The Mycetoma Research Center,
University of Khartoum
WHO Collaborating Center
on Mycetoma & Skin NTDs

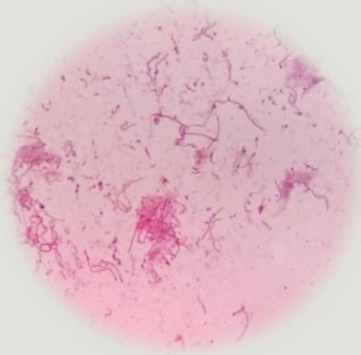
Standard Operating Procedures for Gram Staining for Identification of Myctoma Causative Microorganisms From Direct Specimens and Cultures

Background

Gram staining, devised by Hans Christian Gram in 1884, is a vital microbiological technique used for the initial identification and classification of bacterial species based on their cell wall properties. It categorises bacteria into Gram-positive and Gram-negative groups, each displaying distinct staining behaviours due to differences in their cell wall composition.

Gram-positive bacteria retain the crystal violet-iodine complex, appearing purple or blue. In contrast, Gram-negative bacteria, with thinner peptidoglycan layers and outer membranes, allow for counterstaining with safranin, resulting in pink or red colouration.

Purpose & Scope



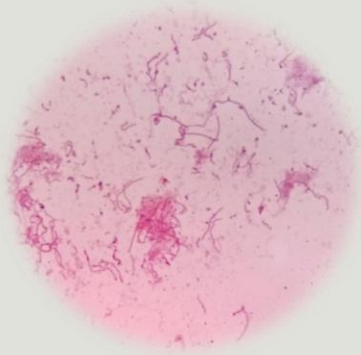
Purpose

The purpose of this Standard Operating Procedure is to outline the protocol for Gram staining to aid in the identification of causative microorganisms of mycetoma, facilitating accurate diagnosis and treatment selection.

Scope

This SOP is to be used by the laboratory personnel involved in using the Gram staining technique for the diagnosis of mycetoma causative microorganisms from clinical specimens.

Responsibilities

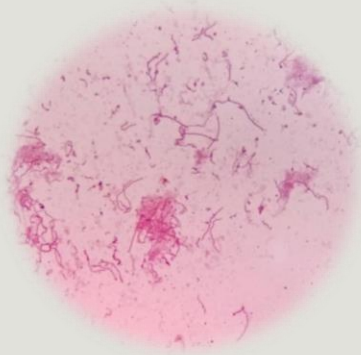


Laboratory Technicians: Responsible for performing the Gram staining procedure according to this SOP.

Laboratory Supervisor/Manager: Responsible for overseeing the implementation of this SOP and ensuring compliance.

Quality Assurance Officer: Responsible for monitoring and evaluating the quality control measures outlined in this SOP.

Safety Precautions



The mycetoma isolates pose no risk of infection. Although there have been no documented instances of hospital-acquired cross-infection, it remains crucial to exercise caution and observe safety protocols. Adequate storage facilities must be employed to ensure the safe utilisation of these grains for various diagnostic purposes.

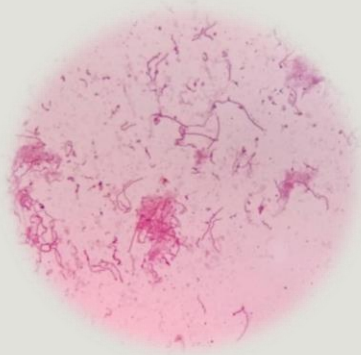
- Wear appropriate personal protective equipment (PPE), including gloves, laboratory coat, and safety goggles.
- Work in a Biosafety Level 2 (BSL-2) laboratory.
- Handle potentially infectious materials with care.
- Follow the established biohazard waste disposal procedures.

Sample Collection

Collect samples from mycetoma culture isolates using sterile techniques, ensuring minimal contamination.

Label containers with patient information and sample details.

Materials and Equipment



Bacterial culture.

Solution of crystal violet, gram iodine, 95% ethanol and safranine.

Clean glass slide.

Inculcating loop.

Bunsen burner.

Filter paper.

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Microscope.

Microscope slides

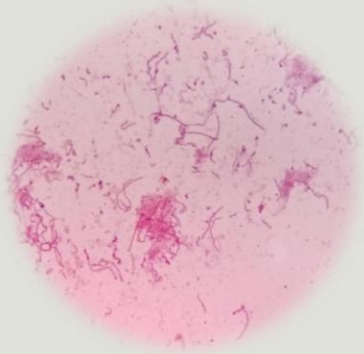
Inoculation loops or swabs

Immersion oil.

Staining rack.

Distilled water.

The Procedure



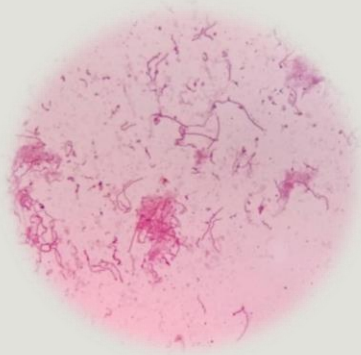
Prepare heat-fixed smears from the bacterial culture.

Label the slide's corner with a pencil.

Place a loopful of normal saline onto the center of a clean glass slide.

Aseptically transfer a minute amount from the culture isolate using an inoculating loop and mix it into the normal saline droplet.

The Procedure



Prepare heat-fixed smears from the bacterial culture.

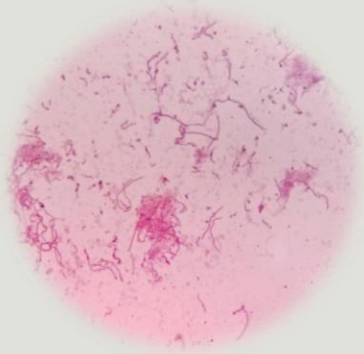
Spread this mixture across half an inch of the slide.

Allow the slide to dry.

Pass the slide through a Bunsen burner flame three times to heat-fix the bacteria .

Direct Smears from clinical specimens should be fixed with 95% ethanol.

Staining Procedure



Place the slide in the staining rack.

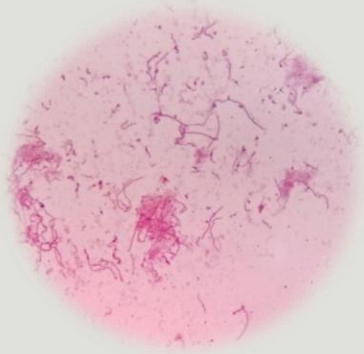
Flood crystal violet to cover the slide smears and allow it to stand for 30 seconds.

Rinse with water for 5 seconds.

Apply gram iodine and allow it to stand for one minute.

Rinse with water for 5 seconds.

Staining Procedure



Decolour using 95% ethanol for 15-30 seconds.

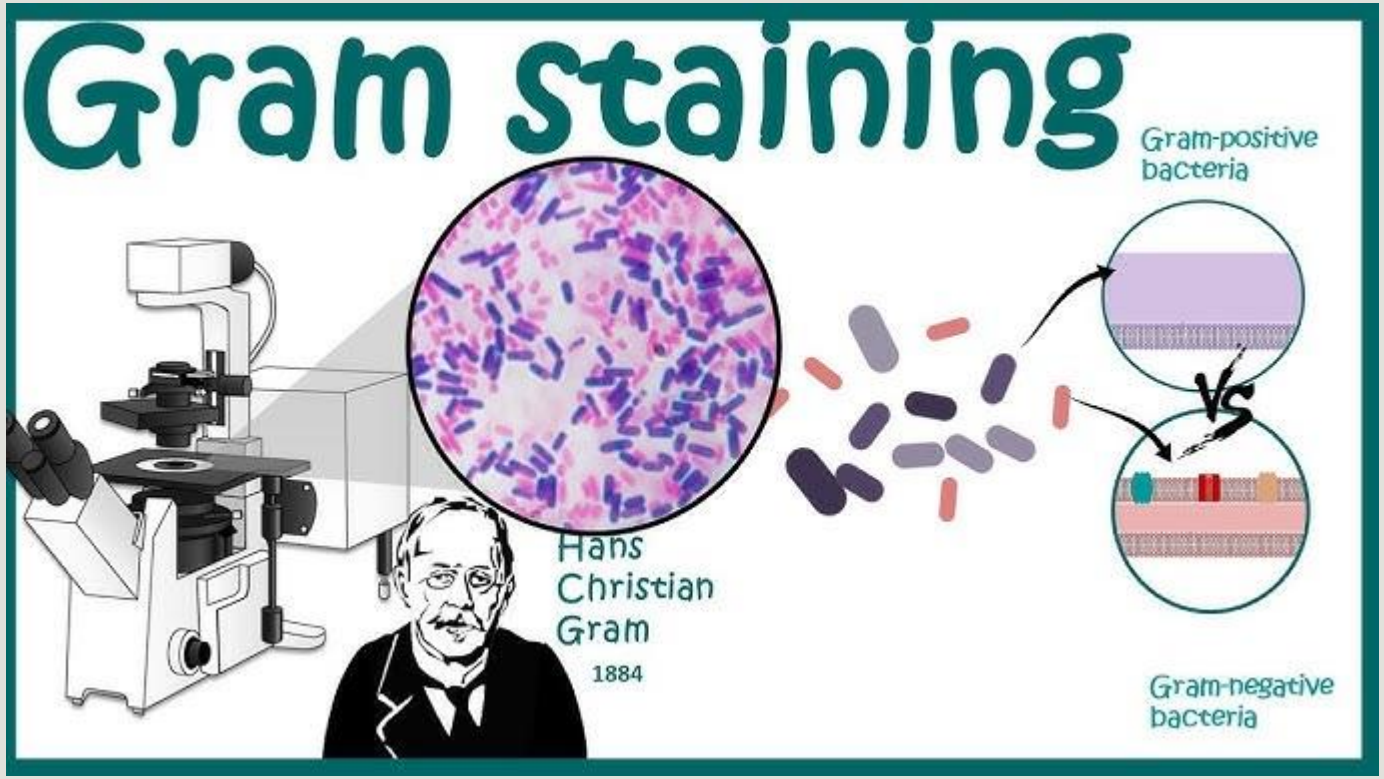
Rinse with water for 5 seconds.

Counterstain with safranin for approximately 80 seconds.

Rinse with water for 5 seconds.

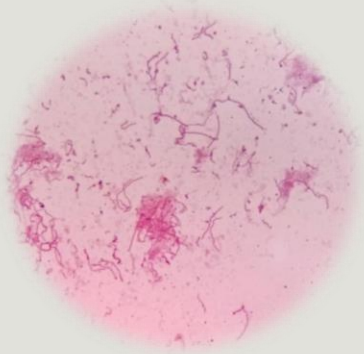
Blot dry using Filter paper.

Staining Procedure



https://www.youtube.com/watch?app=desktop&v=CbMGr9wFV2w

Observation



Observation

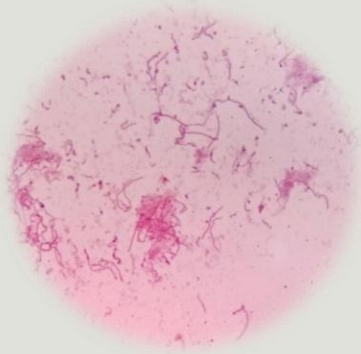
Examine the stained smear under a microscope using ×100 oil immersion lens .

Observe the morphology and staining characteristics of the microorganisms present.

Results

Each causative microorganism has a distinct positive or negative appearance.

Identification



Gram-positive bacteria will appear purple or blue, while Gram-negative bacteria will appear pink or red.

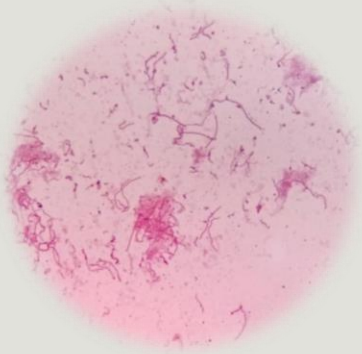
Identify the type of microorganism based on its staining pattern and morphology.

Reporting

Record observations of Gram staining results, including the presence of Gram-positive or Gram-negative bacteria.

Generate a report summarising the findings.

Limitation



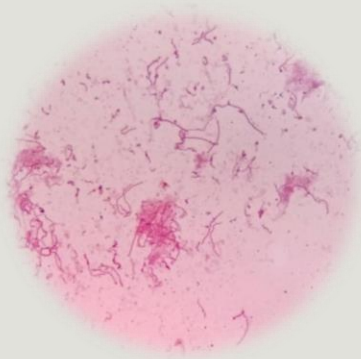
The duration of the decolourisation process (using ethanol/acetone) is crucial.

Thin smears necessitate less time compared to thick smears.

Excessive decolourisation will result in everything on the slide turning red, whereas

inadequate decolourisation will cause everything on the slide to remain purple.

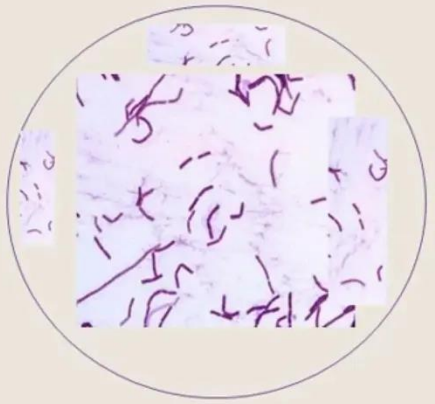
Quality Control Measures



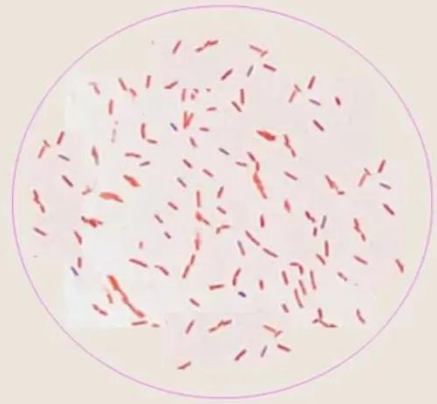
Quality control procedures should be conducted on a weekly basis.

Quality control involves examining prepared slides containing

Gram-positive and Gram-negative organisms. Staphylococcus aureus is Gram-positive, and E. coli is Gram-negative.

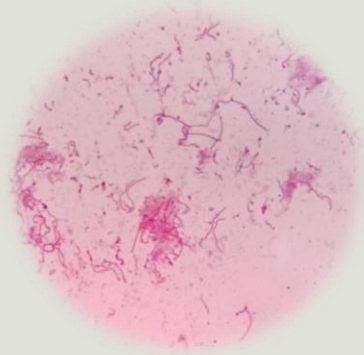


Gram-positive
Violent colour



Gram-negative
Pink colour

Quality Control Measures



Regular checks on staining reagents are performed for quality and expiration dates.

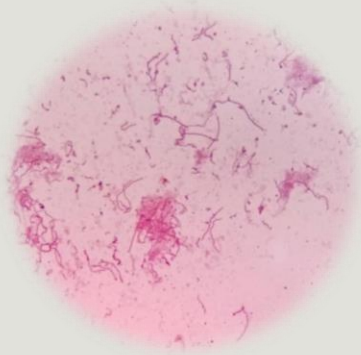
Include positive and negative control slides to ensure staining consistency.

Document the QC outcomes on the provided Gram stain QC sheet in Annex 1.

Additionally, any unacceptable QC results should be recorded on a nonconformity form to initiate problem analysis and corrective measures.

Review the QC findings monthly.

Procedures to follow if results exceed acceptable limits



Prepare new control slides.

If the results remain unacceptable, verify the quality of the reagent, ensure adherence to the staining protocol, and confirm the use of the correct reference strains.

Storage

Store the stained slides in slide boxes or folders for future reference.

Document Control

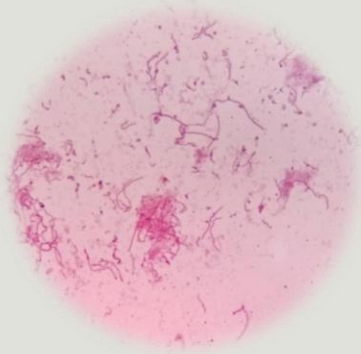
All personnel must record the details of the Gram staining procedures and any deviations from the standard protocol and document the results for future reference in the laboratory logbook.

Any revisions or updates to this SOP must be documented and communicated to relevant personnel.

Maintain detailed records of Gram staining procedures performed, including staining parameters and observations made.

Ensure documentation complies with institutional and regulatory standards.

Attachment



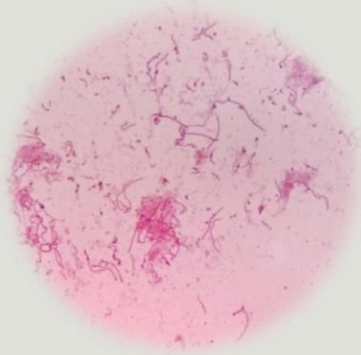
Annex 1: Quality Control Sheet Gram Stain.

Acceptable results:

- Gram positive: blue cocci (Staphylococcus aureas)
- Gram negative: red bacilli (E. Coli)

Record all unacceptable quality control results on nonconformity to start the problem analysis and corrective action process.

References



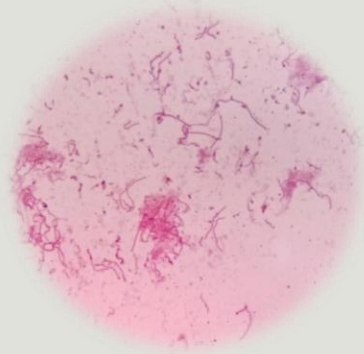
United States pharmacopoeia. https://www.usp.org/

William B. Whitman, Michael Goodfellow, Peter KämpferHans-Jürgen Busse. Bergey's Manual of Systematic Bacteriology, Volume 5: The Actinobacteria, 2012.

Murray PR, Baron EJ, Jorgenson JH. Pfaller MA, Yolken RH. eds., Manual of clinical microbiology 8th edition. ASM Press, 2003, 2113 pages, 2 vol, 2003. ISBN: 1-555810255-4.

Matthew J. Binnicker. Manual of Clinical Microbiology, 13th Edition. https://www.clinmicronow.org/doi/book/10.1128/9781683670438.MCM

Patient Safety Monitoring & International Laboratory Evaluation portal. Accessed on February 20, 2013. Available at: http://resources.psmile.org/resources/process-control/section-specificinformation/microbiology/bacteriology/Pro6.7-A-12%20Gram%20Stain%20%20.doc/view 1



Revision History

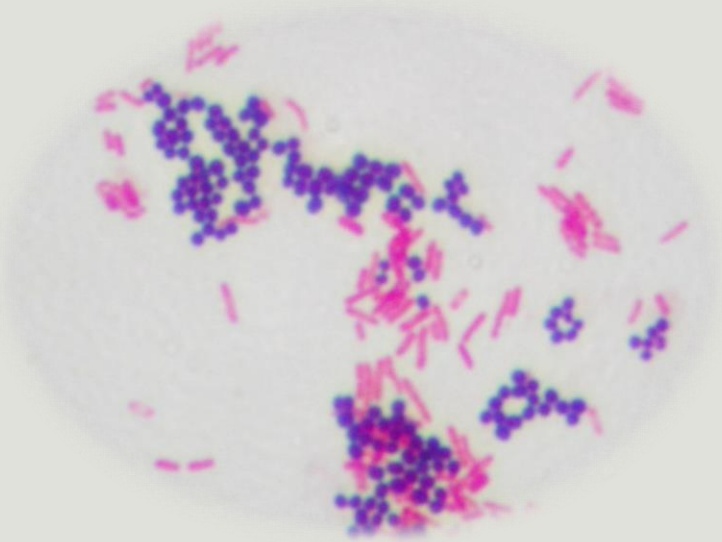
Version 1.0: [2017] - Initial SOP created.

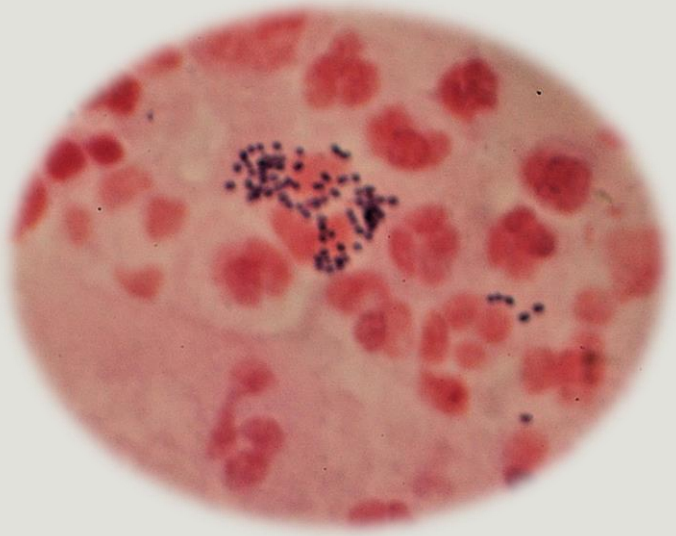
Version 1.1: [2019] - Minor revisions for clarity and accuracy.

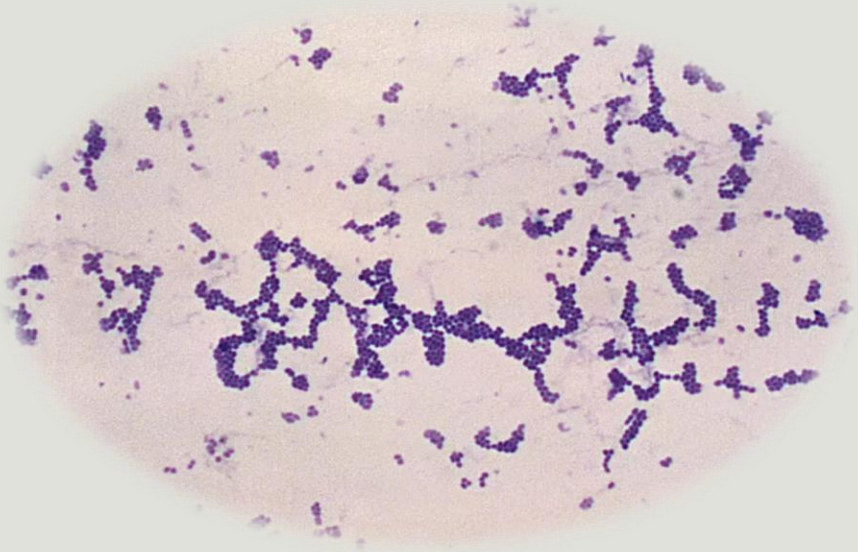
Distribution

Copies of this SOP shall be distributed to all personnel involved in grain culture.

Ensure that all personnel are trained and familiarised with the procedures outlined in this SOP before performing the grains culture technique.







Different Gram staining images

Approval

This Standard Operating Procedure has been prepared, reviewed and approved by:

Mrs Lubna Sulayman Elnour	Technologist	Lubna
Miss Nema Ahmed Alfaki	Technologist	Nema
Dr Abdallah Osman Ahmed	Senior scientist	Abdallah
Prof Ahmed Fahal	Center Director	Fahal

On May 3 2024.

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