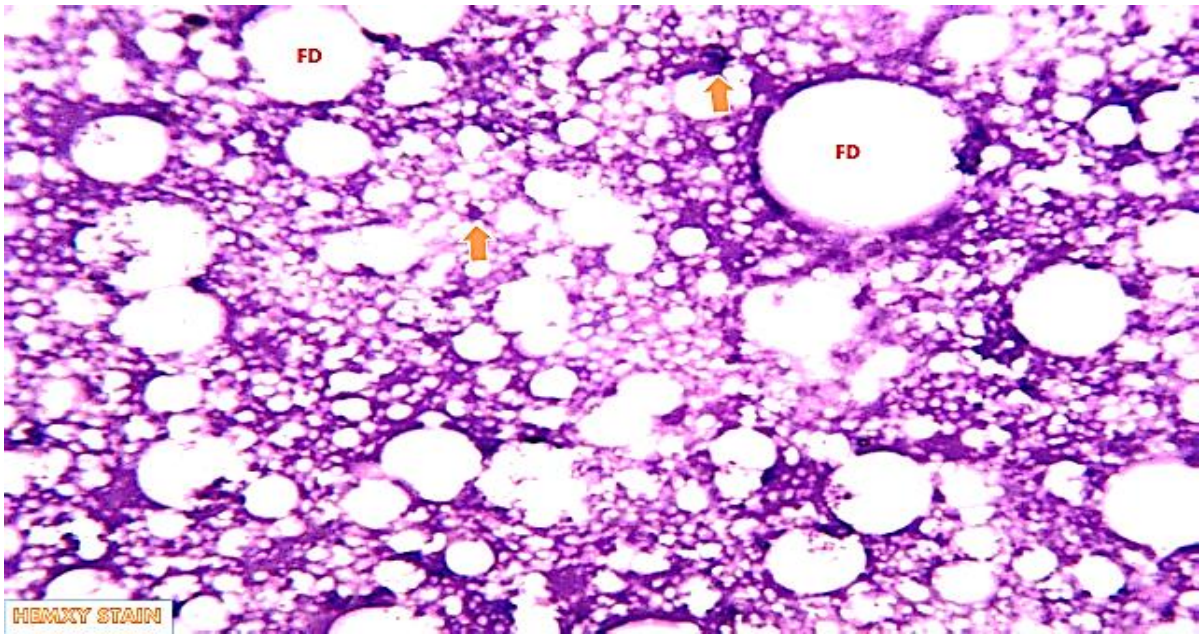

Figure 3: Hematoxylin stained cow milk smear demonstrating cytoarchitectural features (HX ×250).

Non- distinctly stained somatic cells (orange arrows); Strong purplish stained background masking most of the somatic cells; numerous fat droplets (FD) with varying sizes.

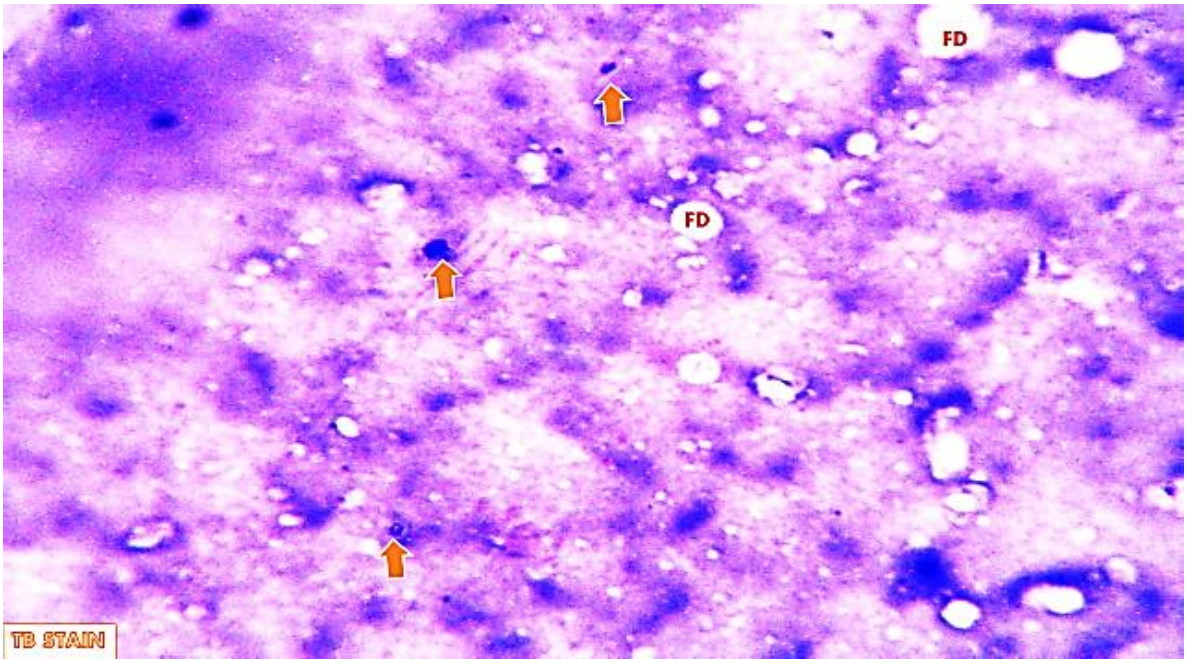

Figure 4: Toluidine Blue stained cow milk smear demonstrating cytoarchitectural features (TB ×250).
A slight homogenous background staining with relatively distinct somatic cell (orange arrows); slightly distinct fat droplets (FD)

DISCUSSION

In this study, a modified staining protocol for three stains on smeared samples was assessed for staining outcomes.

Observation of the MB staining, shows aggregation of distinctly stained somatic cells with intact cellular structures, and well preserved fat droplets which did not present any diagnostic interference to the staining of the somatic cells is suggestive of reactive affinity with the cytoarchitectural features of the smear. This result is similar to the report of Zajác et al. [5] and Harmon [23] that cow milk somatic cells stained with methylene blue shows the lightly retention of the stain and uniformly by only the milk protein, with no mottling or network effect on the background which makes the somatic cells to be visible and distinct, [11, 24, 25] using the Panótico staining technique, observed no changes in cell morphology of smear from a bovine milk. Gupta et al. [12] and Penney et al. [26] stated that MB stain is a cationic stain commonly used for histological staining of tissues and cells. Briggs and Bain [13] reported that MB binds to negatively charged parts of the cells, such as DNA and RNA, this could be a possible reason for the strong staining affinity of MB on somatic cells observed in this study.

Non-distinctly stained somatic cells, with a strong purple color stained background masking most of the somatic cells, limiting visualization observed with HX staining is suggestive of poor reactive affinity with the cytoarchitectural features of the smear. This observation agrees with a similar study reported by Viana et al.[11] where, the staining of Wright and Panóptico demonstrated poor cellular morphologic features with smudged smeared film. In the conventional hematoxylin progressive or

regressive staining for tissues sections as described by Kiernan [14] and Kumar et al.[15], once the desired level of nuclear staining is achieved, excess hematoxylin reaction is moderated by differentiating in an acid–alcohol medium and "blued" in an alkaline solution to give a distinct blue coloration. This differentiation and bluing process in hematoxylin staining which was not observed in this study could be responsible for the poor demonstration of the somatic cells observed with HX staining in this study.

In this study, the TB stained smear presented with slightly homogenous background staining having a relatively distinct somatic cell and reduced affinity for fat droplets. A similar finding was reported by Moraes et al. [16] for the evaluation of microscopic protocols for somatic cell counts in sheep milk; staining with TB demonstrated poor cytoarchitectural features with blurry background making localization and counting challenging. In contrast, Gokceoglu et al. [17] reported excellent reactive affinity with TB staining in centrifuged sediments of smear sample from cow milk. Toluidine blue is an acidophilic metachromatic dye that selectively stains acidic tissue and cells components and, has affinity for nucleic acids. The possible reason for the poor background staining affinity of TB on somatic cells observed in this study could be associated to the low pH of the stain solution.

In this study, it is imperative to mention that the modified staining protocol with MB stain demonstrated somatic cells satisfactorily with preserved cellular features and clarity of background compared to the other two staining protocols using hematoxylin and toluidine stains. Thus, suggesting the MB staining protocol as a preferred method or technique for the demonstrating of somatic cells; aiding direct microscopic somatic cell counts easier and less challenging. On the other hand, the hematoxylin staining protocol demonstrated more of the fat droplets compared to MB and TB stains. There is a likelihood that including the differentiation and bluing steps in conventional hematoxylin staining protocol may enhance the visibility of the targeted (somatic) cells with a clearer background. However, this may pose the challenge of increasing the time or duration in the staining protocol. The blurry background appearance observed with TB staining protocol associated with low pH of the staining solution could be enhanced by carefully increasing the pH (that is, alkalization of the solution).

CONCLUSION

This study identified the modified staining protocol with MB stain for demonstrating somatic cells as satisfactory with well-preserved cellular morphology and background clarity. Thus, this protocol is recommended for adoption in our of research and diagnostic laboratories to safe time in emergency cases.

Competing interests: The authors declare no conflict of interest

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