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Assessing Gram-stain error rates within the pharmaceutical microbiology laboratory

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Abstract

Gram-staining remains the fundamental method for determinative bacteriology, dividing bacteria into Gram-positive and Gram-negative organisms. This test provides information as to the origin of any contamination and is a pre-requisite for many microbial identification methods. Despite the longevity of the test, the test is highly reliant upon analyst technique and therefore errors occur. While there are a few studies looking at errors in the clinical context, research has not been extended to the pharmaceutical microbiology laboratory context. In this study, we present a review of over 6,000 Gram-stains and establish an error rate of around 3%, with the most common reason for error being an over-decolourisation step resulting in organisms that should be Gram-positive appearing as Gram-negative. The analysis enables others to benchmark their facilities against.

Introduction

Gram-staining, a differential staining technique, is of fundamental importance for the identification and classification of bacteria and it is widely used in the medical and industrial fields of microbiology. Despite being a long-established method, the technique remains open to occasional errors. However, it is rare for the error rate to be quantified and assessed for error types (with the few published studies being confined to the clinical setting) [1,2]. Errors related to issues with technique and interpretation. The interpretation of a Gram-stain slide is inherently subjective. While interpretation can be overcome through good training there remains several factors which can affect whether a correctly stained sample is obtained, including specimen fixation, staining protocol, and slide analysis. In order to assess the level of errors made in a typical laboratory, this paper assesses the different types of errors that can occur within a pharmaceutical facility. Data was drawn from a pharmaceutical facility located in south-east England.

The implications of errors with the Gram-stain can influence the selection of the test method (and test kit) for the next stage of identification, whether this is a manual biochemical identification method (such as API) or a semi-automated method (such as Vitek or Omnilog). Getting an identification wrong could lead to an incorrect root cause analysis (which impacts on all types of pharmaceutical processing, including sterile products) and potential errors relating to batch release (especially with non-sterile pharmaceuticals where understanding the pathogenic nature of the organism is a key requirement).

The Gram-stain

The Gram-stain is an important tool in the process of bacterial identification by dividing bacteria into two groups (the so-called Gram-positives and Gram-negatives) and in allowing their morphological types (coccoid or rod shaped) to be clearly seen [3]. The developer of the differential stain was the Danish bacteriologist Hans Christian Joachim Gram [4]; it was during his study of red corpuscles in 1884 that Gram developed his method for staining bacteria [5].

The polychromatic staining method has gone through several iterations since its initial description [6]. Today, the staining technique involves the following steps [7]:

- Taking a microbial colony and emulsifying it onto a slide.
- Fixing the smear to the slide through the application of heat.
- Staining bacterial cells with crystal violet.
- Fixing the stain (by using a mordant, chemicals that help “set” a dye, like iodine).
- Using a solvent to remove the stain from some types of bacteria, such as acetone or ethanol.
- Applying a counter stain.

The idea for a counterstain was developed some years later by a German pathologist named Carl Weigert who used safranin as a counter stain, which stained the cells red. Gram himself never used a counter stain [8].

The first step involves taking single (pure) colonies from an agar plate of still growing bacteria (often 18 – 24 hours old) and heat-fixing the cells (which kills them) onto a microscope slide. Following this the cells are then stained with a basic dye, crystal violet, which stains all bacterial cells blue. In aqueous solutions, crystal violet dissociates into CV+ and Cl – ions that penetrate through the wall and membrane of both Gram-positive and Gram-negative cells. The CV+ interacts with negatively charged components of bacterial cells, staining the cells purple [9].

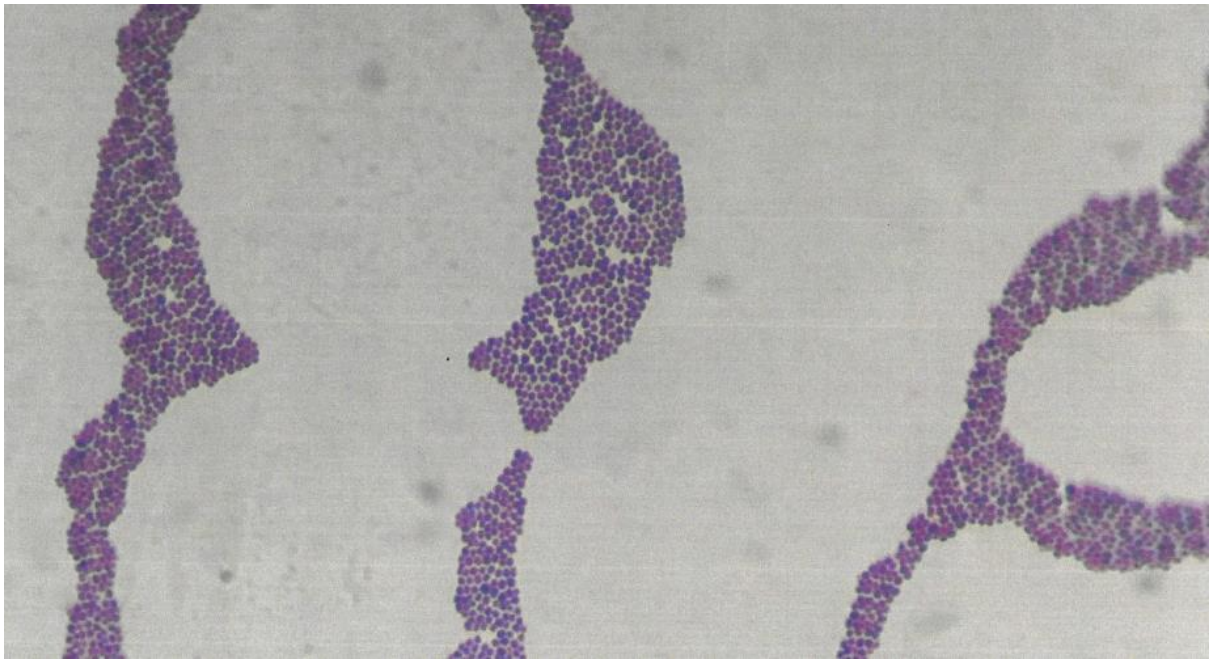

Figure 1: Positive Gram-stain (*Micrococcus luteus*) (Image: Tim Sandle)

The second step involves adding an iodine-potassium iodide solution. The iodine solution enters the cells and forms a water-insoluble complex with the crystal violet dye. When added, iodine (I- or I3-) interacts with CV+ to form large crystal violet-iodine (CV-I) complexes within the cytoplasm and outer layers of the cell. For the third step, cells are treated with alcohol or acetone solvent in which the iodine-crystal violet complex is soluble. Following solvent treatment, only Gram-positive cells remain stained. The outer membrane of the Gram-

negative cell (lipopolysaccharide layer) is lost from the cell, leaving the peptidoglycan layer exposed. Gram-negative cells have thin layers of peptidoglycan, one to three layers deep with a slightly different structure than the peptidoglycan of gram-positive cells. With ethanol treatment, Gram-negative cell walls is dissolved, and this allow the large CV-I complexes to be washed from the cell, whereas the highly cross-linked and multi-layered peptidoglycan of the Gram-positive cell is dehydrated by the addition of the solvent. The multi-layered nature of the peptidoglycan along with the dehydration from the ethanol treatment traps the large CV-I complexes within the cell [10].

After decolourisation, the Gram-positive cell remains purple in colour, whereas the gram-negative cell loses the purple colour and is only revealed when the counterstain is added. After the decolourisation procedure, cells are treated with a counterstain, i.e., a positively-charged red acidic dye such as safranin, in order to make Gram-negative (decolourised) cells visible (sometimes basic fuchsin is substantiated for safranin; fuchsin, generally, stains bacteria more intensely than safranin. In addition, some bacteria are poorly stained by safranin, such as *Haemophilus* spp., *Legionella* spp., and some anaerobic bacteria). Counterstained Gram-negative cells appear red, and Gram-positive cells remain purple. The slide is then examined microscopically using a x 1000 magnification (via a x10 eyepiece and a x100 objective) under oil immersion (to improve the refractive index) [11].

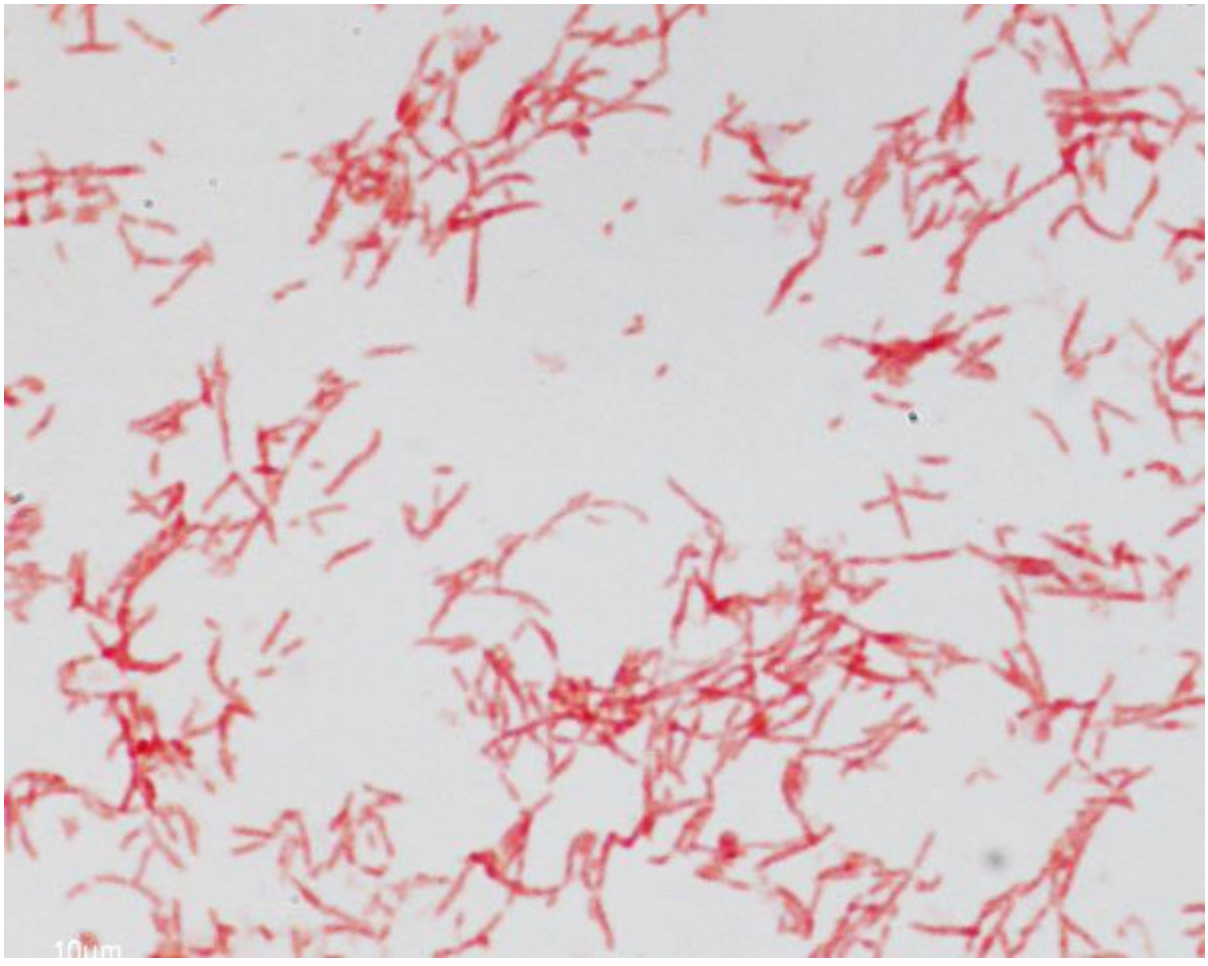

Figure 2: Negative Gram-stain (Bacteroides species) (Image: Tim Sandle)

Some bacteria cannot be stained using the Gram-method, these include bacteria that exist almost exclusively within host cells such as intracellular bacteria (such as *Chlamydia*); those that lack a cell wall (like the Mycoplasmas); and these of insufficient dimensions to be resolved by light microscopy (such as the Spirochetes). In these cases, alternative stains can be considered [12].

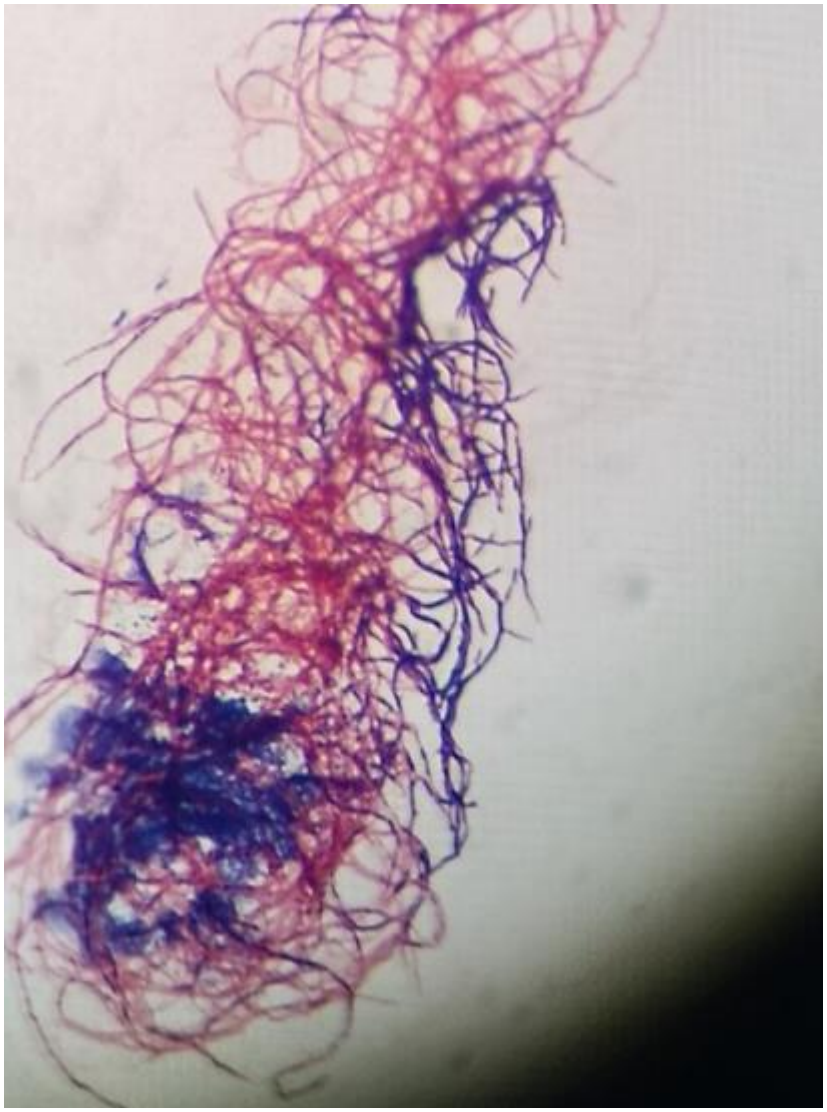

Figure 3: Mixed Gram-stain (thermophilic Actinomycete) (Image: Tim Sandle)

Gram-stain errors

For an effective Gram-stain, the starting bacterial cell number needs to be sufficient (ideally taken from a pure colony). To be visible on a slide, organisms that stain by the Gram method must be present in concentrations of about 10^4 to 10^5 cells. Aside from this, errors can occur with the Gram-stain and where errors lead to misidentifications, these can have serious consequences.

The primary error is through mis-identifying Gram-positives as Gram-negatives and Gram-negatives as Gram-positives. Of these two mis-categorizations the former is more common. Gram-positive bacteria may lose their ability to retain crystal violet and stain Gram negatively due to cell wall damage of bacteria due to antibiotic therapy or excessive heat fixation of the smear or through the use of an Iodine solution which is too old, that is where the iodine turns yellow instead of being brown in colour. However, the most common issue relates to over-decolourisation of the slide smear. The key to the technique relates to the length of time that the solvent is applied during the 'decolourisation' step, for too long an exposure removed the stain from both groups of bacteria [13]. A prolonged exposure to the decolourising agent will remove all the stain from both types of bacteria. Some Gram-positive bacteria may lose the stain easily and therefore appear as a mixture of Gram-positive and Gram-negative bacteria (Gram-variable). Further, the phenomenon of 'Gram-variability' (or 'Gram indeterminate') is also shown by ageing cells where logarithmic growth has been suspended, remains a problem. What is happening is the thinning of the cell wall peptidoglycan layer, which occurs as some cultures age. A further factor is where Gram staining imposes great stress on the integrity of some organisms leading to a weakening of the protein S-layer [14].

It is also possible for Gram-negatives to appear as Gram-positives. This can occur when the smear is too thick, resulting in Gram-negative bacteria not being fully decolourised during the decolourisation steps and appearing as Gram-positive bacteria. Other errors include:

- Smear improperly heat-fixed.
- Iodine solution is weak.
- Failure to rinse between reagents.

Detection errors can also occur through the failure to use positive and negative control smears on the slide, which means that procedural inaccuracies may go undetected.

Assessment of Gram-stain error rate

This paper presents a study of Gram-stain error rates across a two-year period, across multiple laboratory analysts. The analysts worked at a major pharmaceutical facility located in the south-east of England. Over 6,000 separate Gram-stain results were assessed for errors and the errors were categorised, in an attempt to establish common causes for the errors. With the study presented in this paper, errors were assessed by [15]:

- Comparing the Gram-stain result to plate morphology
- Comparing the Gram-stain result to later species identification
- Assessing the Gram-stain by supervisory check and later repeat test

The types of errors were categorized into:

1. Misread stains (positive stains misread as negative and vice versa).
2. Mixed cultures.
3. Using subcultures that were aged (plated out >24 hours).
4. Over decolourisation.
5. Inadequate fixation (e.g. iodine solution is too weak).
6. Disorganisation resulting in slides being mislabelled and the incorrect result being recorded for a given slide.

7. Small specimen size.
8. Low number of bacterial cells.

Study results

During the course of the study, results from a total of 6,303 specimens (performed by ten different analysts) were reviewed for discrepant smear Gram-stain results, as set out in Table 1. These tests were conducted over a two-year period (2016 to 2018). The incidence of discrepant specimens was 216 (or 3.2% of the samples processed). The range across the analysts was between 0% and 6.4%, with the mean error rate standing as 2.9%.

Table 1: Error rate by analyst

Analyst	Number of Gram-stains prepared (n=6,303)	No. (& percentage) of discrepant results
1	1,030	36 (3.5%)
2	528	21 (3.9%)
3	374	14 (3.7%)
4	1,120	72 (6.4%)
5	905	33 (3.7%)
6	55	0 (0%)
7	327	11 (3.4%)
8	861	5 (0.6%)
9	1,005	22 (2.1%)
10	98	2 (2%)

In terms of the types of errors, Table 2 classifies the errors as Gram-stain results initially recorded as Gram-negative (but where the bacterium was actually Gram-positive); where the Gram-stain results initially recorded as Gram-positive (but where the bacterium was actually Gram-negative); and where the result could not to be said to be conclusively either Gram-negative or Gram-positive due to other factors (such as age of culture or due to a mixed culture).

Table 2: Types of errors recorded by analyst according to incorrect Gram-stain outcome

Analyst	No. (& percentage) negative results that should be positive	No. (& percentage) positive results that should be negative	No. (& percentage) indeterminant stains
1	32 (3.1%)	4 (0.4%)	0 (0%)
2	15 (2.8%)	3 (0.6%)	3 (0.6%)
3	14 (3.7%)	0 (0%)	0 (0%)
4	60 (5.3%)	10	2
5	33 (3.7%)	0 (0%)	0 (0%)
6	0 (0%)	0 (0%)	0 (0%)
7	10 (3.1%)	0 (0%)	1 (0.3%)
8	4 (0.5%)	1 (0.6%)	0 (0%)
9	11 (1.1%)	6 (0.6%)	5 (0.5%)
10	2 (2%)	0 (0%)	0 (0%)
Total	181	24	11

With Table 2, the percentages are based on the number of tests performed by the analyst as per Table 1. Table 2 shows that the most common error was with producing a Gram-negative result in error when the bacterium was actually Gram-positive. This represented 181 of the 216 errors (83%). This type of error is consistent with over-decolourisation of the stain. The second error, recording Gram-negative organisms erroneously as Gram-positive organisms accounted for 24 incidents (of the 216 errors), 11% of the samples. In a few cases (11 of 204 or 5%) the result was indeterminant.

Table 3 provides a further breakdown of the reason for the errors, based on assessment by supervisors and noting the performance of any repeat tests conducted.

Table 3: Detailed breakdown of error root causes, by number

Analyst	Misread stains	Mixed cultures	Aged subcultures	Over decolourisation	Inadequate fixation	Disorganisation	Insufficient culture
1	1	2	1	32	0	0	0
2	1	3	2	14	0	1	0
3	0	0	0	12	1	0	1
4	2	4	2	60	0	4	0
5	1	1	0	31	0	0	0
6	0	0	0	0	0	0	0
7	2	0	0	9	0	0	0
8	2	1	0	2	0	0	0
9	1	5	4	11	0	0	0
10	0	0	0	2	0	0	0
Total	10	16	9	173	1	5	1

Based on the analysis presented in Table 3, the primary reason for errors relates to over-decolourisation, at 173 of the 216 identified discrepant Gram-stains. This is followed by the starting culture being mixed, at 16 and by misreading of stains (that is stains that were correctly performed but which were misread by the analyst). The rank of errors is:

1. Over decolourisation
2. Mixed cultures
3. Misread stains
4. Aged subcultures
5. Disorganisation
6. Inadequate fixation and Insufficient culture

In terms of the types of organisms most likely to be susceptible to errors, this data was not formally collated. However, the general pattern suggest that the over-decolourisation of *Bacillus* species and related genera represented the largest bacterial group prone to Gram-stain error. Where unchecked, this could lead to *Bacillus* species being mis-identified as Gram-negative organisms (such as Pseudomonads). In terms of sample types, such misidentifications tended to relate to environmental monitoring samples drawn from cleanroom environments where medical products were prepared.

Discussion

Gram staining of bacterial cells is neither an accurate nor elaborate technique, but nevertheless it is practically useful to distinguish two big domains of eubacteria species: Gram-positive bacteria that include most of the Firmicutes, and Gram-negative bacteria that include the remainder [16]. Even in the emergence of more sophisticated microbial identification methods, Gram-staining is required in many circumstances. Moreover, even where manufacturers of microbial identification technologies state that Gram-staining is not required (as with some molecular arrays) [17] or analysis of headspace volatile compounds [18], should an atypical or discrepant result arise being able to refer back to the Gram-stain invariably proves useful when conducting an investigation. Misidentification poses the risk of sending any investigation down the wrong path which can cause further delays (and hence impact on batch release) or present the person tasked with batch release (the Qualified Person in Europe) with incorrect information and hence the risk of an incorrect decision being made.

The data presented in this paper demonstrates that despite the long-history of the Gram-stain, errors with the technique still occur, even with analysts who are well-trained and who would be considered advanced practitioners.

In comparing the error rate to a pharmaceutical microbiology laboratory, the clinical error rate was found to be 3.2% based on 6,303 specimens (based on discrepancy from culture) [19]. Notably there were some differences with the analysts in terms of their performance over the two-year assessment period. The errors divided into specimens with no organisms reported on Gram-stain but where significant growth on culture was observed; reported organisms on a Gram-stain that were not recovered in culture; and discrepant results due to

reader error and so on, as set out in Tables 2 and 3. The primary reason for error was due to over-decolourisation.

In terms of best practices designed to reduce error rate, this can be tackled by having clearly defined standard operating procedures and putting analysts through robust training regimes. Analyst competency takes on and even greater importance where the centralisation of microbiology testing occurs, and activities become staffed by non-traditional microbiologists [20]. Training can be supported by participation in proficiency schemes [21]. In addition, an automated Gram-stainer may standardize the protocol; although such technology is not without its limitations [22,23]. Errors can also be reduced by using controls with each slide (one organism designed to produce a positive reaction and another organism designed to produce a negative reaction). Controls can either be prepared in the laboratory using known cultures (such as those traceable to a national culture collection) or by using commercial slides with the controls pre-prepared as dried-on smears. An additional practice to assess the difference between Gram-positive and Gram-negative organisms is the potassium hydroxide test, which assesses the difference in the cell wall, and this can supplement microscopic analysis [24]. Errors can also be reduced through ensuring that the time steps for each stage of the staining procedure are adhered to, such as by using a stopwatch.

The research presented has some limitations in that the error rates obtained using this approach may only apply to the specific laboratory (rather than a multi-facility assessment) and within one country. It may be that the actual error rate for Gram-stains in other pharmaceutical microbiology laboratories may differ from the data presented here (as indeed may laboratories with different functions, such as clinical laboratories). Furthermore, the data presented represents a two-year window, which itself may or may not be truly representative. Looking at the total data does not permit an evaluation as to whether the performance of analysts improves over time (such as progressing from a trainee to a more experienced analyst). However, the data presented in this paper is generally comparable to other studies in the clinical setting. Samuel and colleagues recorded an error rate of around 5% across different clinical facilities [19], which was a similar finding to Brizzi *et al* (also 5%) [25]; with Munson and associates finding a 6% error rate [21]. In different research, Rand and Tillan recorded a lower error rate of around 1% [26].

The data set out can go towards establishing a benchmark for the incidence of errors during the performance of Gram-stains in the pharmaceutical and other disciplines and the types of errors identified can be considered and addressed through laboratory training programmes, with an aim to seek future improvements with the Gram-stain technique.

References

1. Sogaard M, Norgaard M, Schonheyder HC (2007) First notification of positive blood cultures and the high accuracy of the gram stain report. *J Clin Microbiol* 45:1113–1117
2. Strand CL (2006) Positive blood cultures: can we always trust the Gram stain? *Am J Clin Pathol* 126:671–672
3. Musher DM, Montoya R, Wanahita A. (2004) Diagnostic value of microscopic examination of Gram-stained sputum and sputum cultures in patients with bacteremic pneumococcal pneumonia. *Clin Infect Dis* 39:165–169
4. Sandle, T. (2004) Gram's Stain: History and Explanation of the Fundamental Technique of Determinative Bacteriology, *IST Science and Technology Journal*, No. 54 ,pp3-4
5. Gram, H. C. J. (1884): Über die isolirte Färbung der Schizomyceten in Schnitt- und Trockenpräparaten, *Fortschritte der Medizin, Berlin*, 2: 185-189.
6. Adams E. (1975) Studies in gram staining, *Stain Technol*. 50(4):227-31
7. Coico R (2005) Gram staining, *Curr Protoc Microbiol*. Appendix 3:Appendix 3C. https://doi.org/10.1002/9780471729259.mca03cs00
8. Popescu A, Doyle RJ. (1996) The Gram stain after more than a century, *Biotech Histochem*.;71(3):145-51
9. Beveridge TJ (2001) Use of the gram stain in microbiology, *Biotech Histochem*. 2001 May;76(3):111-8
10. Mueller, K.L. (2015) Detecting Gram-negative bacteria. *Science*. 348 (6240): 1218
11. Moyes RB, Reynolds J, Breakwell DP. (2009) Differential staining of bacteria: gram stain, *Curr Protoc Microbiol*. Appendix 3: Appendix 3C. https://doi.org/10.1002/9780471729259.mca03cs15
12. Sizemore RK, Caldwell JJ, Kendrick AS. (1990) Alternate gram staining technique using a fluorescent lectin, *Appl Environ Microbiol*.;56(7):2245-7
13. Nagendra S, Bourbeau P, Brecher S, Dunne M, LaRocco M, Doern G. (2001) Sampling variability in the microbiological evaluation of expectorated sputa and endotracheal aspirates. *J Clin Microbiol* 39:2344–2347
14. Beveridge TJ (1990) Mechanism of gram variability in select bacteria, *J Bacteriol*. 172(3):1609-20
15. Rand KH, Tillan M (2006) Errors in interpretation of Gram stains from positive blood cultures. *Am J Clin Pathol* 126:686–690
16. Aizawa, S-I. (2014) Introduction. In Aizawa, S-I. (Ed.) *The Flagellar World: Electron Microscopic Images of Bacterial Flagella and Related Surface Structures*, Academic Press, Oxford, UK, pp1-11
17. Rhein J, Bahr, R.J., , Hemmert AC, Cloud J L, *et al* (2016) Diagnostic performance of a multiplex PCR assay for meningitis in a HIV-infected population in Uganda. Diagn *Microbiol Infect Dis* 84:268–273
18. Dolch, M. E., Janitza, S., Boulesteix, A-L., *et al* (2016) Gram-negative and -positive bacteria differentiation in blood culture samples by headspace volatile compound analysis, *Journal of Biological Research-Thessaloniki*, 23 (3): https://doi.org/10.1186/s40709-016-0040-0
19. Samuel, L. P., Balada-Llasat, J-M., Harrington, A. and Cavagnolo, R. (2016) Multicenter Assessment of Gram Stain Error Rates, *Journal of Clinical Microbiology*, 54 (6): 1442 - 1447

20. Church, D. L., Don-Joe, C. and Unger, B. (2000) Effects of restructuring on the performance of microbiology laboratories in Alberta. *Arch. Pathol. Lab. Med*. 124:357-361
21. Munson, M., Block, T., Basile, J., Hryciuk, J. E., Schell, R. F.(2007) Mechanisms To Assess Gram Stain Interpretation Proficiency of Technologists at Satellite Laboratories, *Journal of Clinical Microbiology*, 45 (11): 3754-3758
22. Wilkins, J. R., and Mills, S. M. (1975) Automated single-slide staining device. *Appl. Microbio*l. 30:485-488
23. Baron, E.J., Mix, S., and Moradi, W. (2010) Clinical Utility of an Automated Instrument for Gram Staining Single Slides, *Journal of Clinical Microbiology*, 48 (6): 2014-2015
24. Roberts, J. and Sandle, T. (2008): Assessment of the Potassium Hydroxide Test as a Complimentary Test for the Gram-stain, *Pharmig News*, No. 31, pp1-4
25. Brizzi K, Hines EM, McGowan KL, Shah SS (2012) Diagnostic accuracy of cerebrospinal fluid gram stain in children with suspected bacterial meningitis. *Pediatr Infect Dis J* 31:195–197
26. Rand, K. and Tillan, M. (2006) Errors in Interpretation of Gram Stains From Positive Blood Cultures, *American Journal of Clinical Pathology* 126(5):686-90