International Medicine and Health Sciences Congress (IMHSC'24)
5-7 November 2024, Male-Maldives

Optimization of Gram Stain for Its Application in Tissues

Karol García-Aguilar
Department of Pathology
School of Medicine, Autonomous University of Nuevo Leon
Monterrey, Nuevo Leon, Mexico
karolgarciaag@gmail.com

Yareth Gopar-Cuevas
Department of Pathology
School of Medicine, Autonomous University of Nuevo Leon
Monterrey, Nuevo Leon, Mexico
yaregopar@gmail.com

María-de-Lourdes Chávez-Briones
Department of Pathology
School of Medicine, Autonomous University of Nuevo Leon
Monterrey, Nuevo Leon, Mexico
mdelchavezb@gmail.com

Marta Ortega-Martínez
Department of Pathology
School of Medicine, Autonomous University of Nuevo Leon
Monterrey, Nuevo Leon, Mexico
marta.ortegamrt@uanl.edu.mx

Gilberto Jaramillo-Rangel
Department of Pathology
School of Medicine, Autonomous University of Nuevo Leon
Monterrey, Nuevo Leon, Mexico
gilberto.jaramillorn@uanl.edu.mx

***Abstract*—Gram stain remains one of the most universally used techniques for microbiological diagnosis. In clinical practice, situations may arise in which the establishment of the etiology of certain bacterial infections is limited to histological findings. Although conventional Gram stain works very well to identify bacteria in smears from liquid samples, its application is not appropriate for tissue sections, because connective tissue and other elements of biopsies stain easily and nonspecifically with the dyes used in the technique, which complicates the visualization of bacteria. Therefore, the objective of this work was to optimize Gram stain to be able to detect bacteria in histological sections where the morphology of the tissue can be appreciated, thus increasing the possibility of observing the histopathological pattern underlying the disease. For this, sections from patients previously diagnosed with mycetoma or pyelonephritis were used. These sections were stain used in the standard protocol of Gram staining technique. Subsequently, the sections were immersed in tap water for two to three hours (depending on the tissue) to release excess dye. This step constitutes our optimization parameter. With the optimization carried out on the Gram stain in this work, it is possible to detect and differentiate bacteria and at the same time appreciate the morphology of the tissue, thus increasing the possibility of observing the histopathological pattern underlying the disease. Therefore, this technique could be of great support in the clinical setting for the histopathologist in the diagnosis of infections due to this type of agents.**

Keywords*—*Gram stain, biopsies, bacterial infections, diagnosis

I. Introduction

Today, Gram stain remains one of the most universally used techniques for microbiological diagnosis due to its simplicity, speed, and effectiveness. Its ability to demonstrate bacterial morphology (cocci, bacilli, coccobacilli) and distinguish between gram-positive bacteria (stained purple) and gram-negative bacteria (stained red to pink) based on the chemical and structural composition of the cell walls of both varieties, make it a great tool for the detection of presumed pathogenic agents, development of an initial diagnosis and preliminary therapeutic approach [1-3].

In clinical practice, situations may arise in which the establishment of the etiology of certain bacterial infections is limited to histological findings [4]. Although conventional Gram stain works very well to identify bacteria in smears from liquid samples, its application is not appropriate for tissue sections, because connective tissue and other elements of biopsies stain easily and nonspecifically with the dyes used in the technique, which complicates the visualization of bacteria [5, 6]. Commonly, the standard stain for the histopathological evaluation of tissue sections is hematoxylin and eosin (H&E) staining, since it allows recognition of the pattern of inflammation associated with the bacterial microorganism in question. Despite some infectious agents or their pathological effects can be seen with such staining, such a technique is not designed to differentiate between gram-positive and gram-negative bacteria and, therefore, makes its detection difficult [7, 8].

To solve these problems, modified Gram stains have been developed, which vary in the destaining agent and counterstain used. Among them is the Brown-Hopps technique, in which gram-positive bacteria are stained blue, gram-negative bacteria are stained purple-red, and the cell nuclei acquire a reddish brown tone. The background (the tissue) stains yellow, allowing to highlight the presence of bacteria [9]. Becerra and collaborators modified the traditional Gram stain with a very similar approach, managing to increase the contrast between the infected tissue and the bacteria present in it [6]. The disadvantage is that with both stains important morphological details are lost that would allow the inflammatory response to be associated with the infection.

Therefore, the objective of this work was to optimize Gram stain to be able to detect bacteria in histological sections where the morphology of the tissue can be appreciated, thus increasing the possibility of observing the histopathological pattern underlying the disease.

II. Methodology

Serial sections of skin and kidney biopsies were used from patients previously diagnosed with mycetoma or pyelonephritis. These sections were deparaffinized in xylol and hydrated in decreasing concentrations of alcohol and finally in water. Conventional Gram stain reagents (crystal violet, lugol, acetone/isopropanol, and safranin) were applied at concentrations and times used in the standard protocol, alternating washes with tap water between each step [10]. Subsequently, the sections were immersed in tap water for two to three hours (depending on the tissue) to release excess dye. This step constitutes our optimization parameter. Finally, the sections were dehydrated and mounted.

To compare the results obtained with our optimized Gram stain, Brown-Hopps staining was also performed on the serial sections described above. The Brown-Hopps staining reagents (crystal violet, lugol, acetone, and safranin) were applied, alternating washes with tap water between each step. Subsequently, Gallegos' differentiating solution was placed on sections and then rinsed with water. Then, in the following order, the samples were treated with acetone, picric acid-acetone solution, and acetone-xylol solution [9]. Finally, the sections were rinsed with xylol and then mounted.

A Primo Star brightfield microscope was used with an Axio-Cam ICc1 camera attached (both from Carl Zeiss Microscopy GmbH, Oberkochen, Germany). High-resolution color images of the sections were captured at magnifications of 400x and 1000x.

III. Results

Figure 1 shows images of the optimization carried out on mycetoma samples. Sections were immersed in water for two to three hours after being stained with the Gram technique. As time passed, was possible to observe the gradual release of excess dye captured by the tissue, which improved contrast, and allowed bacteria to be detected and tissue morphology to be appreciated.

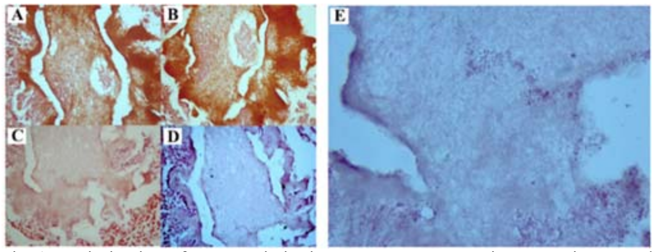

Fig. 1. Optimization of Gram stain in tissues. Mycetoma sections were immersed in water at progressive times after being stained with the Gram technique. A is the shortest time and D is the longest time. As the time passed, it was possible to notice the gradual release of excess dye captured by the tissue. E is the image D at higher magnification. The presence of gram-positive cocci is observed in the tissue and simultaneously the tissue architecture is appreciated. A-D: 400x; E: 1000x.

Figure 2 shows sections of pyelonephritis to which the Gram stain optimized in this work was applied and sections to which the Brown-Hopps stain was applied. The morphology of the tissue is best appreciated with the optimized Gram technique, with which it is also possible to detect bacteria efficiently.

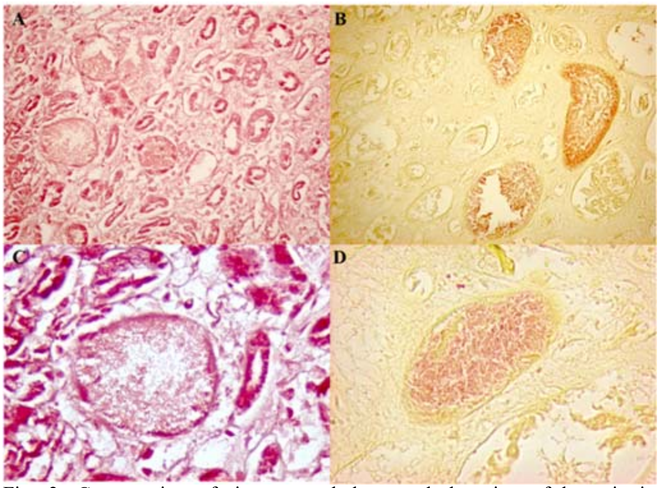

Fig. 2. Conservation of tissue morphology and detection of bacteria in pyelonephritis sections with optimized Gram stain. A Optimized Gram stain. The tissue morphology and lesions caused by the infection can be seen. B Brown-Hopps stain. Notably, the contrast enhancement of this technique sacrifices recognition of tissue morphology. C Optimized Gram stain at higher magnification. Gram-negative bacilli are seen in the pyelonephritis lesion. D Brown-Hopps stain at higher magnification. Gram-negative bacilli are distinguished in the pyelonephritis lesion. A and B: 400x; C and D: 1000x.

Finally, Figure 3 depicts that with the optimized Gram stain bacteria can also be detected in samples that are not damaged by infection, while also showing the tissue architecture satisfactorily.

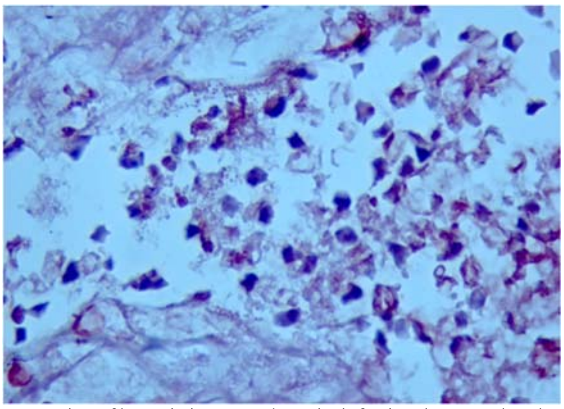

Fig. 3. Detection of bacteria in areas where the infection does not alter the tissue architecture. Optimized Gram stain on a mycetoma section. The presence of gram-positive cocci is observed. 1000x.

IV. Discussion

The Gram technique remains one of the main tools for bacterial detection and differentiation [11]. It has been observed that its application to tissue sections is not very effective, since the connective tissue and other elements of the biopsies stain very easily and in a non-specific way, thus making it difficult to visualize the bacteria and causing important morphological details to be lost [5, 6]. With the optimization carried out on the Gram stain in this work, it is possible to detect and differentiate bacteria and at the same time appreciate the morphology of the tissue, thus increasing the possibility of observing the histopathological pattern underlying the disease.

A critical step in optimized staining is immersing the tissue sections in water for 2 to 3 hours. This procedure releases the excess dye that the tissue captures and improves the contrast, which allows its morphology to be appreciated and at the same time to distinguish the bacteria present in it. It is interesting to note that many of the improvements in experimental techniques come, as in this case, from changes that appear to be simple or even obvious. For example, Becerra et al. modified the conventional Gram stain simply by adding saffron alcohol when dehydrating the tissue after staining it with the traditional Gram technique, considerably favoring bacterial detection [6].

Thus, in addition to standard stains such as H&E, the optimized Gram technique could be applied to detect bacteria in cases where infection is suspected. It is important to mention that the clinical interpretation of the Gram stain in tissues must take certain precautions, especially in situations in which there are areas of extensive necrosis where the bacteria present may be dead or with damage to their cell wall, which could reflect another staining pattern [12].

We believe that our technique has potential application in histopathological diagnosis, as currently pathologists continue to use H&E staining for the detection and classification of bacteria, which may lead to incorrect or inconclusive diagnoses [6]. The use of the optimized Gram stain in tissues could largely avoid these problems, and in many cases it could be of great support when choosing the initial antibiotic therapy and thus avoid the unnecessary use of broad-spectrum antibiotics or, in some cases, it could indicate the need for urgent treatment [2]. It should be noted that, regardless of the stain technique used, the morphology of the tissue will be variably lost in areas where the damage caused by the infectious agent, or by the host's immune response to it, alters the tissue architecture.

V. Conclussion

The Gram stain optimized in this work allows the unambiguous detection of gram-positive and gram-negative bacteria in tissues, while allowing the appreciation of tissular morphology. Therefore, this technique could be of great support in the clinical setting for the histopathologist in the diagnosis of infections due to this type of agents.

References

[1] P. R. Murray, K. S. Rosenthal, and M. A. Pfaller, Medical microbiology, 8th ed. Philadelphia: Elsevier, 2016, pp. 106-108.

[2] L. J. Boyanova, Direct Gram staining and its various benefits in the diagnosis of bacterial infections, Postgrad. Med., vol. 130, pp. 105-110, January 2018.

[3] T.J. Beveridje, Use of the gram stain in microbiology, Biotech. Histochem., vol. 76, pp. 111-118, May 2001.

[4] K. Imrit, M. Goldfischer, W. Jie, J. Green, J. Levine, J. Lombardo, et al., Identification of bacteria in formalin-fixed, paraffin-embedded heart valve tissue via 16S rRNA gene nucleotide sequencing, Clin. Microbiol., vol. 44, pp. 2609-2611, July 2006.

[5] R. W. Horobin, and J. D. Bancroft, Troubleshooting histology stains, 1st ed. New York: Churchill Livingstone, 1998.

[6] S. C. Becerra, D. C. Roy, C. J. Sanchez, R. J. Christy, and D. M. Burmeister, An optimized staining technique for the detection of Gram positive and Gram negative bacteria within tissue, BMC Res. Notes., vol. 9, p. 216, April 2016.

[7] G. W. Procop, and M. Wilson, Infectious disease pathology, Clin. Infect. Dis., vol. 32, pp. 1589-1601, June 2001.

[8] G. L. Woods, and D. H. Walker, Detection of infection or infectious agents by use of cytologic and histologic stains, Clin. Microbiol. Rev., vol. 9, pp. 382-804, July 1996.

[9] R. C. Brown, and H. C. Hopps, Staining of bacteria in tissue sections: a reliable gram stain method, Am. J. Clin. Pathol., vol. 60, pp. 234-240, August 1973.

[10] R.B. Moyes, J. Reynolds, and D.P. Breakwell, Differential staining of bacteria: gram stain, Curr. Protoc. Microbiol. Appendix 3:Appendix 3C, November 2009.

[11] H. A. Alturkistani, F. M. Tashkandi, and Z. M. Mohammedsaleh, Histological stains: a literature review and case study, Glob. J. Health Sci. vol. 8, pp. 72-79, June 2015.

[12] R. W. Brown, Histological preparations: common problems and their solutions college of american pathologists, 1st ed. Northfield: College of American Pathologists, 2009, pp. 49-56.