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SURAT KETERANGAN

Nomor: 1227/UNUSA/Adm-LPPM/IX/2021

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No. Pemeriksaan	:	2021.27.09.446

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Submission date: 21-Jul-2021 02:41PM (UTC+0700)
Submission ID: 1622276002
File name: Prayekti_2021_IOP_Conf._Ser.__Earth_Environ._Sci._819_012075.pdf (409.37K)
Word count: 2224
Character count: 11870

Variations in the incubation time of the Staphylococcus aureus, Bacillus sp and Escherichia coli cultures on the results of the gram stain visualization

Variations in the incubation time of the Staphylococcus aureus, Bacillus sp and Escherichia coli cultures on the results of the gram stain visualization

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Abstract. Gram staining is a routine procedure performed in microbiology laboratories. Gram staining of bacteria was used to obtain data on the shape and group of gram bacteria. The results of gram staining can give confusing results so that the next process will be invalid. Several things were found in the laboratory, pure culture staining that should show gram-positive, but the results showed gram-negative or the presence of a mixture of gram-positive and negative in one field of view. The aim of this study was to determine the effect of incubation time on the results of gram bacteria staining. This research is an experimental study using pure cultures of Staphylococcus aureus, Bacillus sp, and Escherichia coli at 16, 18, 20, 24, and 26 hours of culture. The parameter observed was the color that was shown after gram staining at each bacterial culture age. The data obtained is qualitative data which is then processed into quantitative and then statistically analyzed. Statistical results using kruskall walis showed a significant difference between treatment groups based on incubation time. The results of the most uniform gram staining visualization were found at the incubation time of 16 and 18 hours.

1. Introduction

Information on gram bacteria plays an important role in the identification process because of the presence of a system of grouping bacteria into two, namely gram positive and gram negative. After the information on the gram and shape of the bacteria is known, various biochemical tests can be performed to obtain bacteria identity (1). The result of Gram stain can be used as an early diagnostic of infection in the urine specimen. Prasetyowati (2) concluded that bacteriuria on gram urine stains can be used as a diagnostic criterion for UTI in children. In contrary, the use of gram stain for joint fluid specimens cannot be used as an initial diagnosis in patients requiring surgery for drainage and cleaning. Although Gram stain cannot be used as a reason for making surgery decisions, any finding of microorganisms in the results of joint fluid staining, it describing certainty for positive joint fluid culture (3).

False positive results on gram staining, among others, are due to the presence of dead bacteria in the reagent (4), while false negative results can be due to coagulation (5). In general, the confusion of results can be evaluated from reagents and sample handling. However, in pure cultures where the species are known, evaluation can be done from the incubation time of the bacterial culture to be stained. Some finding in the laboratory, pure culture staining which should give gram-positive result, show vary in color of the stain in one field of view which can potentially confuse the pure culture examination.

2. Material and Methods

2.1. Bacterial strains and preparation

The bacteria used are a collection from the Microbiology Laboratory of the Nahdlatul Ulama University Surabaya. Bacteria used in this research were Escherichia coli, Bacillus sp., and Staphylococcus aureus. All bacteria maintain in Nutrient Agar (MERCK) at 37oC in incubator (Memmert). Preparation of the test bacteria was carried out by subculturing the bacteria on fresh Nutrient Agar medium. Incubation time for bacterial culture subsequently were 16 hours, 18 hours, 20 hours, 24 hours and 26 hours.

2.2. Gram Staining

Bacterial smears at object glass were labelled according to culture incubation time. Each of bacterial smears then undergo gram staining process using Hucker methods. Staining process were caried out based on Gram Staining Protocols (6). Results of gram staining were observed using brightfield microscope (Olympus CX23) under oil immersion (Olympus). Colour chart based on RGB were used for gram staining evaluation.

3. Result and Discussion

Gram stain was first used by Hans Christian Gram in 1884 (6). The gram staining method is then used to differentiate bacterial groups based on their cell wall components (7). In general, bacteria are divided into gram-positive and negative groups. The gram-positive group had a thick peptidoglycan layer on the cell surface, while the gram-negative group had a thin peptidoglycan layer (7, 8). The thickness of the peptidoglycan will affect the binding of dyes to the bacterial cell wall. Bacteria that have thick peptidoglycan will form a complex bonding between the main dyes and iodine which is strong against the decolorizing agent so that it displays a purple color (8, 9).

In this study, gram-positive and gram-negative bacterial cell walls were stained at different incubation times. Gram positive were represented by Staphylococcus aureus and Bacillus sp., while gram negative was represented by Escherichia coli. The results of staining the three bacteria at different incubation times are illustrated in Tables 1, 2 to 3. Different incubation times gave various staining results, but still in accordance with the gram type of bacteria tested. Staphylococcus aureus and Bacillus sp.-stained results were similar. Treatments with an incubation time of 16 and 18 hours produced the same color visualization between the two gram-positive bacteria.

Table 1. Gram result for Staphylococcus aureus based on different incubation time

Bacteria Species	Incubation time (hour)	Colour visualisation (RGB)	Description
Staphylococcus aureus	16	2607 (86 2 130)	Dark purple
	18	276 (42 32 72)	Dark Blue
	20	253 (170 21 166)	Purple
	24	260 (170 21 180), 256 (186 10 176), 269 (64 32 86), 270 (179 175 216), 271 (153 146 205), 272 (131 119 194), 273 (58 23 115)	Vary from violet to dark purple
	26	255 (112 49 108), 256 (216 196 222), 257 (200 165 209), 258 (142 78 151), 259 (101 15 110), 2623 (84 13 98), 2613 (91 4 111)	Vary from violet to dark purple

At the incubation time of 20 hours, the results varied in one field of microscopic view as shown in the staining of Bacillus sp. Meanwhile, Staphylococcus aureus still gave uniform results. The color visualization of Escherichia coli at the incubation time of 16 and 18 hours gave uniform results, while the results of 20 hours showed slight variation. Gram stain is considered to be a faster process for determining the presence of bacterial infection in a sample than doing culture (9,10). However, the variation in results in gram staining can lead to misinterpretation when carrying out the initial identification of bacteria and establishing the disease diagnosis.

This is especially the case for some tissue samples. Confusion will appear if the resulting color resembles the tissue around bacteria (11). Gram reading errors also occur with positive blood agar cultures. Readability errors mainly occur with gram-positive cultures which read as gram-negative (12).

Table 2. Gram result for Bacillus sp. based on different incubation time

Bacteria Species	Incubation time (hour)	Colour visualisation (RGB)	Description
Bacillus sp.	16	2607 (86 2 130)	Dark Purple
	18	276 (42 32 72)	Dark blue
	20	2567 (178 143 209), 299 (53 154 211)	Predominantly violet
	24	258 (142 78 151), 2582 (150 61 190), 276 (42 32 72), 277 (188 205 299), 278 (158 185 227), 279 (84 131 214), 280 (15 44 126)	Vary from light blue – dark blue to violet
	26	260 (170 21 180), 276 (42 32 72), 277 (188 205 299), 278 (158 185 227), 279 (84 131 214), 280 (15 44 126), 2563 (191 151 211)	Vary from light blue – dark blue to violet

Table 3. Gram result for Escherichia coli based on different incubation time

Bacteria Species	Incubation time (hour)	Colour visualisation (RGB)	Description
Escherichia coli	16 Jam	226 (187 0 117)	Red
	18 jam	2385 (196 21 170)	Rhodamine red
	20 Jam	1787 (255 64 85), 1785 (230 76 100)	Red
	24 Jam	1787 (255 64 85), 1788 (221 46 60), 1790 (208 70 61), 184 (228 97 127)	Vary of Red
	26 jam	1785 (230 76 100), 206 (190 15 84) , 199 (167 29 69)	Vary of Red

Table 4. Kruskall wallis test for visualisation from each bacterial group

No	Bacterial Species	Test	Significant
1	*Staphylococcus aureus*	Kruskall Walis	0,000

2	*Bacillus sp.*	Kruskall Walis	0,025
3	*Escherichia coli*	Kruskall Walis	0,000

The three tested bacteria in this study gave varied gram staining results in the 24- and 26-hours treatment. Staphylococcus aureus and Bacillus sp. has 7 color variations at the 24- and 26-hours incubation time treatment, while Escherichia coli shows 4 color variations at 24 hours and 3 color variations at 26 hours. Statistical analysis using kruskall walis showed varied results for each treatment group. Changes in the cell wall of gram-positive bacteria occur with age of bacterial culture. Bacillus brevis reached the stationary phase at more than 20 hours, and the results of gram staining visualization showed a mixture of gram-positive and gram-negative on microscopic observation (13).

In line with this study, when the culture of Bacillus sp. reached an incubation time of 20 to 26 hours, there were variations in the gram color. However, the color variations still range in the purple color range. The various color visualizations produced in this study could be due to changes that occur in the bacterial cell walls along with the incubation time. These changes can be due to the recycle of the building blocks of bacterial cell walls (14,15), the defense of bacterial cells during the stationary phase (16), and the turn over process in the bacterial cell wall (15)

4. Conclusion

Gram stain of the test bacteria showed varying results at each incubation time. However, in general, the uniformity of visualization can be demonstrated at the 16- and 18-hour incubation times.

Acknowledgment

This research was supported by the Institute for Research and Community Service of the Nahdlatul Ulama University Surabaya

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