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COMMENTARY

One Small Step for the Gram Stain, One Giant Leap for Clinical Microbiology

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The Gram stain is one of the most commonly performed tests in the clinical microbiology laboratory, yet it is poorly controlled and lacks standardization. It was once the best rapid test in microbiology, but it is no longer trusted by many clinicians. The publication by Samuel et al. (J. Clin. Microbiol. 54:1442–1447, 2016, http://dx.doi.org/10.1128/JCM.03066-15) is a start for those who want to evaluate and improve Gram stain performance. In an age of emerging rapid molecular results, is the Gram stain still relevant? How should clinical microbiologists respond to the call to reduce Gram stain error rates?

The Gram stain has been a mainstay in the fields of microbiology and infectious diseases since 1884, when Danish physician Hans Christian Gram, working with Carl Friedlander, first dripped reagents onto lung tissue samples and found differential staining of what later was determined to be *Diplococcus pneumoniae* and *Bacillus pneumoniae*, now known as *Streptococcus pneumoniae* and *Klebsiella pneumoniae*, respectively (1). Although many modifications have been offered, the addition by Hucker in 1921 of ammonium oxalate to stabilize the crystal violate solution proved the most important (2). The Gram stain's clinical utility peaked between 1940 and 1960 (3). These were early days in the practice of infectious diseases that featured clinician-microbiologists who developed Gram staining skills while holding positions in outpatient clinics and inpatient wards. Fresh specimens were collected, smears were prepared and stained, and microscopy was performed, all by the clinician in what we today call a point-of-care setting. Pierce Gardner, an Associate Professor of Medicine at Harvard Medical School in 1974, wrote about the Gram stain and its interpretation (4): "The responsibility for interpretation of the Gram-stained smear should not be delegated. Technicians often are highly skilled in the recognition of bacteria but may have had little training in the interpretation of background material and cell types as they appear in Gram-stained smears of clinical specimens. Furthermore, the laboratory technician is usually not privy to important clinical facts. . .which may influence the interpretation of the smear. Therefore, it is our feeling that the Gram-stained smear should be considered part of the physical examination of the patient with an acute bacterial infection and belongs in the repertoire of all physicians delivering primary care in acutely ill patients." The Gram stain was king.

Antimicrobial resistance requiring sophisticated testing practices appeared soon after the introduction of antimicrobials in 1940 and was accompanied by novel equipment and technologies that changed the practice of clinical microbiology. These changes and others resulted in separate disciplines of infectious diseases and clinical microbiology (3, 5). The clinician began to rely on a central microbiology laboratory for stain, culture, and antimicrobial testing results. Interns in the clinics and at bedsides received Gram stain training from senior house staff. Without oversight from laboratory physicians and scientists, Gram stain skills deteriorated. As laboratory testing expanded, so did government regulation to ensure accurate results by trained technologists. Gram stain errors by clinicians were increasing (6). Because of an increase in the frequency of Gram stain errors performed by clinicians, microscopes and reagents were removed from near-patient locations. Regulations (CLIA 88) now mandate quality control and proficiency testing for laboratories, including those in physician offices, that perform and report Gram stain results, although performance requirements are minimal. The typical core microbiology laboratory has been moved miles from the bedside clinician. The evolution of the Gram stain from a clinician-performed, bedside test to a remotely performed test assigned, in many cases, to the least experienced laboratory worker with the minimal training needed to pass basic proficiency requirements, has taken the Gram stain from "the best rapid test in microbiology" to a subjective, poorly controlled microbiology test. Where do we go from here?

Samuel and colleagues have taken a small but very important step to improve the Gram stain through their multicenter assessment of Gram stain error rates (7). In a simple but effective approach, they compared Gram stain and culture results for over 6,000 specimens processed at 4 different tertiary care medical centers. As limitations, they only used specimens that included relatively high counts of bacteria by stain or culture, included specimens without an anaerobic culture component, attached no clinical significance to their Gram stain result, and because of the retrospective nature of the study they were able to perform a secondary review of only 87% of the paired stain-culture results that were discrepant. But, for a test that has almost no published performance data, this is a giant leap for clinical microbiology. We now know that in a controlled environment, discrepancies between stain and culture occur approximately 5% of the time, with

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only one-quarter of these discrepancies being the result of reader error. In other words, a projected 73 (1.2%) Gram stains out of 6,115 total Gram stains performed resulted in Gram stain reader error. This is an important benchmark. College of American Pathologists-accredited microbiology laboratories are required to have a policy that addresses "correlation of direct Gram stain results with final culture results" (Microbiology Checklist 7.28.15 requirement number MIC21530). These data now have a target, allowing us to evaluate performance and set goals for improvement.

Although Gram stain performance and error rate data are sparse, available publications offer insights. Urine Gram stains have been standardized compared to culture, showing that 1 to 2 bacterial cells per 1,000× field correspond to a bacterial quantity by culture of 100,000 CFU per ml (8). Cytocentrifugation smear preparation improves sensitivity of body fluid Gram stains by up to 2 logs, compared to unconcentrated smears, and with improved leukocyte morphology (9). Sputum Gram stain results suffer from variability of smear preparation as demonstrated by the use of replicate smears, for which 56% of sputum Gram stains resulted in at least one bacterial morphotype disagreement (10). Sputum Gram stains predict pneumococcal pneumonia in bacteremic patients 63% of the time, as long as specimens are collected before the initiation of antimicrobial therapy and are not grossly contaminated with oropharyngeal flora (11). Cerebrospinal fluid (CSF) Gram stains have high sensitivity (98%) for common pathogens when the culture is positive and may detect bacteria in the smear following antimicrobial administration when the culture is negative (12). The Gram stain is a reliable indicator of sterile abscesses, abscesses that grow a single isolate in pure culture, and those with mixed anaerobes in culture (13). Gram stains of positive blood culture broths correlate to subculture identifications 99.3% of the time (14). Review of the literature provides anecdotes and an understanding of what a Gram stain can do in specific applications, but we do not know how well, overall, the test performs in busy, sophisticated, centralized laboratory settings.

Based on the findings of Samuel et al., should laboratories evaluate and attempt to improve Gram stain performance and clinical utility (7)? Some say "no," since molecular testing, e.g., molecular arrays that identify bacteria and yeasts in CSF specimens within 1 to 2 h, is replacing the need for a Gram stain (15). Such molecular arrays and related molecular approaches will soon be applied to wound and sputum samples. Why do a Gram stain? The properly performed and accurately interpreted Gram stain provides an early diagnosis, supports antimicrobial stewardship, assesses specimen quality, identifies indicators of pathogenesis, guides the technologist in culture workup, and, when interpreted by a medical microbiologist, can display a pattern recognized as indicative of infectious pathology specific to each organ system. In this age of personalized medicine, there is no more personalized test than the Gram stain for those with an infection. This is how we build on the contributions by Samuel et al. (7). As a profession, we can standardize the Gram stain procedure by defining specimen assessment, i.e., what portion of the specimen is used to prepare the smear, defining smear preparation resulting in a monolayer of cells and not clumped material, defining how to recognize and perform microscopic examination of the best areas in the smear, including the minimum number of low- and high-power fields to be examined, and defining terms used for quantitation for microorganism morphology and inflammatory cells. A clinically useful Gram stain result reports more than bacterial shapes and inflammatory cells that are present. Surgical pathologists and cytologists report an interpretation based on pattern recognition. The Gram stain smear is a study in pattern recognition. The laboratory team should be trained to recognize normal and abnormal patterns for specific sites and report this finding accurately and consistently to the clinician. Once the bacteria in a Gram-stained smear can be reported accurately and reproducibly >99% of the time, in a common format used throughout our health care systems, and with professional interpretations based on pathological patterns, clinician confidence will be reestablished and the Gram stain will once again be the best rapid test in clinical microbiology. Let's get started!

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