CASE REPORT

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Gram stain evaluation in dermatovenereology clinic and microbiology laboratories: case series

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ABSTRACT

Introduction: The normal vaginal flora consists of aerobic and anaerobic bacteria; Lactobacillus *sp.* is the main microorganism in the vagina. In bacterial vaginosis, there is a decrease in *Lactobacillus sp.* and an increase in pathogenic bacteria. Direct gram staining of vaginal fluid is the standard microbiological method for diagnosing bacterial vaginosis. Gram staining allows us to distinguish between gram-positive and gram-negative bacteria based on differential staining with crystal violet iodine complex and safranin. This study aimed to evaluate and compare the gram stain results between the dermatovenereology clinic and microbiology laboratories.

Case description: Direct gram staining of vaginal or cervical swabs was carried out on women aged 19 years, women aged 29 years, and women aged 40 years. The gram staining was carried out at the dermatovenereology clinic and microbiology laboratory with different results between the two locations.

Conclusion: It is important to evaluate the process of taking vaginal fluid samples, making smears/slides, and gram staining so that the quality of the examination results is maintained

Keywords: bacterial vaginosis, Gram staining, *Lactobacillus*, vaginal discharge.
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INTRODUCTION

The normal vaginal flora consists of aerobic and anaerobic bacteria; *Lactobacillus sp.* is the main microorganism (95%) of all bacteria in the vagina. This is no different in the cervical area.[1–3] *Lactobacillus sp.* maintains an acidic pH, and it ensures the presence of hydrogen peroxide in the vagina, thereby providing defenses against infection. In bacterial vaginosis, polymicrobial syndrome occurs which causes a decrease in *Lactobacillus sp.* and an increase in pathogenic bacteria such as *Gardnerella vaginalis*, *Mobiluncus sp.*, *Bacteroides sp.*, *Prevotella sp.* and *Mycoplasma sp.*[1] Bacterial vaginosis is a lower genital tract disorder that commonly occurs in women of reproductive age and is a common cause of vaginal discharge and odor. Women who smoke, use intravaginal products or are active in sexual activity are at risk factors that increase the occurrence of bacterial vaginosis.[1,4]

Bacterial vaginosis can be diagnosed clinically and microbiologically. The most common diagnostic methods for bacterial vaginosis are the Amsel criteria and the Nugent score. The Amsel criteria are clinical diagnostic criteria published in 1983 in the American Journal of Medicine. There are four parameters used in the Amsel criteria in determining bacterial vaginosis, namely: (1) Discharge of thin, white or yellow, homogeneous fluid; (2) "Clue cells" (vaginal squamous epithelial cells covered with bacteria) from the results of microscopy; (3) pH of vaginal fluid > 4.5; (4) A fishy smell after adding 10% potassium hydroxide (KOH) solution (whiff test). The diagnosis of bacterial vaginosis is established if three of the 4 existing criteria are present. However, some literature states that the diagnosis of bacterial vaginosis can be made if two of the four criteria are present. The Amsel criteria have a sensitivity of 37%-70% and a specificity of 94%-99%. Amsel criteria are fast, cheap, and simple compared to Nugent scores. The Nugent score as a microbiological criterion for diagnosing bacterial vaginosis has disadvantages such as being time-consuming, expensive, and requiring laboratory equipment and specialists, but the Nugent score is the gold standard in diagnosing bacterial vaginosis because of its high sensitivity. Nugent score is assessed by direct gram staining of vaginal fluid. Direct gram staining of vaginal fluid is a standard method in the laboratory where it is used to determine the relative concentration of *Lactobacillus sp.* and corresponding pathogenic bacteria. The resulting score is used to determine whether an infection is present. Based on the Nugent score, if the score is 0-3, it is considered normal vaginal flora; if the score is 4-6, it shows intermediate change in vaginal flora; and if the score is >7, it is considered bacterial vaginosis.[1,4,5] Gram staining allows us to distinguish between gram-positive and gram-negative bacteria based on differential staining with crystal violet iodine complex and safranin.[6]

This article reports the results of gram staining carried out at the dermatovenerology (DV) clinic and the microbiology laboratory along with the calculation of the Nugent score from 3 cases where there were differences in the results obtained. As far as the author knows, the dermatovenereology clinic and microbiology laboratory, which is the location of this case, often performs gram staining examinations and has not conducted similar studies before. Therefore, the author decided to write this article.

CASE DESCRIPTION

Gram staining was carried out directly from vaginal or cervical swab specimens, of which 2 swabs were made from each swab which were stained in two different places (DV polyclinic and microbiology laboratory) on the same day. Samples were taken from 3 different patients at different times. The patients have given informed consent. The results of gram staining are described below.

Case 1

An 18-year-old woman underwent vaginal and cervical swab examinations. Vaginal Gram staining was performed at two locations, yielding consistent results in both: the presence of epithelial cells [+] and yeast [+] (Figure 1). However, cervical Gram staining showed differing results between the two locations. At the DV polyclinic, the findings included epithelial cells [+], polymorphonuclear cells (PMN) [+], and the absence of intracellular Gram-negative diplococci (DGNI) [-]. In contrast, the microbiology laboratory results indicated epithelial cells [+], PMN cells [+], and the presence of DGNI [+] (Figure 1). The Nugent score was 1, which is consistent with normal vaginal flora.

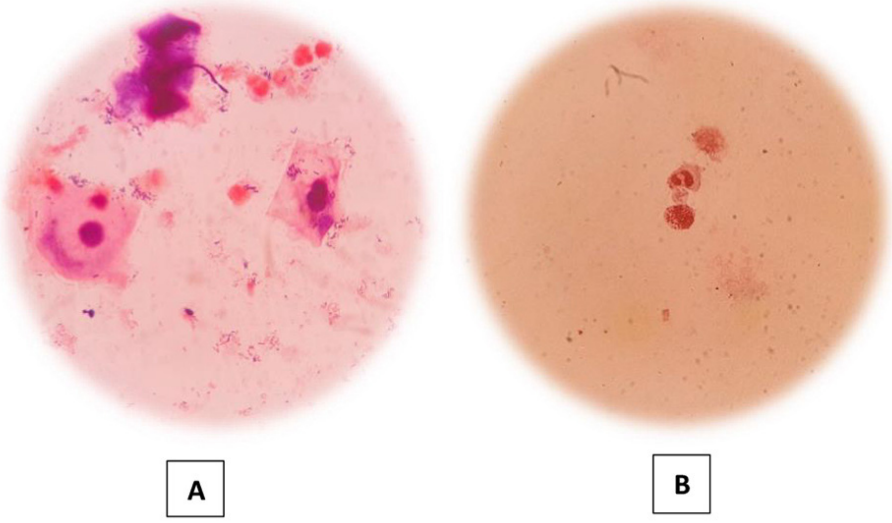

Figure 1. (A) DV polyclinic: Gram staining of a vaginal swab shows epithelial and yeast cells; (B) Microbiology laboratory: Gram staining of cervical swab showed DGNI.

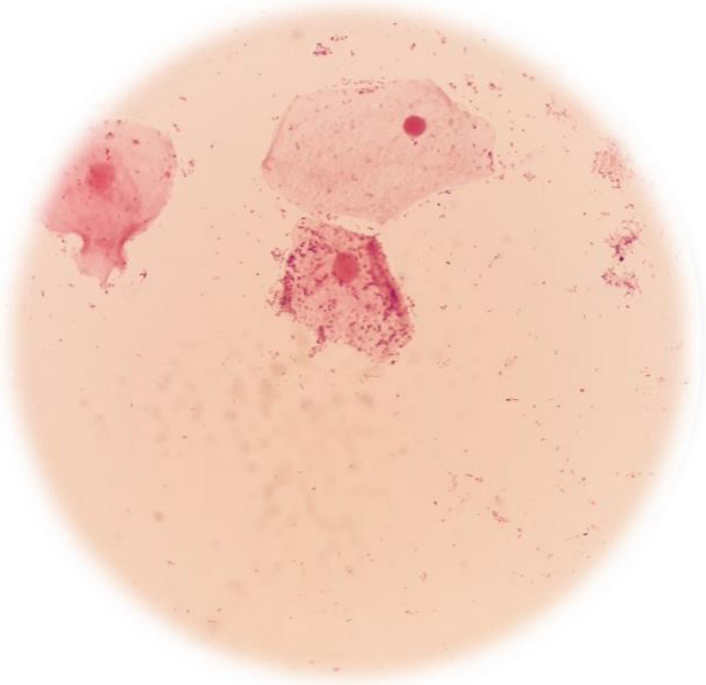

Figure 2 DV polyclinic: Gram staining of a vaginal swab shows epithelial and clue cells.

Case 2

A 29-year-old woman underwent vaginal and cervical swab examinations. Gram staining of the vaginal and cervical swabs yielded differing results between the two testing locations. For the vaginal swabs, the DV polyclinic results showed epithelial cells [+], polymorphonuclear cells (PMN) [+], clue cells [+] (Figure 2), and Gram-positive rod bacteria [+]. In contrast, the microbiology laboratory findings revealed epithelial cells [+], PMN cells [-], clue cells [-], and Gram-positive rod bacteria [-]. Similarly, for the cervical swabs, Gram staining at the DV polyclinic indicated epithelial cells [+], PMN cells [+], and intracellular Gram-negative diplococci (DGNI) [-]. However, in the microbiology laboratory, the cervical swab results showed epithelial cells [-], PMN cells [-], and DGNI [-], with only residual Gram-staining artifacts observed. The Nugent score was 10, which is consistent with a diagnosis of bacterial vaginosis.

Case 3

A 40-year-old woman underwent a vaginal swab examination. Gram staining of the vaginal swab was performed at two locations, yielding differing results. At the DV polyclinic, the findings included epithelial cells [+], polymorphonuclear cells (PMN) [+], clue cells [+], and Gram-positive rod bacteria [+]. In contrast, the microbiology laboratory results showed epithelial cells [+], PMN cells [-], clue cells [-], and Gram-positive rod bacteria [+]. The Nugent score was 1, indicating normal vaginal flora. No imaging was available for this case.

DISCUSSION

Gram stain was discovered by Hans Christian Gram in 1884. It allows us to differentiate gram-positive bacteria from gram-negative bacteria, making it one of the most important stains for bacteria. This method helps to examine bacteria in samples and determine their morphological characteristics. Gram staining is based on differential staining with a complex crystal violet iodine and safranin.[6] Gram staining steps include (1) Using crystal violet dye for initial/primary staining; (2) Adding iodine to form a crystal violet-iodine complex, which is used to prevent the dye from being easily removed (dye fixation/mordant); (3) Decolorizing agent (alcohol/ethanol/acetone) was used to remove the initial dye and (4) Counterstain using safranin dye. Gram-positive bacteria will appear purple because the cell walls retain the complex crystal violet iodine even after having a decolorization with alcohol. Meanwhile, gram-negative bacteria will appear red because the cell walls are decolorized with alcohol and then bind to safranin.[6–8]

The basic principle of gram staining involves the ability of bacterial cell walls to retain crystal violet dye during solvent treatment. There are several theories regarding the mechanism of gram staining seen from the permeability of the cell wall or cell wall components. The cell wall permeability theory by Burke and Barnes is based on the idea of the passage of certain molecules across the bacterial cell wall. in Gram-positive bacteria, molecules that would pass through the cell wall, are inhibited when they are suspended in higher concentrations of ethanol, whereas Gram-negative bacteria are affected to a lesser extent. It shows that during the color removal process with alcohol, the size of the pores in the cell walls of gram-positive bacteria will decrease so that the crystal violet iodine complex cannot come out of the cell walls. Thus, it can be concluded that gram staining can be influenced by changing the permeability of cell walls with acids, alkalis, and even water.[7,9,10] Kaplan & Kaplan (1933) and Bartholomew et al. (1959) report that the color fixation process with iodine causes the size of the pores in the cell wall of gram-positive bacteria to shrink so that the crystal violet iodine complex is trapped.[11,12] The structure of the cell wall in gram-positive bacteria is dominated by peptidoglycan, while in gram-negative bacteria, it is dominated by lipids. During the decolorization process, alcohol dissolves the lipid layer on gram-negative bacteria so that the crystal violet iodine complex can come out.[7,13]

Gram stain has been used for several purposes which are (1) directly to examine the specimens submitted for microbiological examination. It provides doctors with initial information about pathogenic bacteria so that appropriate initial therapy can be given which can affect the patient's prognosis; (2) to assess the quality of the specimen (sputum specimen sample); (3) to help determine the next identification method; (4) to be able to provide an overview of the characteristics of the types of bacteria that grow on culture media; (5) to detects unusual/rare pathogens (anaerobic infections, *Actinomycosis*, *Campylobacter fetus* bacteraemia, *Listeria* bacteraemia and meningitis, gastric non-*Helicobacter pylori*, fungal specimens) and to be able to differentiate true infection/contamination.[14,15] Direct Gram stain is the stain performed on smears prepared from clinical specimens directly. Direct Gram stain can be performed on a variety of clinical specimens such as normally sterile clinical specimens such as blood cultures, cerebrospinal fluid, pleural, peritoneal, and joint fluids as well as sputum, swabs from the nostrils, throat, rectum, wound, and cervix can be used. This method is simple, easy, cost effective and can help reveal suspected bacterial pathogens and even differentiate between bacterial and fungal infections. In order to get the maximum benefit from direct gram staining, collection, and transportation of specimens must be carried out according to recommendations, including taking appropriate specimens from the site of infection, carrying out before administering antibiotic therapy (if possible), collecting specimens in sterile containers, using appropriate transport media if the specimen cannot be sent immediately to the laboratory (cerebrospinal fluid specimens must be sent immediately) and providing complete data (patient diagnosis, current therapy) of the patient.[7,15]

Errors that can occur during gram coloring include (1) Technical reasons: (a) Excessive decolorization and (b) Excessive heating during fixation. This can change cell morphology and make cells decolorize more easily; (c) Low concentration of crystal violet. This can cause stained cells to decolorize easily. Standard 0.3% solution can be used but decolorization time does not exceed 10 seconds; (d) Excessive washing with water between steps (more than 5 seconds); (e) The amount of iodine in the solution is insufficient. The iodine concentration is generally 0.33–1%; if the concentration is low, it is difficult to form violet iodine crystal complexes, so the color is easily lost, and 60% of the iodine lost can give uncertain results. Exposure to air and high temperatures can affect the loss of iodine from the solution. Iodine solution (concentration 0.33%) will lose > 50% in a closed container and > 90% in an open container within 30 days; (f) Prolonged decolorization;[14] (2) Clinical specimens which are contaminated/poor quality; (3) The patient who has used antibiotics before; (4) Mixed infection/polymicrobial; (5) characteristics of the bacteria themselves; (6) misinterpretation.[15] Further considerations in carrying out gram staining include (1) Gram stain results that should be used in conjunction with other clinical and laboratory findings; (2) Gram stain results that assist in determining additional procedures (examples: special stain, direct antigen test, use of selective media) to confirm the findings indicated by gram stain; (3) Compliance in carrying

out procedures and interpretation criteria that are needed in order to obtain accurate results (training and skills of microscopy experts can affect the level of accuracy); (4) It is recommended that specimens in the form of pus/pus be examined with additional staining; (5) If gram staining results are positive and culture results are negative, this may be due to contamination of the specimen with normal commensals, an artifact of the staining reagent, previous use of an antimicrobial agent, or failure of the organism to grow in normal culture conditions (media/atmosphere/etc. not in accordance).[14]

Gram stain reagent quality control is important before examining and reporting smear results. Quality control is carried out once a week by reading slides containing smears of gram-positive and gram-negative bacteria. ATCC 25923 *Staphylococcus aureus* is used for gram-positive bacterial smears and ATTC 25922 *Escherichia coli* is used for gram-negative bacterial smears. Whereas the gram staining results of Staphylococcus aureus must appear in the form of blue cocci, and *Escherichia coli* must appear in the form of red rods. If the results do not match what is required, then try again to color a new control slide. If the results are still not suitable, then check whether the staining procedure has been carried out correctly, check whether the bacterial strain used matches the recommended reference, and check the quality of the gram staining reagent.[16,17]

Direct gram staining is very specific in detecting cases of sexual transmission, such as gonorrhea and bacterial vaginosis. The sensitivity and specificity of direct gram staining is about > 97% for the diagnosis of gonococcal urethritis in men, and even the concordance between direct gram staining and PCR is around 99.4% for the diagnosis of gonorrhea in men. Direct gram stain sensitivity is lower in women (≤ 32%) than in men (≥ 95.4%). A negative Gram stain does not exclude the presence of urethritis in symptomatic patients.[15,18] Direct gram staining of vaginal fluid is a microbiological method that provides specific results in diagnosing bacterial vaginosis and there is a positive correlation between microscopy results and clinical diagnosis of bacterial vaginosis. Gram staining is more recommended for detecting bacterial vaginosis than culture, but Gram staining or culture alone is not recommended as a method for diagnosing bacterial vaginosis without considering the patient's clinical condition because the organisms found are often elements of the normal vaginal flora.[15,19]

Handling of vaginal fluid specimens from which smears are made for gram staining includes (1) Collect vaginal fluid specimens on cotton-tipped swabs; (2) Prepare a slide and label it with the date of collection with a marker that cannot be erased, including during the decolorization process for gram staining; (3) Have vaginal fluid swabs for bacterial vaginosis examination that must be immediately smeared onto a glass slide; (4) Have An ideal smear (visually seen) that should cover at least two-thirds of the surface area of the slide; (5) Have the smear that must be dry before being sent to the laboratory; (6) Have the smear that must be packaged well to protect the slide from breaking during transit, and keep it at room temperature during shipping; (7) After arriving at the laboratory, have the smear that is heat fixed before being stained with Gram and (8) Have smears that have been gram stained that can be read using microscopy and interpreted.[17,20,21] Criteria for rejecting smear slides include (1) Slides are not adequately labeled/unlabelled/unreadable; (2) Slide damaged; (3) The slide does not have a specimen inoculum; (4) Slides sprayed with a fixative, such as pap smear cytology spray fixative. This can cause gram staining results to be less good; (5) Slides containing < 2 epithelial cells per field of view; This can indicate that the sample is taken from the wrong area, namely the cervix, not the vagina and (6) The results of the gram stain slide only contain cellular debris/only contain epithelial cells.[17]

In this case, only the gram staining results of the vaginal swab from the first patient are the same between the two locations. Meanwhile, other gram staining results show different results between the two Gram staining locations. This difference may occur (1) when vaginal fluid collection is inappropriate; (2) in this case, one swab of vaginal fluid is scratched on two glass objects so that the results may be uneven between the two scratches on the glass object; (3) the smear made is erased during the process of being taken from the DV polyclinic to the microbiology laboratory or is not properly verified so that the results when staining are not perfect. Based on the Nugent score of the three cases, two of them (case number 1 and 2) are declared bacterial vaginosis and one (case number 3) is declared normal vaginal flora. Nugent score calculation results in the three cases based on Gram staining in the microbiology laboratory.

LIMITATIONS

This case had several limitations, including the following: (1) re-sampling on the same day was not performed due to concerns about causing discomfort to the patient; (2) only a single smear was prepared for testing at both the DV clinic and the microbiology laboratory, preventing repeat Gram staining; and (3) photo documentation was incomplete.

CONCLUSIONS

Based on the three cases analyzed (with patient consent), discrepancies were observed between the Gram staining results of vaginal fluid performed at the DV clinic and the microbiology laboratory. This highlights the importance of routine evaluation and quality control in the sampling, smear preparation, and Gram staining processes to ensure the accuracy and reliability of examination results, thereby minimizing the risk of misdiagnosis while considering the patient's clinical condition.

DISCLOSURES

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Patients' informed consent
All patients have agreed and provided signed informed consent regarding the publication of this case.

Conflict of Interest
There is no conflict of interest in this study.

Author Contributions
Conceptualization, methodology and writing original draft preparation: Kusumadewi YP; Formal analysis:

Kusumadewi YP, Kholidah SN; Data curation: Kusumadewi YP, Kholidah SN, Susetiati DA, Nuryastuti T; Validation: Susetiati DA, Nuryastuti T; Writing, review and editing: Kusumadewi YP; Approval of final manuscript: all authors.

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