Gram Stain of Unknown Bacterium from a Selective Agar Plate

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Introduction

In 1882, Hans Christian Gram, a Danish bacteriologist, introduced the gram stain for pneumonia-causing organism identification. It categorizes bacteria into gram-positive and gram-negative groups using specific dyes (Tripathi & Sapra, 2023). Gram-positive bacteria, with a thick peptidoglycan wall, retain the primary dye (crystal violet), appearing purple. Gram-negative bacteria, having a thin peptidoglycan layer and an outer cell membrane, lose the primary stain and retain the counterstain, often safranin, resulting in a pink coloration (Smith & Hussey, 2005). Figure 1 illustrates the gram staining process based on information from Smith & Hussey (2005).

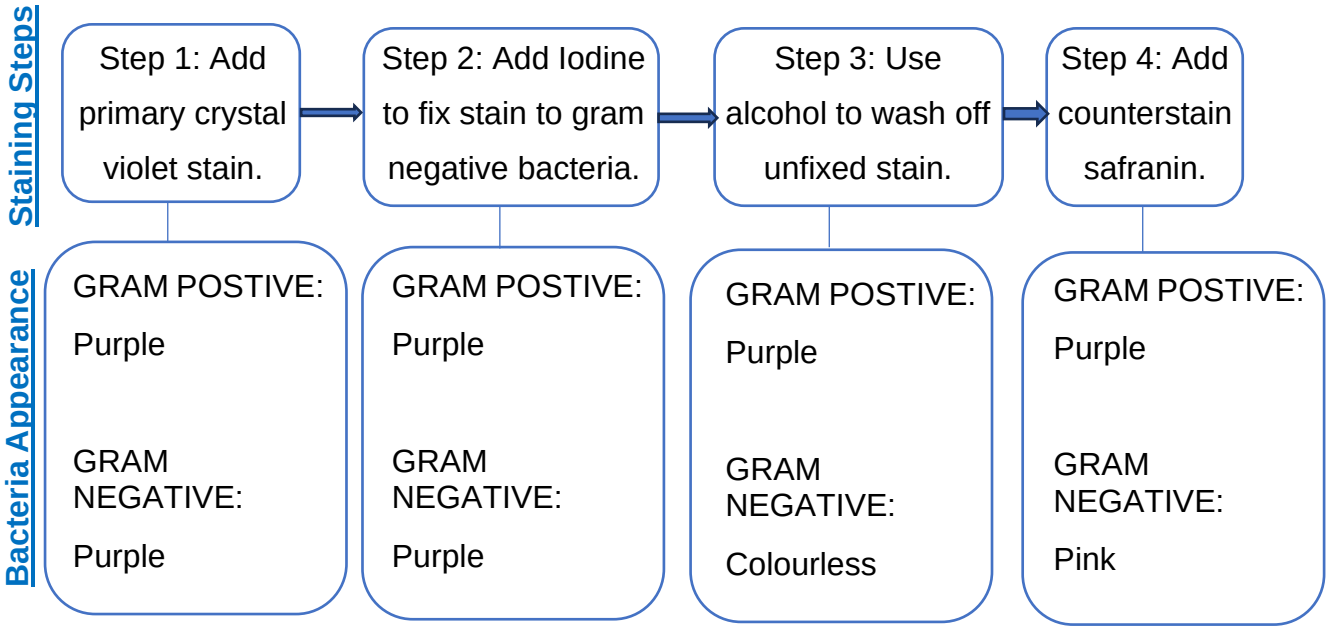

Figure 1: Staining steps used in gram staining and the appearance of the bacteria at each step as described by Smith & Hussey (2005).

As well as observing the stain that is retained, it is also possible to examine the basic shapes and organisation of the bacteria to gather more information. For example, gram-positive cocci arranged in clusters could indicate a *Staphylococcus* infection such as *Staphylococcus aureus.* Gram-negative diplococci could indicate a *Neisseria meningitidis* which causes meningitis (Tripathi & Sapra, 2023).

The Gram staining technique serves as a valuable tool in clinical microbiology, allowing for the identification of bacterial types within blood cultures. Its rapidity is a key advantage, offering clinicians swift preliminary insights. This quick turnaround proves particularly critical in the management of patients suspected of fast-acting infections, such as septic arthritis and meningitis (Thairu et al., 2014). Meningitis can be a life-threatening condition, with pneumococcal meningitis, caused by *Streptococcus pneumoniae*, exhibiting a mortality rate of approximately 30%. This pathogen is characterized by the presence of gram-positive cocci. Notably, it is the most common cause of meningitis in adults. Hence, the Gram stain's ability to quickly detect such pathogens plays a pivotal role in the timely and effective treatment of these infections (Young & Thomas, 2018).

The aim of this practical is to examine unknown bacterium against known controls using gram staining. The results will then be assessed and discussed to determine the outcome, validity, and likelihood of the results being reliable.

Method

Health and safety and PPE procedures were followed, including wearing gloves and a lab coat. Precautions were taken when handling the samples due to their infectious nature. COSHH assessments for the chemicals used were considered also. This practical was undertaken following method guidelines supplied by Lowery (2023).

The slides were prepared to ensure they were dust-free and grease-free by immersing them in 2% DECON-90 overnight, then rinsing with distilled water. A dry cloth was used to remove dust particles. Gloves and forceps were employed to prevent any fingerprint marks.

The specimens were then gathered, consisting of two unknown samples and four known controls. Unknown A was taken from a positive blood culture and Unknown B was taken from a wound swab that was cultivated on blood agar.

The prepared slides were wiped with 95% ethanol, dried, and kept covered until use. A bunsen burner was used to heat a platinum loop and left to cool. A droplet of distilled water was applied to the slide using the loop. After re-flaming the loop, a colony was extracted from the agar plate, spread on the slide, and emulsified in the water droplet. The loop was heated again. The smear was left to air dry and passed through the Bunsen burner flame three times for 1-2 seconds each time to achieve heat fixation.

This process was repeated for all samples, with clear labelling for each. Each slide was then gram stained. Gloves and forceps were employed for slide handling. The slide was positioned on a staining rack and covered with crystal violet stain for 30 seconds, followed by rinsing under a light stream of water to remove excess dye. Lugol's iodine was applied, and the slide was left for 30 seconds before rinsing with water. Alcohol was streamed over the slide for 10 seconds, followed by another water rinse. The slide was then returned to the rack and covered with safranin counterstain for 1 minute, which was subsequently washed off with water. After gentle blotting to dry, the slide was examined under a microscope using an oil immersion lens.

Results

The results from this practical are shown below in table 1.

Table 1: A table to show the observations of each sample following gram staining.

Sample	Retained Colour	Gram Negative or Positive?	Cell Shape	Organisation and Observations
Pseudomonas aeruginosa	Pink	Negative	Rods	Short rods, no obvious organisation. Some short chains. Distinctive Smell
Staphylococcus aureus	Purple	Positive	Cocci	Clusters
Bacillus subtilis	Purple	Positive	Rods	Chains, some quite long (~10-15 cells). Unstained internal regions visible.
Escherichia coli	Pink	Negative	Rods	Short rods, no obvious organisation. Some short chains.
Unknown A	Purple	Positive	Cocci	Chains, some quite long (~10-15 cells)
Unknown B	Purple	Positive	Cocci	Clusters

Four controls were used in the practical to validate the performance. *Pseudomonas aeruginosa* is a gram-negative, rod-shaped bacterium (Diggle & Whiteley, 2020). *Staphylococcus aureus* is a gram-positive, cocci-shaped bacterium that forms clusters (Taylor & Unakal, 2023). *Bacillus subtilis* is gram-positive, rod-shaped bacteria that often chain (Errington & Aart, 2020). *Escherichia coli* is rod-shaped and gram-negative (Koch, 2005). This aligns with expected control outcomes. Nevertheless, unstained regions were observed in *Bacillus subtilis*, possibly due to insufficient staining or

excessive smear thickness (Mitra et al., 2023). All slides followed the same preparation so this should be considered when assessing the results.

Both unknown samples retained the purple stain and therefore are gram positive. Both were shown to be cocci-shaped bacteria. Unknown A was organised in chains whereas unknown B was organised in clusters. There were no further observations such as the unstained regions shown in the *Bacillus subtilis* stain.

Discussion

The results for the controls used offer a degree of validation for the accuracy of the unknown samples results. The observed retained stains, cell shapes, and organisations correlated to what is expected of the controls suggests a degree of reliability in the staining process. Nevertheless, the presence of unstained regions within the Bacillus subtilis control contradicts the notion of staining technique reliability. Despite this, there were no unstained regions in the unknown samples which could indicate that they were stained correctly.

Both unknown samples resulted as gram positive cocci. They differ in their organisations with unknown A being organised in chains and unknown B forming clusters. Sizar er al. (2022) state that gram positive cocci can possibly be a *Staphylococcus* or *Streptococcus* infection. *Staphylococcus* bacteria tend to grow in clusters whereas as *Streptococcus* tend to grow in chains. This correlates with the results for the unknowns.

Group A *Streptococci (Streptococcus pyogenes)* are the most common type to be associated clinically in humans, commonly causing pharyngitis and tonsilitis, and *Streptococcus pneumoniae* is the leading cause of community acquired pneumonia so these should be considered. *Streptococcus pyogenes* often grows in chains whereas *Streptococcus pneumoniae* often appear as diplococci but can also form

chains, meaning that both are possibly the bacteria present in Unknown A (Patterson, 1996). Further information on the clinical details of the patient may be beneficial in identifying the probable bacteria species. Alternatively, a bacterin test can be performed. This test differentiates group A *Streptococci* from other groups, therefore potentially ruling out *Streptococcus pneumoniae* (Patterson, 1996). Rapid antigen tests for group A Streptococci are also an available alternative. These are beneficial as they are cost effective and have quick turnaround times (Fraser at al., 2020).

Unknown B is likely to be a *Staphylococcus* infection because it is gram positive cocci and forms clusters. Sizar et al., (2022) dictate that *Staphylococcus* can be divided into two groups, coagulase-positive (*Staphylococcus aureus)*, and coagulase-negative (*Staphylococcus epidermis* and *Staphylococcus saprophyticus*). A coagulation test can therefore be a beneficial further test to further confirm the *Staphylococcus* species. This can be done by adding EDTA-treated plasma to a slide prepared with a suspension of the bacteria cells. If clumping of cells is observed, this indicates the bacteria is coagulase-positive and therefore can be identified as *Staphylococcus aureus* (Katz, 2010). Unknown B was a sample taken from a wound swab and *Staphylococcus aureus* is one of the most frequent bacteria that is found in chronic wounds (Serra et al., 2015). Also, *Staphylococcus aureus* can be easily cultivated in blood agar (Turista & Puspitasari, 2019).

The gram stain can prove to be time consuming and requires manual interpreting of ever result. Other methods can prove to be more effective such as automated identification systems. Studies have shown that the bioMérieux VITEK 2 can correctly identify over 94% of tested gram-positive bacteria in a clinical setting by comparing the results of 43 biochemical tests against databases and identification cards. This method requires less manual input and interpretation, therefore limiting the chance of errors (Funke & Funke-Kissling).

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