Original Article

Impact of Reagent Age and Storage Temperature on the Diagnostic Accuracy of the Gram Stain: A Quality Control Study in a Microbiology Laboratory at Tertiary care setting in North Gujarat, India

Komal Patel[1], Keyur Chauhan[2], Pankaj Raval[3]

[1]Professor and Head, Department of Microbiology, Bhagyoday Medical College, Kadi, Gujarat, India. [2]Assistant Professor, Department of Microbiology, Bhagyoday Medical College, Kadi, Gujarat, India. [3]Assistant Professor, Department of Microbiology, GMERS Medical College, Vadnagar, Gujarat, India

Abstract

Background: Gram staining is still one of the most commonly applied diagnostic assays in the clinical microbiology laboratory. Technical skill is not alone responsible for the quality of Gram stain results; the stability and correct storage of staining reagents are also crucial. Staining quality may be affected by reagent degradation resulting from exposure to inappropriate storage temperatures and/or the passage of time, which can cause errors in diagnosis. The study was a cross-sectional test that involved a total of ninety-two isolates of various bacterial species. This study revealed that the diagnostic accuracy of Gram staining is affected by the age of the reagent and by the storage temperatures. Quality control recommendations for routine Microbiology laboratory for the use of Gram staining were made. **Material and Methods:** A quality control study was performed in the laboratory using the standard strains of bacteria such as staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Bacillus subtilis. Reagents used were crystal violet, Gram's iodine, decolorizer and safranin stored at 0, 25, 37°C for 0, 1, 3 and 6 months respectively. The reagents were used to stain standardized bacterial smears. The quality of staining was done by evaluating the preservation of the morphology, staining intensity, clarity of background, and correct Gram reaction. The accuracy of diagnosis was estimated by comparing with expected staining characteristics. **Results:** Fresh reagents showed the greatest accuracy in diagnosis (99.2%). They could be used for six months without noticeable loss of performance and were stored at the low temperature of 4°C. Samples of reagents held at 37ºC showed a significant deterioration, with diagnostic accuracy down to 86.5% after six months. The biggest degradation was found with the crystal violet and the iodine reagents. Aged reagents showed reduction in staining intensity, irregular Gram reaction and background artifacts. **Conclusion:** The performance of gram stain is greatly affected by the age of reagents and the temperature of the storage. Refrigerated storage helps to maintain the stability of the reagents while maintaining the diagnostic value. To maintain reliable microbiological diagnosis both routine quality control procedures and scheduled replacement of reagents are necessary.

Keywords: Gram stain, quality control, stability of reagents, storage temperature, laboratory diagnosis, microbiology laboratory.

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Introduction

Gram staining is one of the most widely used and important staining technique in clinical microbiology, and is routinely used as one of the initial tests to be carried out on clinical specimens in the routine diagnostic laboratory.[1,2] It is very useful in providing information about bacterial morphology, arrangement and the presence of inflammatory cells in clinical specimens. Patient management would be impacted by the results, making it very important to have accurate and good quality Gram stain results.[3-6]

The four main reagents used in staining are crystal violet, Gram's iodine, decolorize and counterstain which is safranin. The integrity and performance of these reagents are directly related to staining results. Hence, ensuring the efficacy of the staining reagents by frequent monitoring of the staining procedures and performance of the reagents has been a priority for quality assurance programs.[7-12]

So, although the Gram staining is widely practiced, there are few data available that provide recommendations for a reagent storage time and replacement schedule that would support quality control in microbiology laboratories under normal use.[13] Laboratory standards like the ISO 15189 or the WHO Laboratory Quality Guidelines place a high degree of importance on reagent validation and monitoring of reagents in the laboratory.[15,16] It is essential to understand how environmental conditions affect the stability of laboratory reagents to maintain consistent staining quality and provide reliable laboratory results.

Address for correspondence: Dr. Komal Patel, Professor and Head, Department of Microbiology, Bhagyoday Medical College, Kadi, Gujarat, India.
E-mail: drkomalpatel3011@gmail.com

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Objectives: To evaluate the impact of reagent age and storage temperature on the diagnostic accuracy of Gram staining and to establish quality control recommendations for routine microbiology laboratories. The findings are expected to contribute to laboratory quality management programs and improve diagnostic reliability in routine microbiology laboratories.

Materials and Methods

A prospective laboratory-based quality control study was conducted over a six-month period from 1/1/2023 to 30/6/2023 in a Microbiology Laboratory at Tertiary care setting in North Gujarat, India following standard microbiological quality assurance principles.[17] American Type Culture Collection (ATCC) reference strains commonly recommended for Gram stain quality control were used.[18]

*Staphylococcus aureus ATCC 25923 (Gram-positive cocci)
* Bacillus subtilis ATCC 6633 (Gram-positive bacilli)
* Escherichia coli ATCC 25922 (Gram-negative bacilli)
* Pseudomonas aeruginosa ATCC 27853 (Gram-negative bacilli)

Fresh Gram staining reagents including crystal violet, Gram's iodine, acetone-alcohol decolorizer, and safranin were prepared according to standard microbiological laboratory procedures described in established microbiology manuals.[3,7] Reagents were stored under three temperature conditions: Group A: Refrigerated storage (4°C),Group B: Room temperature storage (25°C),Group C: Elevated temperature storage (37°C).The selected storage temperatures were based on commonly encountered laboratory conditions and previous studies evaluating reagent stability.[8,19]

Standardized bacterial suspensions equivalent to 0.5 McFarland standard were prepared according to CLSI recommendations.[18] Uniform smears were prepared on clean grease-free glass slides and heat-fixed before staining. Gram staining was performed according to the conventional method originally described by Gram and subsequently standardized in diagnostic microbiology manuals.[1,3] Stained smears were independently examined by two experienced microbiologists. Assessment criteria included correct Gram reaction, staining intensity, morphology preservation, background clarity, and overall staining quality as recommended in laboratory quality control guidelines.[16-20]

Data analysis was performed using SPSS software. Mean quality scores and diagnostic accuracy percentages were calculated. Statistical significance was determined using one-way ANOVA, with $p < 0.05$ considered statistically significant.

Results

A total of 480 Gram-stained smears were evaluated during the study. Each bacterial strain was stained using reagent sets stored under different temperature conditions and tested at predefined storage intervals. Freshly prepared reagents demonstrated excellent performance with an overall diagnostic accuracy of 99.2%. Minor interpretation errors were attributed to observer variation rather than staining quality.

Reagents stored at 4°C maintained high diagnostic performance throughout the study period. Diagnostic accuracy remained above 95% even after six months of storage. In contrast, reagents stored at 25°C showed gradual deterioration over time. The most pronounced decline was observed among reagents stored at 37°C.

Table 1: Diagnostic Accuracy According to Storage Temperature and Reagent Age

Storage Condition	Fresh(%)	1Month(%)	3Month(%)	6Month(%)
4oc	99.2	98.7	97.8	96.1
25oc	99.2	97.3	94.6	91.4
37oc	99.2	95.1	90.2	86.5

The reduction in diagnostic accuracy with increasing reagent age was statistically significant ($p < 0.05$). Elevated storage temperature accelerated deterioration of staining performance.

Crystal violet retained optimal staining characteristics in refrigerated conditions. However, reagents stored at 37°C exhibited progressive loss of staining intensity after three months. Gram-positive organisms frequently appeared pale purple rather than deep violet, increasing the likelihood of misinterpretation.

Table 2: Mean Staining Quality Scores

Storage Condition	Fresh(%)	1Month(%)	3Month(%)	6Month(%)
4oc	4.9	4.8	4.7	4.5
25oc	4.9	4.6	4.3	4.0
37oc	4.9	4.4	3.8	3.3

Morphological preservation remained satisfactory in all groups during the first month. After prolonged storage at elevated temperatures, increased background staining and reduced cellular definition were observed. Gram-positive cocci appeared less distinct, while Gram-negative bacilli showed variable counterstaining.

Crystal violet and Gram's iodine were identified as the most temperature-sensitive reagents. These reagents demonstrated visible color fading and precipitation after prolonged storage at 37°C. The decolorizer remained relatively stable throughout the study period. Safranin showed minimal deterioration and maintained acceptable staining characteristics under all storage conditions.

Quality Control Observations

Daily staining of control strains revealed increasing variability in staining quality with aging reagents. Slides stained using six-

month-old reagents stored at 37°C frequently required repeat staining due to suboptimal results.

Discussion

Gram staining is still a fundamental step in microbiological diagnosis and continues to be an important procedure in every microbiology laboratory. The current study shows that the performance of Gram stain and diagnostic reliability vary significantly with the age of the reagent and with storage temperature. It was also found that freshly prepared reagents gave the best staining results, in corroboration with other observations that the integrity of reagents is of utmost importance for the correct determination of the bacteria. Such results have also been observed in laboratory quality assurance studies highlighting the need for reagent standardization and monitoring.[1-3]

The present study showed that over 6 months, refrigerated storage helped to maintain the reagent performance well. Good reagent stability and low chemical degradation due to storage at low temperatures maintained the accuracy of the diagnosis greater than 95%. Storage in the storage room at room temperature was likely to cause moderate deterioration after three months, but after a month had passed the deterioration was less. While good diagnostic diagnostics was preserved, there was a slight decrease in staining intensity and background clarity was noted. The results suggest that storage at ambient temperature might be possible for short term use, but might not be recommended for long-term preservation of reagents.[7-9]

The highest loss of performance was recorded with the reagents stored at 37°C, which may affect the molecular structure of the staining compounds because high temperatures increase chemical reactions. Crystal violet and Gram's iodine seemed to be the most thermally unstable. There was an increase in the false Gram-negative interpretation of Gram-positive bacteria with weak primary staining. This is clinically important as incorrect gram will have an impact on the choice of treatment for the bacterial infections. Pathogen misclassification can result in delayed treatment and patient outcomes.[10-12]

The study also revealed that crystal violet and iodine make significant overall contributions to the quality of the stains. The primary stain complex is made up of crystal violet and the iodine is used as a mordant to secure dye uptake in the wall of Gram-positive bacteria. Among the various reagents, Safranin was the most stable, which is the most important criterion for Gram differentiation.[13-15] Other laboratory tests have shown that counterstains do retain satisfactory performance after prolonged storage.[16]

The QC results highlight the need for periodic testing with reference bacterial strains. Known Gram positive and Gram negative organisms are checked on a daily basis which enables the detection of deterioration of reagent before serious diagnostic error is made. The ISO 17025-series laboratory accreditation standards suggest that staining procedures be periodically checked as a component of a broader quality management system.[17-19]

The results obtained also have practical applications for the laboratories in tropical areas. The ambient temperatures in many areas are often above 30°C, which can lead to rapid degradation of reagents. It is therefore recommended that refrigerated storage might be useful in such environments.

There are numerous caveats to be noted, though. Firstly, this was a laboratory study with reference strains and not clinical specimens. Second, there were only four types of bacteria that were considered. Further research with a variety of organisms and clinical specimens would provide more validation of results. In spite of these restrictions the study offers valuable evidence to the temperature controlled storage and replacement of Gram stain reagents at regular intervals. Implementation of such measures can enhance diagnostic accuracy and enhance laboratory quality assurance programs.

Conclusion

The present QC study showed that the age of a reagent and the storage conditions of the reagent are significant factors that impact Gram stain diagnostic accuracy. Optimal results were obtained with fresh reagent, a progressive decrease in staining performance was observed with increasing storage time, especially at high temperatures. The reagent stability and the diagnostic accuracy remained at more than 95% over the 6-month evaluation period in refrigerated storage at 4°C. Reagents kept at 37°C showed significant reductions in staining quality and diagnostic reliability, in contrast. Crystal violet and Gram's iodine were found to be the most temperature-sensitive parts of the Gram staining. Consistent and reliable Gram stain results require routine quality control with standard reference strains, proper storage of reagents, and regular replacement of reagents.

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Conflicts of interest

There are no conflicts of interest.

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