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Utilization of Alternative Buffer Solutions for Staining Thin Blood smears by the Giemsa, Wright stain and Romanowsky method

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ABSTRACT: To make a reliable diagnosis on haematological examination, it is necessary to examine further the morphology of the blood cells previously stained in the procedure for staining thin blood smears using a buffer solution with a standard pH of 6.4 to 6.8. One of the problems that may occur in the laboratory is that the buffer reagents are damaged, past the expiration date or running out, so alternative buffers are needed that are cheap, fast and easy to obtain. This study aims to evaluate the staining results from alternative buffers. Study used a quasi-experimental method, and using alternative buffers from bottled mineral water following SNI-01-0553 2006. The colour produced by some of these alternative buffers is almost equivalent to blood cells stained with phosphate buffer. The percentage of assessment results in the alternative buffer codes B, C, D, E and F compared with control (A) were 62.67%, 92.00%, 82.67%, 80.00% 88, 00%, and 68.00%. The use of alternative buffers for staining thin blood smears using the Giemsa, Wright stain, and Romanowsky method can be done with mineral water as an alternative buffer for sample codes C, D, E and F, while B and G cannot be used. This alternative buffer can be applied by laboratory personnel in urgent situations in limited equipment and material facilities.
Keywords: Alternative buffer; blood cell stain; erythrocyte; leukocyte

INTRODUCTION

A peripheral blood smear is a laboratory examination that involves cytology of peripheral blood cells smeared on a slide. As a basis for examination, the peripheral blood smear is invaluable in the characterization of various clinical diseases[1].

An excellent thin blood smear preparation must meet the requirements; namely, the width and length do not meet the entire glass, the tail is not shaped like a torn flag, the thickening appears to be gradually thinning from head to tail, not perforated, not broken, not too thick[2]. In the staining procedure for thin blood smears using Giemsa, Wright stain and Romanowsky dye, a buffer solution is used with a standard pH of 6.4 to 6.8. However, when laboratory personnel do staining in a simple laboratory or field, the buffer is mineral water packed and others whose pH is unknown because it is more practical and economically cheaper. Adding azur B dye will colour acidic cell components, while eosin Y will colour alkaline. The bonding of the two dyes will create a contrast with the result that the nucleus is purple and the cytoplasm is blue. Buffer solution with a low pH of less than 6.8 causes leukocytes to not completely absorb Giemsa dye because it is too acidic so that nuclear chromatin, which should be purple

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only forms part in the middle of the nucleus, and some is red; leukocytes will also show parts that are lacking. On the other hand, the buffer solution with a high pH of more than 6.8 with a strong base causes leukocytes to absorb too much methylene blue so that the cytoplasm becomes more concentrated and the granules darken[2].

One thing that must be considered in a good Giemsa stain is the accuracy of the pH of the buffer as a solvent solution. A buffer with a low pH causes red blood cells to absorb much eosin, so the nuclear chromatin that should be purple becomes pinker. Leukocytes will show parts that are less clear[3]. Previous research has proven that several factors, like temperature and humidity[4,5], Concentration[6], can affect the ability of blood cells to absorb the dye,so the difficulty of observing the results depends on the manufacture and colouring of the preparation. Research conducted by Suryanta et al., 2013, found a significant difference between the effect of the pH buffer on the morphological staining of erythrocytes[7]. According to Rahmah's research in 2018, it was found that there was a significant difference between Aquadest solution, PDAM Water and Phosphate Buffer pH 6.8 on the quality of Wright's thin blood smear[8]. This study seeks to enrich insight and provide another illustration of the use of alternative buffers derived from bottled drinking water in Indonesia, so this study aims to evaluate the results of blood smears with alternative buffers derived from bottled drinking water in Indonesia.

MATERIALS AND METHODS

The research design is an analytical survey. This type of research is laboratory observational, namely to see the quality of thin blood smears using Giemsa, Wright stain and Romanowsky staining methods.

Materials

The materials used are Azur B and Eosin Y dyes, the alternative buffer is six different types of mineral water pack, following SNI-01-0553 in 2006[9], and control blood samples, control blood used is with the following inclusion criteria morphology and the number of leukocytes and erythrocytes were average. The number of repetitions in this study for each alternative buffer sample is 5.

pH Measurements

Multi-Parameter PCD650 Eutech was used as pH measurements tools in this study. First, wash an electrode with aqua dest, dry it with tissue, and then calibrate the tools with pH controls buffer solution. Prepare a mineral water sample solution, homogenize, measure the sample solution's temperature and ensure it is the same as the temperature of the calibrator buffer solution. For measurement, clean the electrode with aqua dest again, then dry it with a tissue. Insert the electrode into the sample solution, press the "Measure" button, let it stand 10-15 seconds, and then wait until the final result of the pH measurement appears[10].

Preparate Preparation

Venous blood samples were taken using a three-cc syringe and inserted into the EDTA tube. The sample is made a peripheral blood smear immediately after being taken. The blood in the EDTA vacuum tube must be shaken up and down, so the blood plasma mixes with the blood cells. Then the blood is taken using a dropper and dripped onto the slide (glass object). Next, the glass object is placed at an angle of 25°- 30° on the drop of blood, then pulled straight to the end of the preparation[11,12].

Blood Smear Staining

Stain Preparation

For Giemsa, the buffer was added and mixed with Giemsa stock with a ratio of 1 (Giemsa stock): 9 (buffer solution); for Wright, the buffer was added and mixed with

a ratio of 1:1, and buffer was added to smear after wright stain solution, For Romanowsky, a combined technique from Giemsa and Wright.

Blood Smear Staining Process

For the Giemsa stain, methanol was dripped onto the slide and left for 5 minutes. Then the remaining methanol is removed. Drops of Giemsa solution (until all the smear is flooded) and wait for 15 minutes. The preparations were rinsed with water and then dried in the air[12].

For wright stain, drip Wright's solution onto the slide (until all the swabs are flooded), Drop the pH 6.4 buffer solution (until all the swabs are flooded) and wait for 5-12 minutes. The smear is rinsed with water, and the back of the soiled smear is cleaned of residual dye. The peripheral blood smear was left to dry in the air[12].

For the Romanowsky stain, drop Wright's solution onto the slide until all the swab is submerged, then let it sit for 2 minutes. Add 10% Giemsa solution until the smear is wholly flooded, then wait for 15 minutes. The preparations were rinsed with water and then dried in air[5].

Assessment of Preparate Results

Readings of blood smear preparations can be made on the top and bottom of the zone close to the tail. Reading technique is one of the determining factors in successfully assessing blood smear preparations[11]. Assessment of the results in this study was carried out by comparing the morphology of erythrocytes, leukocyte cell morphology, erythrocyte cell colour suitability, leukocyte cell colour match and blood cell contrast with the background, then compared with the control preparations, which were stained with phosphate buffer pH 6.8. All of these criteria are worth 1 point with a maximum of 5 points for each preparation. Then the assessment results are presented with a lower value limit of 75% for alternative buffers that meet the colouring requirements.

RESULTS AND DISCUSSION

Table 1. pH Meter Measuring Results

Sample Code	Function	Sample Material	Results
A	Control	Buffer Phosphate	6,80
B	Sample 1	Mineral Water	7,19
C	Sample 2	Mineral Water	6,83
D	Sample 3	Mineral Water	7,07
E	Sample 4	Mineral Water	7,04
F	Sample 5	Mineral Water	6,87
G	Sample 6	Mineral Water	7,16

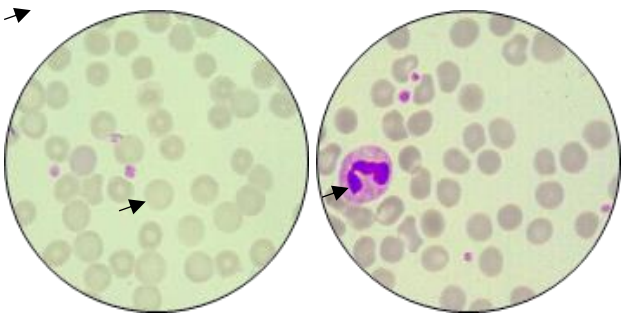

Figure 1. Control Giemsa Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

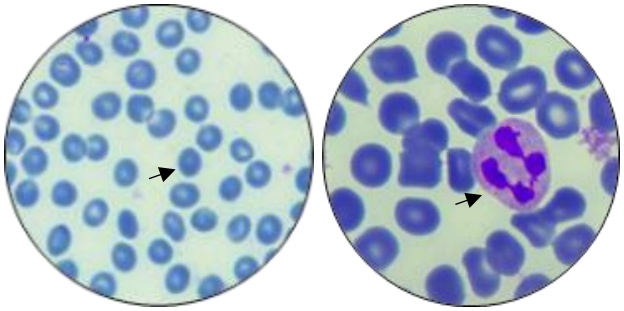

Figure 2. Sample Code B Giemsa Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

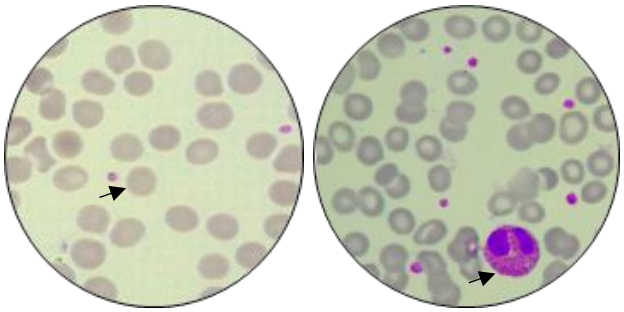

Figure 3. Sample Code C Giemsa Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

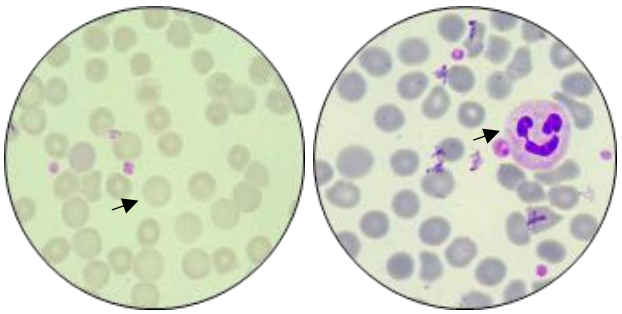

Figure 4. Sample Code D Giemsa Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

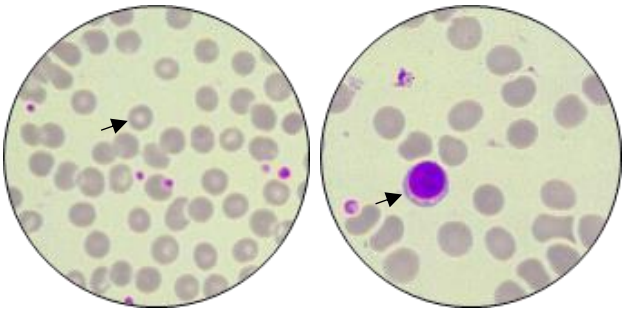

Figure 5. Sample Code E Giemsa Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

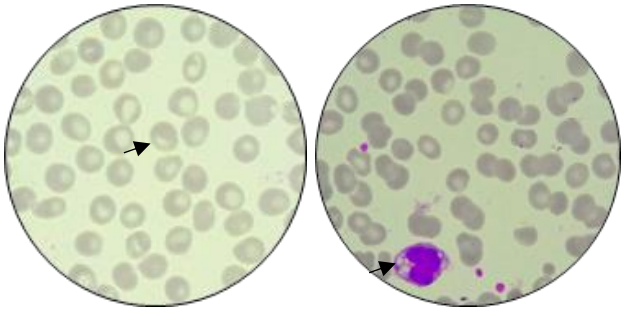

Figure 6. Sample Code F Giemsa Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

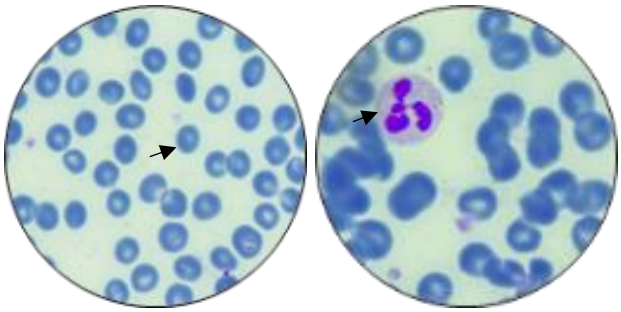

Figure 7. Sample Code G Giemsa Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

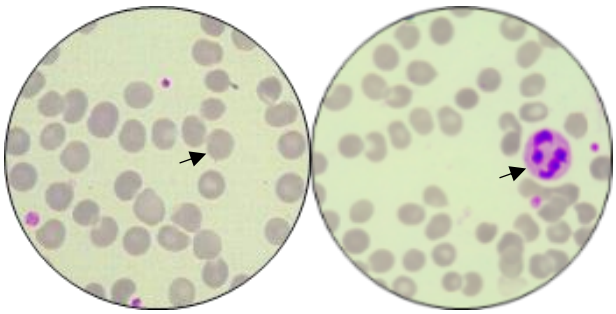

Figure 8. Control Wright Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

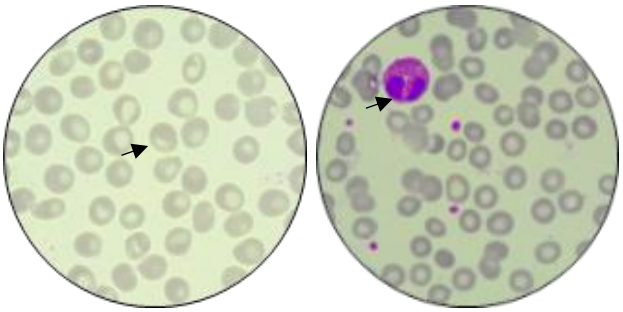

Figure 9. Sample Code B Wright Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

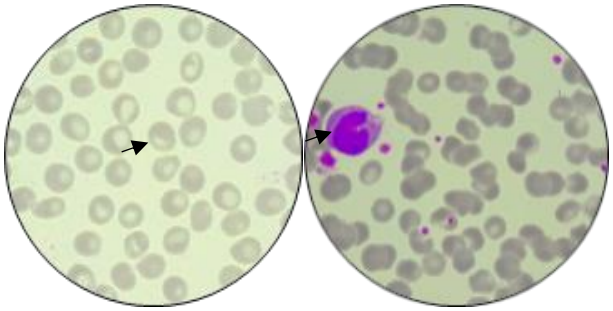

Figure 10. Sample Code C Wright Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

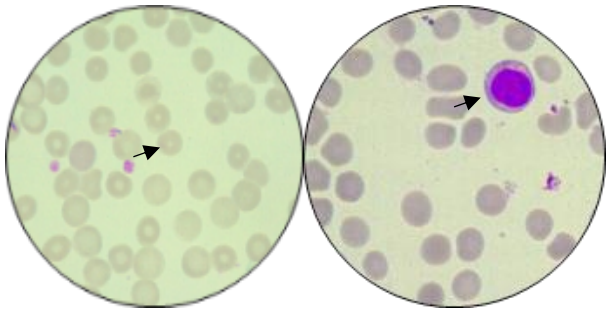

Figure 11. Sample Code D Wright Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

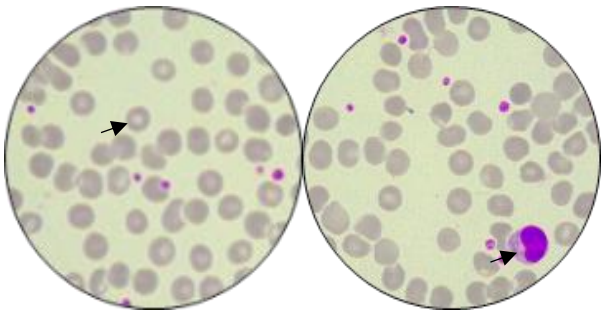

Figure 12. Sample Code E Wright Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

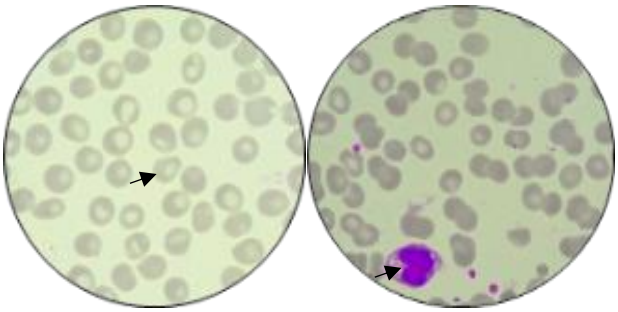

Figure 13. Sample Code F Wright Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

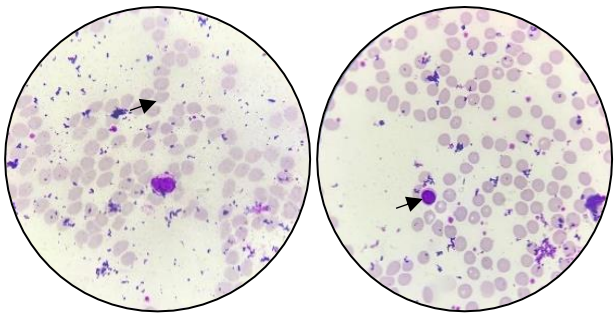

Figure 14. Sample Code G Wright Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

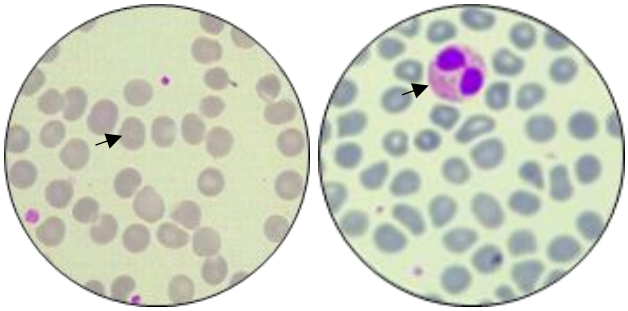

Figure 15. Control Romanowsky Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

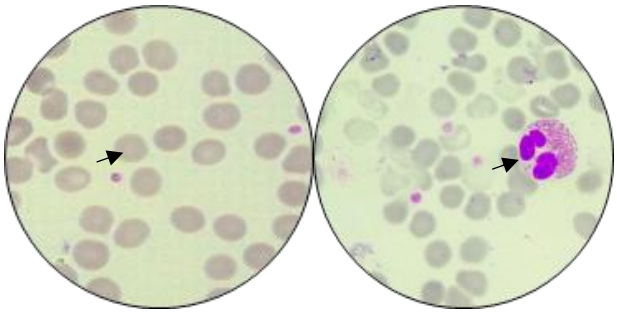

Figure 16. Sample Code B Romanowsky Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

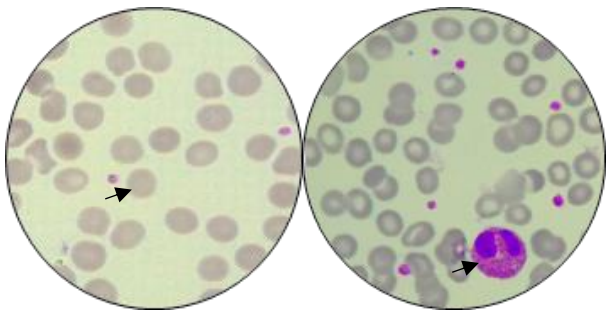

Figure 17. Sample Code C Romanowsky Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

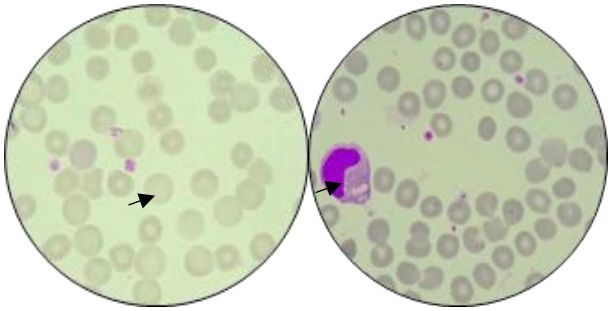

Figure 18. Sample Code D Romanowsky Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

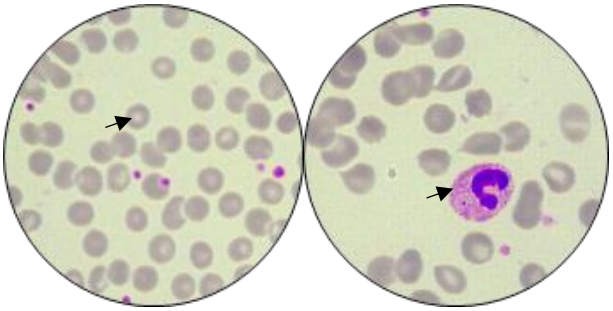

Figure 19. Sample Code E Romanowsky Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

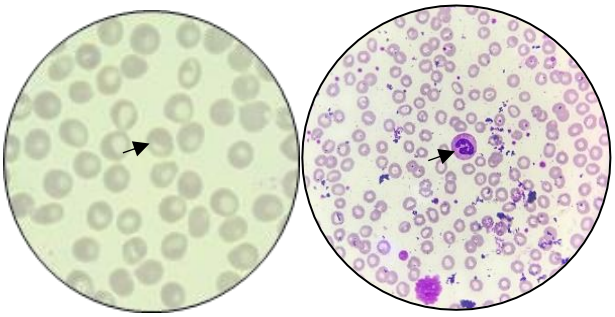

Figure 20. Sample Code F Romanowsky Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

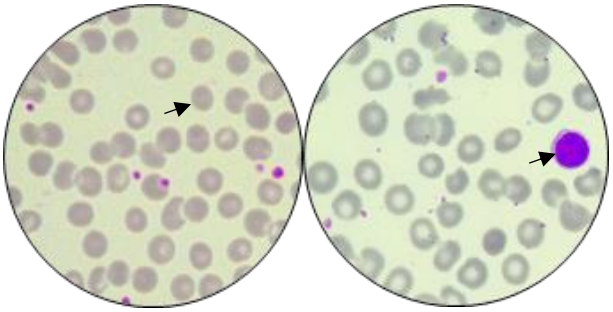

Figure 21. Sample Code G Romanowsky Stain;
Erythrocytes Cell (Left) and Leucocytes Cell (Right)

Table 2. Percentage and Scoring of Staining Results on Erythrocyte and Leukocyte Cell Morphology Giemsa Method based on buffer

Sample Code	Repetition					Percentage
	1	2	3	4	5	
A (Control)	5/5	5/5	5/5	5/5	5/5	100%
B	3/5	3/5	2/5	3/5	3/5	56%
C	5/5	5/5	4/5	5/5	4/5	92%
D	4/5	4/5	4/5	4/5	4/5	80%
E	4/5	4/5	4/5	4/5	4/5	80%
F	4/5	5/5	4/5	4/5	5/5	88%
G	4/5	3/5	3/5	3/5	3/5	64%

Table 3. Percentage and Scoring of Staining Results on Erythrocyte and Leukocyte Cell Morphology Wright Stain Method based on buffer

Sample Code	Repetition					Percentage
	1	2	3	4	5	
A (Control)	5/5	5/5	5/5	5/5	5/5	100%
B	3/5	3/5	2/5	3/5	3/5	56%
C	5/5	5/5	4/5	5/5	4/5	92%
D	4/5	5/5	4/5	4/5	4/5	84%
E	4/5	4/5	4/5	4/5	4/5	80%
F	4/5	5/5	4/5	4/5	5/5	88%
G	4/5	3/5	3/5	3/5	3/5	64%

Table 4. Percentage and Scoring of Staining Examination Results on Erythrocyte and Leukocyte Cell Morphology Romanowsky Stain Method based on buffer

Sample Code	Repetition					Percentage
	1	2	3	4	5	
A (Control)	5/5	5/5	5/5	5/5	5/5	100%
B	4/5	4/5	4/5	4/5	3/5	76%
C	5/5	5/5	4/5	5/5	4/5	92%
D	4/5	5/5	4/5	4/5	4/5	84%
E	4/5	4/5	4/5	4/5	4/5	80%
F	4/5	5/5	4/5	4/5	5/5	88%
G	4/5	4/5	4/5	4/5	3/5	76%

Table 5. Average Percentage of Staining Examination Results on Erythrocyte and Leukocyte Cell Morphology based on buffer

Sample Code	Giemsa Stain	Wright Stain	Romanowsky Stain	Average Percentage
A (Control)	100%	100%	100%	100,00%
B	56%	56%	76%	62,67%
C	92%	92%	92%	92,00%
D	80%	84%	84%	82,67%
E	80%	80%	80%	80,00%
F	88%	88%	88%	88,00%
G	64%	64%	76%	68,00%

The data obtained were then analyzed statistically using a computer data analysis to determine whether there were differences between the seven treatments studied, namely using phosphate buffer pH 6.8 and 6 brands of national and local branded mineral water. The research data that has been tested can be seen from the results of the normality test, which shows that the morphology of erythrocytes and leukocyte cell morphology <0.05, the data obtained are not normally distributed. Then seen from the results of the homogeneity test, there were different variations between groups which could be shown as <0.05. So this study uses the Kruskal Wallis test because the requirements for conducting the Kruskal Wallis test are that the data are not normally distributed and the data variations are not homogenous.

Based on the results of the research conducted, the results of statistical analysis using the Kruskal-Wallis test with a 95% confidence level showed that the significance value of erythrocyte cell morphology and leukocyte cell morphology was 0.021 and 0.008, respectively. The significance value obtained <0.05 means that there is a significant difference between the seven groups of solutions examined.

Then proceed with the Mann-Whitney statistical test, a post hoc analysis of the Kruskal-Wallis test. The Mann-Whitney test aims to determine whether there is a significant difference between the two treatments studied between pH 6.8 phosphate buffer solution with national and local branded mineral water. (A) with bottled drinking water C, D, E, and F. However, the national and local branded mineral water B and G against phosphate buffer pH 6.8 (A) obtained a significance value of <0.05, which means there is a significant difference.

There was a difference in the staining results of each solution due to the difference in the pH value of the solution from each solution. According to Adianto in 2013, one thing that needs to be considered in a good Giemsa stain is the accuracy of the pH of the solution buffer[3]. Based on the pH measurements carried out can be seen in Table 1. The pH of A (control), Sample B, C, D, E, F, and G were respectively 6.80; 7.19; 6.83; 7.07; 7.04; 6.87; and 7.16.

According to Harr in 2002, the solution in Giemsa staining is used as a buffer solution which serves to maintain the pH of the preparation from the addition of acids and bases from the substances contained in Giemsa as well as water and the stagnation carried out during the staining process, so that the cells can absorb the dye that is produced[4]. In the thin blood smear, the Giemsa, Wright stain and Romonowsky method on the morphology of erythrocyte and leukocyte cells using phosphate buffer solution pH 6.8. A (control), B, C, D, E, F, and G solutions, respectively, have a percentage of 100 %, 56%, 92%, 80%, 80%, 88%, and 64% refer to table 5. Based on

the results of Giemsa staining, Wright stain and Romonowsky cell morphology of erythrocytes and leukocytes using solutions B and G having a pH of 7.19 and 7.16, respectively, there are blue erythrocytes and a green background that does not match the Giemsa staining criteria, Wright stain and Romonowsky. The morphology of leukocytes in eosinophils has blue granules, so it does not match the criteria that should be orange in colour. According to Adianto, 2013 a high pH or pH above 6.8 causes leukocytes to absorb a lot of Azur B so that the cytoplasm is more concentrated and the granules are darker.

However, the results of the study were on reading thin blood smears using a microscope after thin blood smears were stained using Giemsa, Wright stain and Romanowsky stains, and the results of the research showed that mineral water branded B and G were dark blues in colour, both erythrocyte cells and leukocyte cells. According to Harr in 2002, the staining results were caused by the pH of the solution because solutions B and G were far from the standard pH of 6.8. So Giemsa staining has poor results compared to other solutions because if the pH of the solution is too high, excessive absorption of alkaline dye (Azure B) causes erythrocytes to turn dark blue[3].

According to Sholekha in 2018, the background is green because it is caused by factors such as imperfect fixation and because the sample is not fixed immediately[13]. Bain 2015 stated that using too high a solution pH causes eosinophil granules to turn dark blue or dark or dark grey because leukocytes absorb a lot of Azure B in excess[14].

The morphology of erythrocyte cells in solution B obtained one blood smear that met the criteria of five blood smear preparations. According to Nugraha in 2017, a factor that can affect the results of staining is the thickness of the blood smear. The thicker the blood smear, complete fixation should be performed. Imperfect fixation makes it difficult for Giemsa, Wright stain and Romonowsjy solutions to penetrate blood plasma to reach erythrocytes during staining, so the results are not optimal[16].

Based on the percentage of good staining results, it can be seen that solutions C and F have the best percentage results, namely 92% and 88%, compared to other solutions. This is because solutions C and F have pH of 6.83 and 6.87, respectively, close to the pH of phosphate buffer (A), which is 6.8. In solutions D and E staining with Giemsa, Wright stain and Romonowsky, the morphology of erythrocytes and leukocytes was still lacking in colour absorption. Apart from the influence of the pH buffer, according to Sholekha 2018 stated this problem could be influenced because the staining time being too short, especially the quality of Giemsa stock, the quality of the solution Giemsa, Wright stain and Romonowsky, the cleanliness of the glass object, the length of fixation with methanol, and the thickness of the preparation[13].

Based on the above discussion, it can be seen that the morphological staining of erythrocytes and leukocyte cell morphology using solutions A, C, D, E, and F is better than solutions B and G, referring to the percentage of good staining results. The limitation of this study is that blood cells focused only on erythrocytes and leucocytes, for the following research can focus on other blood cells and abnormal cells.

CONCLUSION

The use of an alternative buffer for staining thin blood smears using the Giemsa, Wright stain, and Romanowsky methods can be done with mineral water as an alternative buffer for sample codes C, D, E and F, while B and G cannot be used. This alternative buffer can be applied by laboratory personnel in urgent situations in limited equipment and material facilities.

CONFLICT OF INTEREST

The author has declared no conflict of interest.

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