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RESEARCH ARTICLE

EFFECTIVENESS OF MODIFIED MASSON TRICHROME STAINING VS MODIFIED VOF STAINING FOR HARD TISSUE PATHOLOGIES

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ABSTRACT

Background: Differentiating between various types of bone—such as osteoid, calcified bone, woven bone, and lamellar bone-can be challenging with standard histological techniques for decalcified sections. Several stains, including Verde Luz-orange G-acid fuchsin (VOF), Von Kossa, solochrome cyanine, Masson's Trichrome, and silver staining (applied before decalcification), have been developed to address this issue. These stains are quick, easy, affordable, and effective at distinguishing between hard and soft tissue components in various specimens.
Aim: This study aimed to compare the efficacy of modified Masson Trichrome staining with modified VOF staining for diagnosing hard tissue pathology.
Materials and Methods: We analyzed 18 formalin-fixed, paraffin-embedded, decalcified bone samples with known histological diagnoses. These samples were subjected to staining using standard techniques, as well as modified version of Masson Trichrome and VOF stains. The evaluation criteria included stain intensity, clarity, translucency, and contrast. These factors are critical for accurately distinguishing between different types of bone tissue and assessing pathological changes.
Results: Our analysis focused on grading the quality of the staining techniques based on background clarity, morphological detail, nuclear feature definition, and overall differentiation. The modified VOF stain consistently showed superior performance in these categories compared to the modified Masson Trichrome stain. This indicates a higher efficacy in revealing detailed histological features necessary for accurate diagnosis.
Conclusion: Our findings suggest that modified VOF staining outperforms modified Masson Trichrome staining for evaluating hard tissue pathologies. This enhanced staining method provides clearer differentiation and better detail, making it a more reliable choice for histological assessment in clinical and research settings.

Keywords: Histological Techniques, Connective Tissue Stains, Masson Trichrome Staining, Osteoid Differentiation and Hard Tissue Pathologies

INTRODUCTION

Throughout the development of a tooth, hard tissues including enamel, dentin and bone, as well as the existence of mature and immature collagen, make histochemical staining identification difficult. Different percentages of mature bone, immature bone, and osteoid make it difficult to distinguish between them and, consequently, to diagnose [1]. Hard tissue lesions of the head and neck exhibit different

degrees of ossification within the fibrous connective tissue stroma. To name a few, benign fibro-osseous lesions include soft tissue osteomas, central and peripheral giant cell granulomas, osteomyelitis, and osteosarcomas. Other examples are fibrous dysplasia, cemento-osseous dysplasia, and ossifying fibroma [23].

Histological examination is crucial for biological research and disease diagnosis. The examination exposes the state of cells and tissues and allows us to deduce the general condition of the individual as well [4]. The hematoxylin and eosin staining (HE) method of tissue staining is one of the most common methods of evaluating the condition of cells and tissues. Routine histological techniques like hematoxylin and eosin (H & E) for decalcified bone sections may not clearly identify osteoid from calcified bone, woven bone, and lamellar bone; additionally, both calcified and soft tissue may resemble the same shade of pink [5,6]. Early calcifications are frequently overlooked, and it can be challenging to tell the difference between the organic matrix of calcified tissue and soft tissue [7]**.** Another difficulty is the misdiagnosis of bone vs cementum-like tissue [8].

Over the years, various connective tissue stains have been developed and utilized to distinguish between the stroma's hard and soft tissue components. Among the stains identifying stromal hard tissues are Von Kossa, Masson's Trichrome (MT), solochrome cyanine, and silver staining before decalcification [9]. The tetrachrome technique makes use of the idea that aging, primary and secondary mineralization, and structural and chemical changes in bone tissue are all interrelated. Changes allow for differential staining even after decalcification and distinguish bone matrix from the initial non-mineralized osteoid.

Differential staining has been accomplished using the tetrachrome approach by carefully mixing phosphotungstic acid with dyes. Another advantage of using the technique is its ease of use, low cost, lack of particular equipment requirements, ability to stain large sections, and ability to be used in conjunction with polarized light to show the collagenous matrix's underlying lamellar and woven structure [7,9]. The MT stain is primarily used to stain collagen, and as the name suggests, three dyes are used to stain different tissues selectively. The porosity of the tissue influences the staining procedure because the smallest dye molecule colors less porous tissue [10].

Sarasquete and Gutiérrez changed the original trichromic hematoxylin-Gutiérrez' Verde Luz-orange G-acid fuchsin (VOF) stain to tetrachromic VOF stains to more readily differentiate diverse hard tissue components. The tetrachromatic Verde Luz-orange G-acid Fuchsin (VOF) stain is a promising approach for identifying the components of hard and soft tissues. It is a dye combination including orange G, acid fuchsin, light green, and methyl blue that selectively stains distinct tissue components [11]. The current study compares the efficacy of modified Masson Trichrome staining against modified VOF staining to aid in the identification of histological characteristics of hard tissue pathologies.

MATERIALS AND METHODS

The archives of the Department of Pathology were searched to identify 18 tissue blocks with known histopathological diagnoses (decalcified bone samples). Two sections from each block were cut using a soft tissue microtome for staining. This study was conducted following the approval of the Institutional Human Ethical Committee under the reference number IHEC/SDC/UG-2178/24/GPATH/111.

For the VOF stain preparation, 260 mg of light green, 140 mg of methyl blue, 500 mg of orange G, and 600 mg of acid fuchsin were dissolved in 100 mL of boiling distilled water. After allowing the solution to cool completely, 1.5 g of phosphotungstic acid and 3 mL of glacial acetic acid were added. Then, 200 mL of pure ethanol was mixed into the solution. The VOF stain was now ready for use.

Harris's Hematoxylin stain was prepared by dissolving 2.5 g of hematoxylin in 25 mL of pure alcohol. Separately, 50 g of potassium alum was dissolved in 500 mL of warm distilled water in a 2-liter flask. The hematoxylin solution was then combined with the potassium alum solution in the flask, and the mixture was brought to a boil quickly. Gradually, 1.25 g of mercuric chloride or 0.5 g of sodium iodate was added to the boiling liquid. The stain was rapidly cooled by immersing the flask in cold water or a sink filled with chipped ice. Finally, 20 mL of glacial acetic acid was added to the cooled stain, making it ready for immediate use.

To prepare the Eosin solution, a 0.5% or 1% solution of Eosin Y was made in distilled water. A crystal of thymol was added to the Eosin solution to prevent fungal growth. Additionally, a small amount of acetic acid (0.5 mL per 1000 mL of stain) was added to enhance the sharpness of the staining.

Xylene is used to deparaffinize the tissue slices for 20 minutes. Xylene is a typical solvent for removing

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paraffin wax from tissue slices, which allows the stains to permeate the tissues. After that, the deparaffinized pieces are rehydrated using a succession of alcohol solutions. For about 10 minutes, the slides are immersed in decreasing concentrations of alcohol (e.g., 100% alcohol, 95% alcohol, and 70% alcohol). Rehydration aids in the removal of any residual xylene and prepares the tissue for staining. Following rehydration, the portions are cleaned for 3-5 minutes under running tap water. This procedure eliminates any remaining alcohol and prepares the tissue for the subsequent staining phase. For 5 minutes, the slices are stained with Harris' hematoxylin. Hematoxylin is a natural pigment that attaches to cell nuclei, allowing them to be seen under a microscope. The nuclei are stained blue in this stage. The slides are briefly bathed in acid alcohol to distinguish the stained nuclei from the background. Acid alcohol is a combination of alcohol and hydrochloric acid that aids in the removal of superfluous hematoxylin from tissue, leaving only the stained nuclei. The slides are then briefly immersed in ammonia. Ammonia helps to "blue" the hematoxylin-stained nuclei, increasing contrast and clarity. The slides are then placed in the VOF stain for 5 minutes. The VOF stain is a solution-based mixture of light green, methyl blue, and orange G dyes. This stain gives varied colors to different tissue constituents and aids in the visualization of diverse structures. Following VOF staining, the slides are dehydrated in 80% ethanol and absolute ethanol. The slides are dehydrated by soaking them in increasing concentrations of alcohol to eliminate water from the tissue sections and prepare them for mounting. Instead of employing the VOF stain, another batch of 15 slides can be transferred to eosin for a single dip. Eosin is a red dye that emphasizes cytoplasmic structures and collagen fibers in tissues, allowing for more detailed inspection.

After staining with eosin (if desired), the slides are dehydrated in successive grades of alcohol to eliminate water and excess dye. After that, the slides are cleaned with xylene. Clearing chemicals such as xylene aid in the transparency of tissue slices, allowing them to be mounted. The slides are mounted using DPX, a typical mounting media used to preserve stained tissue slices under a coverslip for microscopic analysis. The commercially available Mason trichrome stain kit (Merk) was used for staining the slides. The slides were washed in increasing grades of alcohol and two observers examined the slides under the microscope and graded the intensity, clarity, translucency, and contrast of the two stains. The grading was tallied, and statistical analysis was performed with SPSS 20 software. To assess interobserver agreement, an independent t Test and Kappa statistics were utilized.

RESULTS

When the MMT stain and VOF stain were compared based on staining parameters such as mean background (p-value = 0.762), mean morphology (p-value = 0.421), mean nuclear characteristics (p-value = 0.220), mean staining (p-value = 0.010) and mean differentiation, the VOF stain was shown to be significantly more effective than the MMT stain (Refer Table 1). Even when comparing hard tissue samples under a microscope, VOF stain enabled a clear visualization and identification of stained components such as mature bone and immature trabecular bone by producing vivid and distinct colors. Mean morphology typically pertains to the average morphological characteristics or features observed in stained cells or tissues. This includes the shape, size, and structural properties of the stained structures, such as cells or organelles (Figure1and 2).

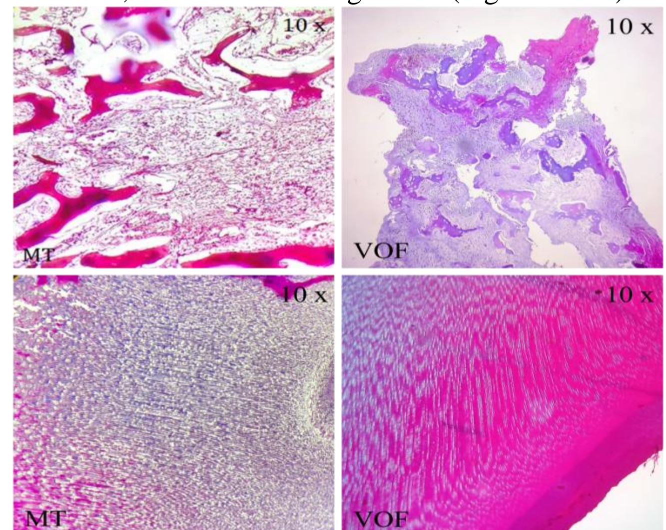

Fig 1. Comparing the features between different stains. The mean background intensity (A), nuclear stain (B), morphology (C), staining characteristics (D), and differentiation (E) of the VOF stain are better than the MMT stain.

Aditya Mantri, Sinduja Palati.Effectiveness of Modified Masson Trichrome Staining vs Modified VOF Staining for Hard Tissue Pathologies. Bulletin of Stomatology and Maxillofacial Surgery. 2025;21(3). 34-40 doi: 10.58240/1829006X-2025.3-34

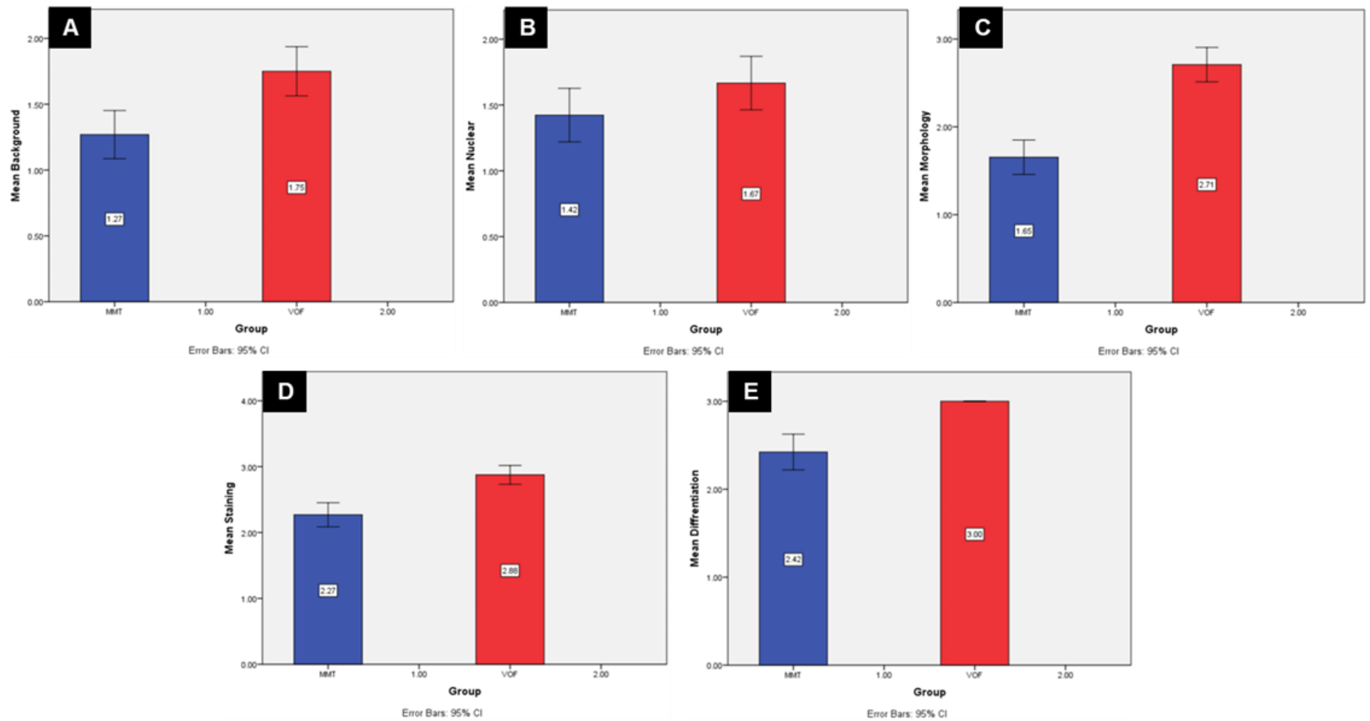

Figure 2. Photomicrograph of a hard tissue sample stained with modified Masson's Trichrome (A). It demonstrates clear and visible differentiation of fine and coarse collagen fibers and their arrangement and it provides contrasting and distinguishable features, allowing examination of tissue architecture, and collagen distribution. Photomicrograph of the hard tissue sample stained with modified VOF staining (B). It provides vivid and distinct colors, allowing for clear visualization and identification of the stained structures. It also provides good contrast between the stained components and the background, making it easier to observe and analyze the targeted structures.

Table1. Comparing the characteristic properties of the stains

Characteristics		**Mean ± Std Deviation**	**p Value**
Background	MMT	1.26 ± 0.45	0.762
	VOF	1.75 ± 0.44	
Morphology	MMT	1.65 ± 0.48	0.421
	VOF	2.70 ± 0.46	
Nuclear characteristics	MMT	1.42 ± 0.50	0.220
	VOF	1.66 ± 0.481	
Staining	MMT	2.26 ± 0.452	0.010
	VOF	2.87 ± 0.33	
Differentiation	MMT	2.42 ± 0.50	0.00
	VOF	3	

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DISCUSSION

The study's findings revealed that VOF stain is superior to MMT stain in terms of its characteristic staining qualities and differentiates the stained structures of the sample better than MMT stain. Bone development in various skulls and lesions might be mature or immature. Immature bone has a larger percentage of osteocytes than mature bone. It has two subtypes: woven bone and coarsely bundled bone [12].

Coarsely bundled bone (immature bone) is distinguished from woven bone (immature bone) by the presence of thick collagen bundles, the majority of which are oriented parallel to one another with osteocytes in between. However, with H and E staining, the distinction may not always be evident [12,13]

Mature bone differs from immature bone in several microscopic ways, including 1) a relatively even acidophilic staining of its matrix, 2) a relatively regular arrangement of its lamellae, and 3) the presence of fewer, more evenly distributed osteocytes in flatter lacunae. However, it may be challenging to categorize hard tissues using such a detailed description because the calcified components may manifest at different phases of maturity [14,15]. As a result, bone and other hard tissue components, as well as soft tissues, are eosinophilic in typical H and E-stained sections, making differentiation difficult [16]. For simple identification, a dye combination can stain hard and soft tissue structures differentially. In these cases, trichrome and tetrachrome staining techniques are advantageous.

JM Tater et al employed histochemical staining with hematoxylin and eosin (H&E) and tetrachromatic Verde Luz-orange G-acid fuchsin (VOF) to distinguish between mature and immature (osteoid production) in ossifying fibroma [17]. After H&E staining, bone and osteoid in ossifying fibromas showed varying degrees of pink staining, as well as soft tissue components including collagen. Collagen was dyed blue by VOF, while osteoid and bone were stained purple-red. The blue, purple, and red VOF staining made it simpler to detect structures inside peripheral and central ossifying fibromas. In addition, H&E staining requires many stages, whereas VOF requires only one [18].

Soft tissue stroma in Masson's trichrome commonly merges with calcification colors while being distinct over H and E and staining in the same slightly different shades of blue, green, and red. However, if the bone is sparse, it becomes challenging to identify mature bone in Masson's trichrome because the mineralized bone takes on the same tones of color as the stroma of the surrounding soft tissue [19].

H&E, Masson's Trichrome, and Modified Gallego's Stains were compared in a research by P Singh et al. to distinguish between healthy and diseased hard tissues. The results were 75, 50, and 45% for MG, MT, and H&E, respectively [20]. The results showed that while H&E stain is often used for regular histopathology exams and provides an overall evaluation of tissue form, MMT stain is particularly useful for assessing collagenous connective tissues and fibrosis [21,22]. A multi-step procedure called MMT stain is used to selectively stain distinct tissue components using a variety of dyes. Muscle fibers turn red, nuclei turn black or brown, keratinized tissues turn black or brown, and collagen fibers turn blue and hard tissue diseases such fibro-osseous lesions and others that are challenging to identify, the MG stain may be a viable differential stain. The MG stain helps clinicians identify ambiguous lesions, determine the type and etiology of lesions, and distinguish between aggressive and nonaggressive lesions. This information helps them comprehend the biological activity of the lesions and develop effective treatment plans [23].

Several investigations have demonstrated that Modified VOF staining uses only one step and takes less time than H and E and Masson's trichrome staining, and it is also less expensive [24]. It stains all hard tissue components, including bone, cementum, and dystrophic deposits, and differentiates them from stromal components. The stain's efficiency is further supported by the fact that the smallest calcified deposits pick up the stain brilliantly against the pale blue collagen background, interpreting it as considerably simpler. Degenerating bone and tumor osteoid also have a distinct staining pattern [24,25]. Because VOF stain appears to be reliable in recognizing hard tissue components of stroma, it may be used to determine the nature of calcified deposits in all suspected cases of normal/abnormal calcifications. This would certainly help with pathology treatments.

CONCLUSION

The study compared the efficacy of Modified VOF staining and Modified Masson Trichrome staining in evaluating hard tissue pathologies. The results showed that Modified VOF staining outperformed Modified Masson Trichrome staining across several

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key parameters, including background clarity, morphological detail, nuclear feature definition, overall staining quality, and differentiation. Modified VOF staining provided superior contrast and translucency, making it easier to distinguish between different tissue components. This enhanced clarity is particularly beneficial for accurately identifying and diagnosing various hard tissue pathologies.

Furthermore, the Modified VOF stain demonstrated a higher mean staining intensity and better differentiation of tissue types, which are crucial for precise histological analysis. These attributes make Modified VOF staining a more reliable and effective method for examining decalcified bone samples and other hard tissues. Given its advantages in terms of clarity, contrast, and overall staining quality, Modified VOF staining should be considered the preferred technique for histopathological evaluation of hard tissue lesions.

In conclusion, this study highlights the superiority of Modified VOF staining over Modified Masson Trichrome staining for hard tissue pathology. The findings suggest that adopting Modified VOF staining in clinical and research settings could improve the accuracy and reliability of histological evaluations.

DECLARATIONS

Conflicts of interest and financial disclosures

The authors declare no conflict of interest and there was no external source of funding

Ethical Approval

The ethical clearance was obtained from the Institutional Ethical Review Committee

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