

A Novel Improved Gram Staining Method Based on the Capillary Tube

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Abstract

In this work, an exploratory study was conducted to examine Gram staining based on the capillary tube. Each Gram staining step for all bacterial strains tested was completed in capillary tubes. The results showed that different Gram staining morphologies were clearly visible in the capillary tubes. The results presented here demonstrated that the improved method could effectively distinguish between Gram-positive and Gram-negative bacteria, and only small volumes of reagents were required in this method. Collectively, this efficient method could rapidly and accurately identify the types of bacteria. Therefore, our findings could be used as a useful reference study for other staining methods.

Key words: Gram staining, capillary tube, bacteria, and glass slide

Since Hans Christian Joachim Gram reported a staining method in 1884 (Gram 1884), such a technique has experienced more than a century of development and has become frequently used in bacteriology. From 1940 to 1960, Gram staining's clinical application reaches its peak (Kass 1987). In recent years, several automated instruments for Gram staining have also been applied for microbiological analysis (Baron et al. 2010; Li et al. 2020). With the development of modern science and technology, some new technologies are expected to replace Gram staining. For example, Sizemore et al. (1990) have developed an alternative Gram staining technique using a fluorescent lectin. Later on, several fluorescent Gram staining methods have been established, and some Gram staining techniques suitable for live bacterial suspension have been described (Mason et al. 1998; Fife et al. 2000; Forster et al. 2002; Kwon et al. 2019). Sharma et al. (2020) have found that acridine orange fluorescent staining is more sensitive than the Gram staining. Besides, Berezin et al. (2017) have established a method for detecting Gram-negative bacteria based on enhanced Raman spectroscopy. Lemozerskii et al. (2020) have also reported a method of bacterial discrimination using an acoustic resonator. However, Gram staining is still a vital detection method in practical application for many microbiologists and clinicians

due to its rapidity and simplicity (Thompson et al. 2017; Jahangiri et al. 2018; Li et al. 2018a).

Over the years, Gram staining has been modified for many times, such as the Brown-Hopps method, Brown-Brenn method, and Gram-Twort method (Brown and Brenn 1931; Brown and Hopps 1973; Peck and Badrick 2017), and these approaches as mentioned earlier are widely used in anatomical pathology laboratories. Through the comparison of various improved methods, it is found that Gram's original four-step method is still used, and some researchers have adopted the three-step method, while its basic principle has not been changed. As reported by Huang and Cui (1996), the three-step Gram staining method combines the two steps of alcohol decolorization and re-staining procedure in one step. Although Gram staining is one of the most commonly used detection methods in clinical microbiology laboratories, many clinicians are skeptical of its results due to differences in operators, low control, and standardization (Samuel et al. 2016; Thomson 2016). Researchers have made efforts to improve the Gram staining's accuracy and reliability over the past few years, such as repeated training and standardization of the staining procedure (Thomson 2016; Siguenza et al. 2019). In this study, we developed a standardized Gram staining procedure for bacterial identification

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using a capillary tube. A modified Gram staining method based on the capillary tube has not yet been reported to the best of our knowledge. Therefore, we proposed a novel improved Gram staining method to improve the accuracy of the detection results and Gram staining efficiency.

Eight bacterial strains, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Bacillus Licheniformis*, *Serratia marcescens*, *Vibrio parahaemolyticus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, and *Streptococcus thermophilus* were provided by the Laboratory of Microbial Engineering, College of Life Science, Luoyang Normal University. *L. bulgaricus* and *S. thermophilus* were inoculated into skim milk culture medium and maintained at 37°C for 12 h. *S. marcescens*, *B. Licheniformis*, *E. coli*, *B. subtilis*, *V. parahaemolyticus*, and *S. aureus* were inoculated into beef peptone agar slants and maintained at 37°C for 16 h.

Capillary tubes with an internal diameter of 0.5 mm and a length of 100 mm were purchased from the Instrument Factory of West China University of Medical Sciences. Gram staining reagent was obtained from the Anhui Chaohuhongci Medical Equipment Co., Ltd.

Procedure: (1) One or two drops of sterile water were placed in the center of a clean glass slide. An inoculating loop was held in a flame until it was red-hot and then allowed to cool approximately 30 seconds. Subsequently, a loop of culture was transferred to the center of the slide. The sample was spread onto the slide using the inoculating loop, and a small volume of bacterial suspension was automatically transferred into the capillary tube.

(2) The capillary tube was then heated by passing over a flame for several times until the liquid was completely evaporated. The capillary tube was naturally cooled in the air for several seconds.

(3) One end of the capillary tube was held upward, and the crystal violet solution was automatically transferred to the capillary tube, followed by standing for 1 minute. The remaining crystal violet solution of the capillary tube was then transferred to absorbent paper.

The capillary tube was washed in a gentle and indirect stream of tap water for a few seconds, and samples were dried on absorbent paper.

(4) One end of the capillary tube was held upward, and Gram's iodine solution was automatically transferred to the capillary tube, followed by standing for 1 minute. Subsequently, the capillary tube was washed using the same procedure as described above.

(5) One end of the capillary tube was held upward, and 95% ethanol was automatically transferred to the capillary tube, followed by standing for 30 seconds. Subsequently, the capillary tube was washed using the same procedure as described above.

(6) One end of the capillary tube was held upward, and the Safranin solution was automatically transferred to the capillary tube, followed by standing for 30 seconds to 1 minute. The subsequent procedure was the same as described above. Besides, conventional Gram staining was carried out according to the instructions from the reagent kit. According to the instructions, Gram-negative cells are in pink to red, and Gram-positive cells show a purple or blue color when observed under a microscope.

The Gram staining is always the “first-stage criteria” in the preliminary identification of bacterial species according to their cell walls (Li et al. 2018b). Eight different bacterial species were examined to investigate our approach, and the strains were selected according to the Gram staining pattern. Gram-negative bacteria *E. coli*, *V. parahaemolyticus*, and *S. marcescens* were examined. Gram-positive bacteria *S. thermophilus*, *L. bulgaricus*, *S. aureus*, *B. licheniformis* and *B. subtilis* were also assessed. Fig. 1, 2, and 3 illustrate the results of Gram staining of *E. coli*, *V. parahaemolyticus*, and *S. marcescens*, respectively. Fig. 4, 5, 6, 7, and 8 show the Gram staining results of *S. thermophilus*, *L. bulgaricus*, *S. aureus*, *B. subtilis*, and *B. licheniformis*, respectively. These results were compared with those obtained using a glass slide for Gram staining. No matter spherical or rod-shaped or not, all bacterial strains could be differentiated into two classifications: Gram-positive and

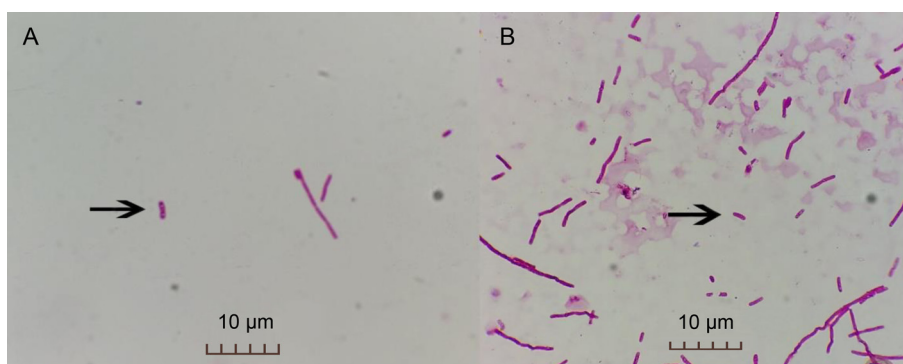


Fig. 1. The Gram staining results of *E. coli*. A – Capillary sample, B – Glass slide sample.

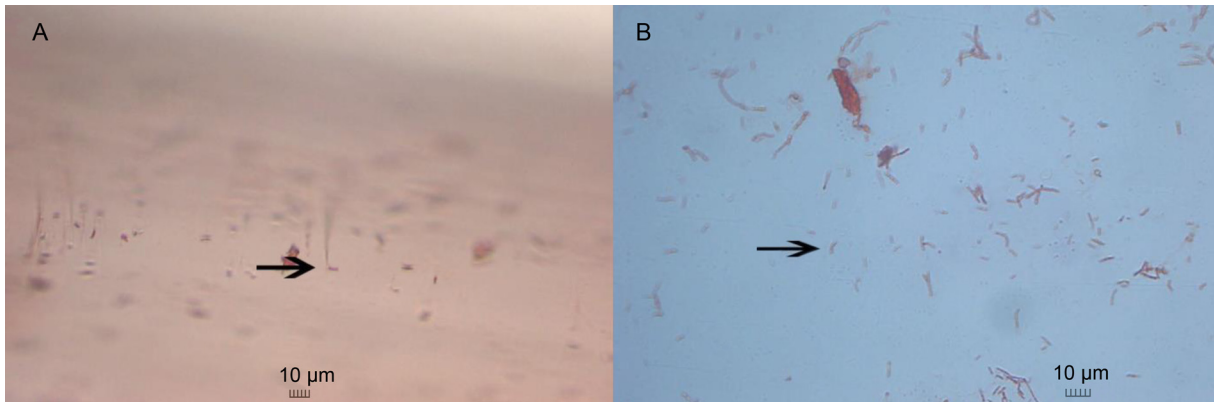


Fig. 2. The Gram staining results of *V. parahaemolyticus*. A – Capillary sample, B – Glass slide sample.

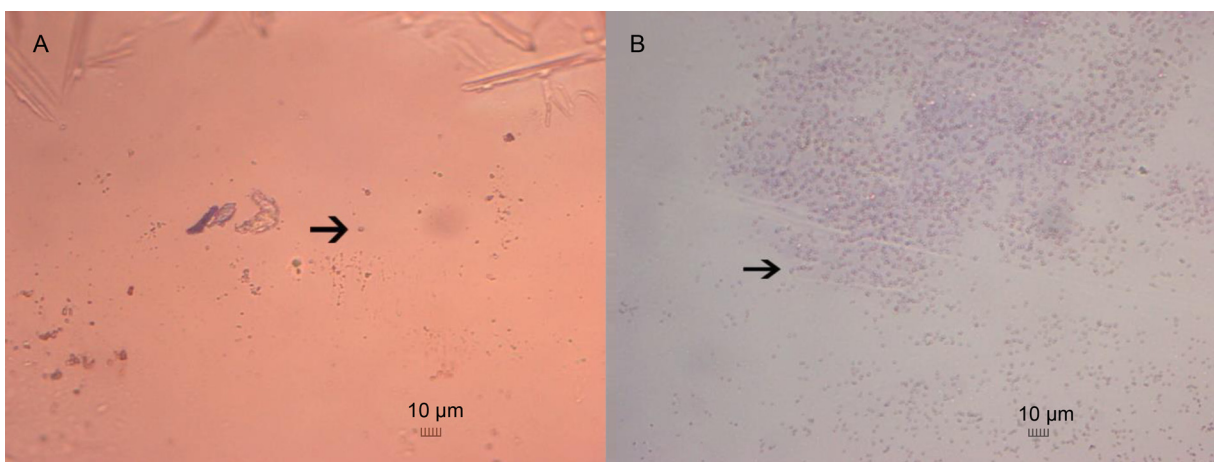


Fig. 3. The Gram staining results of *S. marcescens*. A – Capillary sample, B – Glass slide sample.

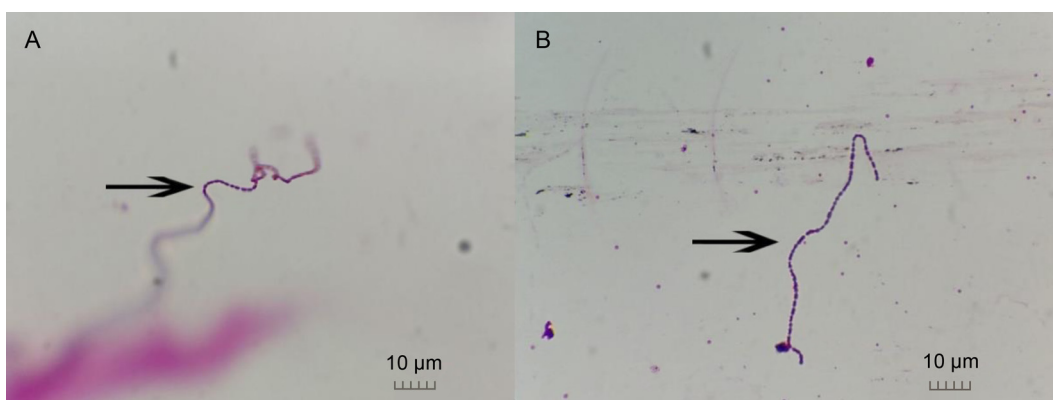


Fig. 4. The Gram staining results of *S. thermophiles*. A – Capillary sample, B – Glass slide sample.

Gram-negative. Comparing these results, we found that the results obtained by the capillary tube method were consistent with the conventional Gram staining approach. It was worth mentioning that in contrast to direct heat fixation of bacteria on glass slides, heat fixation by passing the capillary tube over a flame should be carried out quickly and carefully. If the capillary tube was overheated, it might cause the capillary tube to rupture, and it is easy to blur the field of vision, making it challenging to observe the staining result (Fig. 9). There-

fore, before the experiments, it is better to conduct a preliminary experiment and achieve the desired results.

Several studies (Chimento et al. 1996; Wada et al. 2012; Li Zhu 2018b) have already pointed out that the property of the bacterial cell wall determines whether the organism will be Gram-positive or Gram-negative, and it plays a role in the choice of antibiotics when infection occurs. Since it has frequently been observed that not all bacteria react in the same manner to such staining procedure (Hale and Bisset 1956),

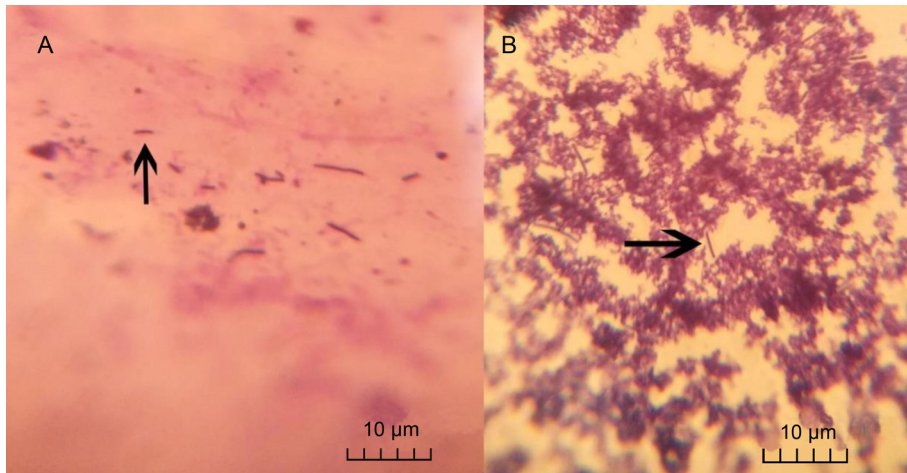


Fig. 5. The Gram staining results of *L. bulgaricus*. A – Capillary sample, B – Glass slide sample.

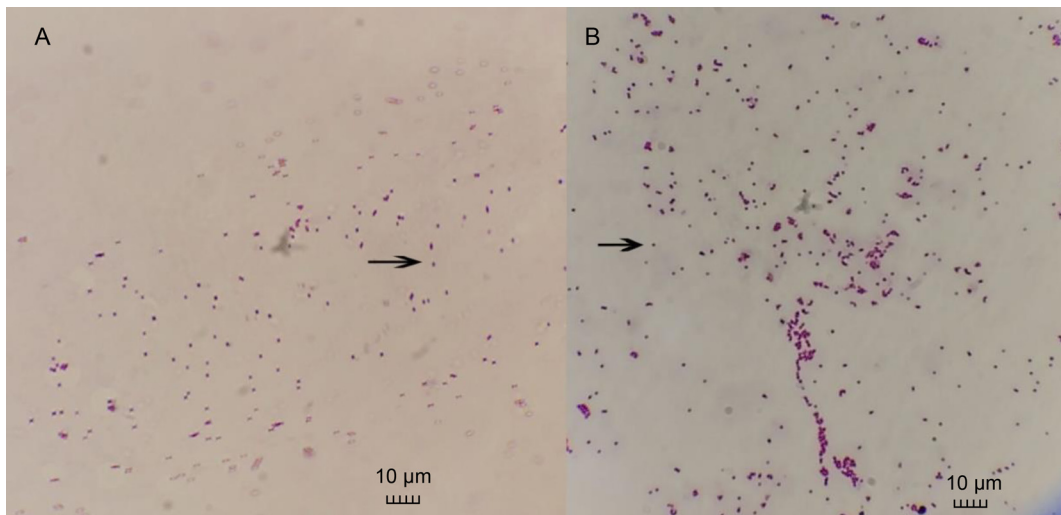


Fig. 6. The Gram staining results of *S. aureus*. A – Capillary sample, B – Glass slide sample.

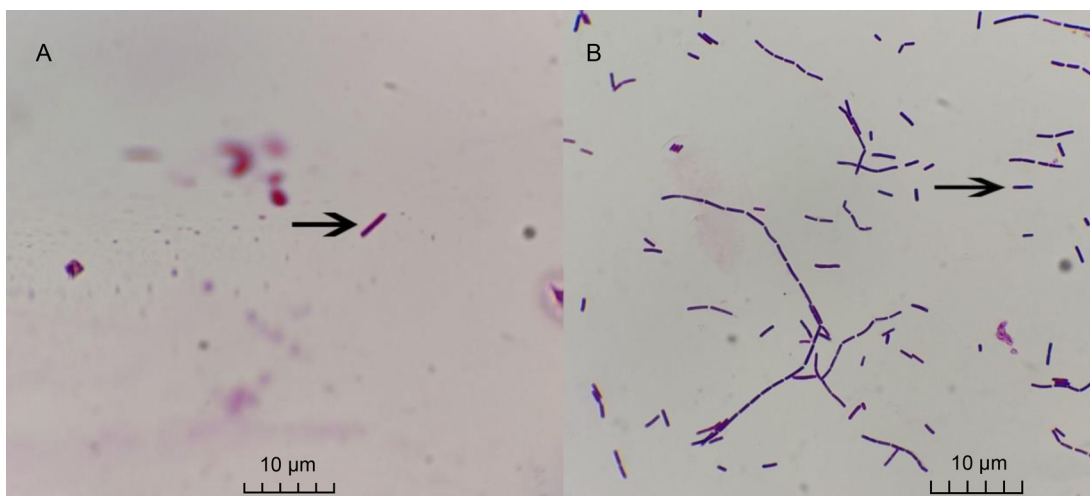


Fig. 7. The Gram staining results of *B. subtilis*. A – Capillary sample, B – Glass slide sample.

it is necessary to make more tests upon a representative selection of Gram-positive and Gram-negative bacteria in future studies.

Molecular biology techniques and high-precision measurement systems have been successfully developed, and they can distinguish bacterial types in clini-

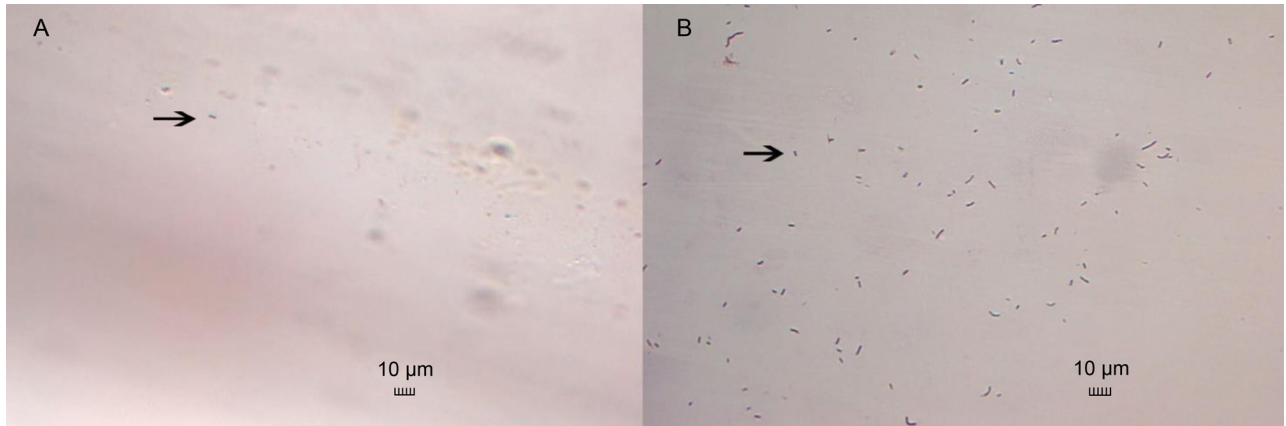


Fig. 8. The Gram staining results of *B. Licheniformis*. A – Capillary sample, B – Glass slide sample.

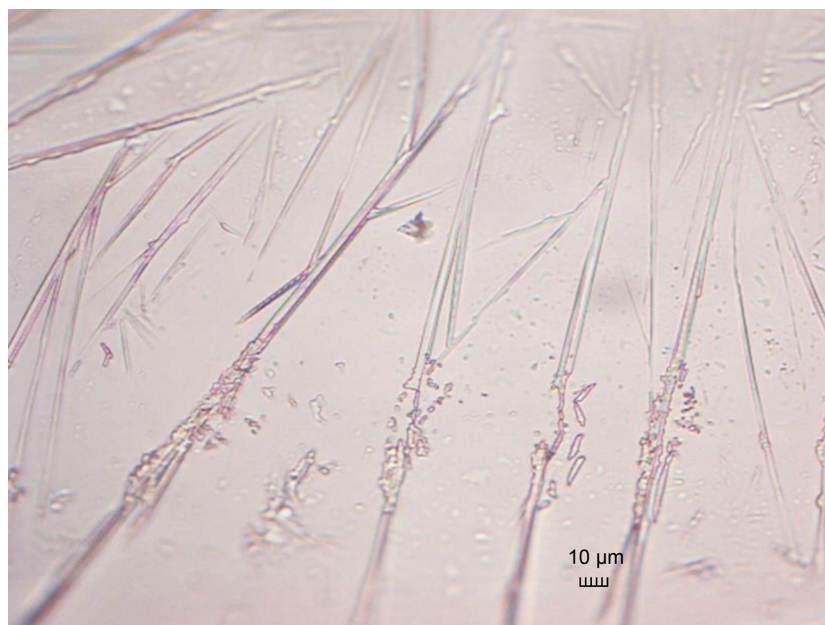


Fig. 9. The microstructure of the overheated capillary tube.

cal samples and improve microbial detection (Klaschik et al. 2002; Dolch et al. 2016; Kim et al. 2018). However, it is still urgently needed to develop a rapid and straightforward Gram staining approach to detect bacteria, especially for those who have only primary experimental conditions. Our results indicated a promising method for bacterial differentiation using the capillary tube as a carrier. Successful differentiation required only small volumes of reagents, and the results were achieved within a few minutes by applying an optical microscope. In addition, the method proposed in this paper had reference value to other staining methods requiring expensive reagents.

In the present study, the improved Gram staining method was developed based on the pure cultures, and it was only a comparison of the staining results between known Gram-negative and Gram-positive bacteria in a glass slide and capillary tube. In order to improve

the accuracy and stability of the results, future study is necessary to detect more bacterial species. In addition, the modified method was not applicable for direct Gram staining of clinical samples. In the future, it may have a positive effect by developing a special method for processing clinical samples.

The experimental results demonstrated that an improved Gram staining method was suitable for differentiating the strains tested in our laboratory. The method could rapidly discriminate Gram-positive and Gram-negative bacteria. Besides, the method only required small volumes of reagents. A much more comfortable and reproducible Gram staining approach can be developed for microbiology research based on our studies.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Literature

- Baron EJ, Mix S, Moradi W.** Clinical utility of an automated instrument for gram staining single slides. *J Clin Microbiol.* 2010 Jun 01; 48(6):2014–2015. <https://doi.org/10.1128/JCM.02503-09>
- Berezin S, Aviv Y, Aviv H, Goldberg E, Tischler YR.** Replacing a century old technique-modern spectroscopy can supplant gram staining. *Sci Rep.* 2017 Dec;7(1):3810–3816. <https://doi.org/10.1038/s41598-017-02212-2>
- Brown JH, Brenn L.** A method for the differential staining of Gram-positive and Gram-negative bacteria in tissue sections. *Bull Johns Hopkins Hosp.* 1931;48:69–73.
- Brown RC, Hopps HC.** Staining of bacteria in tissue sections: a reliable gram stain method. *Am J Clin Pathol.* 1973 Aug 01;60(2):234–240. <https://doi.org/10.1093/ajcp/60.2.234>
- Chimento GF, Finger S, Barrack RL.** Gram stain detection of infection during revision arthroplasty. *J Bone Joint Surg Br.* 1996 Sep; 78-B(5):838–839. <https://doi.org/10.1302/0301-620X.78B5.0780838>
- Dolch ME, Janitza S, Boulesteix AL, Graßmann-Lichtenauer C, Praun S, Denzer W, Schelling G, Schubert S.** Gram-negative and -positive bacteria differentiation in blood culture samples by headspace volatile compound analysis. *J Biol Res (Thessalon).* 2016 Dec;23(1):3. <https://doi.org/10.1186/s40709-016-0040-0>
- Fife DJ, Bruhn DE, Miller KS, Stoner DL.** Evaluation of a fluorescent lectin-based staining technique for some acidophilic mining bacteria. 2000;66:2208–2210.
- Forster S, Snape JR, Lappin-Scott HM, Porter J.** Simultaneous fluorescent gram staining and activity assessment of activated sludge bacteria. *Appl Environ Microbiol.* 2002 Oct;68(10):4772–4779. <https://doi.org/10.1128/AEM.68.10.4772-4779.2002>
- Gram HC.** Ueber die isolirte farbung der schizomyceten in schnitt und trockenpreparaten. *Fortschr Med.* 1884;2:185–189.
- Hale CME, Bisset KA.** A comparison of the staining reactions of the cell walls of *Azotobacter chroococcum* and those of Gram-positive and Gram-negative bacteria. *J Gen Microbiol.* 1956 Dec 01;15(3):423–427. <https://doi.org/10.1099/00221287-15-3-423>
- Huang Y, Cui J.** A simplified Gram stain and its quality control. *Acta Microbiol Sin.* 1996;36:76–78.
- Jahangiri L, Sudagar M, Ashayerizadeh O, Kolangi H, Tabarraei A.** Isolation of probiotic bacteria from guppy *Poecilia reticulata* (Cyprinodontiformes: Poeciliidae). *Turk J Fish Aquat Sci.* 2018;18(6):809–815. https://doi.org/10.4194/1303-2712-v18_6_07
- Kass EH.** History of the specialty of infectious diseases in the United States. *Ann Intern Med.* 1987 May 01;106(5):745–756. <https://doi.org/10.7326/0003-4819-106-5-745>
- Kim SY, Park Y, Kim H, Kim J, Koo SH, Kwon GC.** Rapid screening of urinary tract infection and discrimination of Gram-positive and Gram-negative bacteria by automated flow cytometric analysis using sysmex UF-5000. *J Clin Microbiol.* 2018 May 16;56(8):e02004-17. <https://doi.org/10.1128/JCM.02004-17>
- Klaschik S, Lehmann LE, Raadts A, Book M, Hoefft A, Stuber F.** Real-time PCR for detection and differentiation of Gram-positive and Gram-negative bacteria. *J Clin Microbiol.* 2002 Nov 01;40(11): 4304–4307. <https://doi.org/10.1128/JCM.40.11.4304-4307.2002>
- Kwon HY, Liu X, Choi EG, Lee JY, Choi SY, Kim JY, Wang L, Park SJ, Kim B, Lee YA, et al.** Development of a universal fluorescent probe for Gram-positive bacteria. *Angew Chem Int Ed.* 2019 Jun 17;58(25):8426–8431. <https://doi.org/10.1002/anie.201902537>
- Lemozerskii VE, Gagarina AY, Bondarenko AA, Korol VV, Zimina TM.** Rapid discrimination of Gram-positive and Gram-negative bacilli using an acoustic resonator. *IEEE Conference of Russian Young Researchers in Electrical and Electronic Engineering (EIConRus)* 2020.
- Li H, Li L, Chi Y, Tian Q, Zhou T, Han C, Zhu Y, Zhou Y.** Development of a standardized Gram stain procedure for bacteria and inflammatory cells using an automated staining instrument. *Microbiologyopen.* 2020 Sep;9(9):e1099. <https://doi.org/10.1002/mbo3.1099>
- Li L, Ding F, Sang L, Liu J, Mao D, Liu X, Xu Q.** Study on the oxygen reduction reaction catalyzed by a cold-tolerant marine strain phylogenetically related to *Erythrobacter citreus*. *Bioelectrochemistry.* 2018a Feb;119:51–58. <https://doi.org/10.1016/j.bioelechem.2017.08.011>
- Li Y, Zhu J, Zhang H, Liu W, Ge J, Wu J, Wang P.** High sensitivity Gram-negative bacteria biosensor based on a small-molecule modified surface plasmon resonance chip studied using a laser scanning confocal imaging-surface plasmon resonance system. *Sens Actuator B Chem.* 2018b Apr;259:492–497. <https://doi.org/10.1016/j.snb.2017.12.042>
- Mason DJ, Shanmuganathan S, Mortimer FC, Gant VA.** A fluorescent Gram stain for flow cytometry and epifluorescence microscopy. *Appl Environ Microbiol.* 1998 Jul 01;64(7):2681–2685. <https://doi.org/10.1128/AEM.64.7.2681-2685.1998>
- Peck M, Badrick T.** A review of contemporary practice and proficiency with Gram staining in anatomical pathology laboratories. *J Histotechnol.* 2017 Apr 03;40(2):54–61. <https://doi.org/10.1080/01478885.2017.1327474>
- Thomson RB Jr.** One small step for the gram stain, one giant leap for clinical microbiology. *J Clin Microbiol.* 2016 Jun;54(6):1416–1417. <https://doi.org/10.1128/JCM.00303-16>
- Samuel LP, Balada-Llasat JM, Harrington A, Cavagnolo R.** Multicenter assessment of gram stain error rates. *J Clin Microbiol.* 2016 Jun;54(6):1442–1447. <https://doi.org/10.1128/JCM.03066-15>
- Sharma S, Acharya J, Banjara MR, Ghimire P, Singh A.** Comparison of acridine orange fluorescent microscopy and gram stain light microscopy for the rapid detection of bacteria in cerebrospinal fluid. *BMC Res Notes.* 2020 Dec;13(1):29. <https://doi.org/10.1186/s13104-020-4895-7>
- Siguenza N, Jangid A, Strong EB, Merriam J, Martinez AW, Martinez NW.** Micro-staining microbes: an alternative to traditional staining of microbiological specimens using microliter volumes of reagents. *J Microbiol Methods.* 2019 Sep;164:105654. <https://doi.org/10.1016/j.mimet.2019.105654>
- Sizemore RK, Caldwell JJ, Kendrick AS.** Alternate gram staining technique for a fluorescent lectin. *Appl Environ Microbiol.* 1990;56(7):2245–2247. <https://doi.org/10.1128/AEM.56.7.2245-2247.1990>
- Thompson AB, Wrubel D, Berkowitz FE.** Gram-positive rods on a cerebrospinal fluid gram stain. *Open Forum Infect Dis.* 2017 Oct 01;4(4):ofx240. <https://doi.org/10.1093/ofid/ofx240>
- Wada A, Kono M, Kawauchi S, Takagi Y, Morikawa T, Funakoshi K.** Rapid discrimination of Gram-positive and Gram-negative bacteria in liquid samples by using NaOH-sodium dodecyl sulfate solution and flow cytometry. *PLoS One.* 2012 Oct 15;7(10):e47093. <https://doi.org/10.1371/journal.pone.0047093>