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ORIGINAL ARTICLE

Comparative analysis of different water samples as Buffer in Leishman stain.

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Abstract

Introduction

Staining of blood films remains one of the most essential part of haematology. The different blood cells have different intracellular structure that take up stains according to chemical nature. Leishman stain is one of the Romanowsky stains used in the haematology laboratory for the staining of blood films. It contains methylene blue and eosin dye prepared in an alcohol medium and is diluted with a buffer. Phosphate buffer is used during Leishman stain as it acts as a mordant enhancing staining reactions and it is the recommended and best buffer for staining.

Aim

This comparative study was aimed at determining if different water samples used as buffer show significant effects on the morphology of blood cells during Leishman staining.

Methods

pH analyses of different water sources such as water from borehole, waterboard, rainwater, fridge water, distilled water and air conditioned water were carried out and used as buffers to carry out Leishmann staining on blood films. The stained slides were examined macroscopically and microscopically using 100x objective.

Results

The study has shown that deionized and tap water used as buffer could produce moderately good film with well stained red cells, white cells and platelets. Rainwater, distilled, fridge and air conditioned water showed a significant change in the morphology of blood cells both in

an apparently healthy film and a diseased film

Conclusion

This study demonstrates that deionized water and tap water devoid of chlorine can be used as substitutes in the right pH of 6.8 in a situation where phosphate buffer is not available. It is therefore recommended that quality control should be carried out to check the quality of stains and buffer used for staining films in order to produce quality results.

Keywords: Leishman stain, Buffer, Water

Introduction

Different blood cells in our body have different shapes and sizes, which take up stains according to their structures. Some of the components of the blood cells are basophilic, that is they have great affinity to acidic dyes while some components of blood cells are acidophilic meaning they have affinity to basic dyes and also some components neutral and have high affinity to neutral dyes [1].

Romanowsky stains are types of stains employed for staining of blood cells. Almost all Romanowsky stains have two essential components; acidic and basic dye. Methylene blue is a basic dye with high affinity to the acidic component of the cell which is the nucleus, Eosin is the acidic dye that has high affinity to the basic component of the cell which is the cytoplasm and some granules of the cell [2,3]. Many Romaowsky stains are prepared with methanol which acts as a fixative and also as a cellular stain. These are four types of romanowsky stains commonly used in haematology laboratory for staining blood cells, they include: leishman stain, giemsa stain, field stain and wright stains[4,5].

Leishman stain is a type of Romanowsky stain containing a mixture of methylene blue and eosin dye prepared in an alcohol medium and diluted with buffer of pH6.8. The buffer acts as a mordant, it enhances staining reactions and give better morphology of the blood cells under the microscope[6,7].

Leishman stain is used to stain various components of blood cells and also used to study bacterial cells. It is the basic routine stain for staining and examination of peripheral blood films under the microscope. It is also good for the examination of haemoparasite such as plasmodium species. Phosphate buffer is highly water soluble and has high buffering capacity that inhibits enzymatic activity and will precipitate in ethanol. It is one of the most used buffer employed in the haematology laboratory during Leishman staining [2,8]. Occasionally because of unavailability of phosphate buffer especially in resource-limited areas, available water samples are used as buffer.

These are different water sources that were analysed in this study: Tap water, fridge water, Distilled water, rain water and air conditioned water. Tap water is water supplied through a tap (valve) and distributed through pipes (plumbing). Fridge water is gotten from refrigerator after it has defrosted. Distilled water is steam from boiling water that has been cooled and returned to its liquid state. Rain water is collected from rainfall directly or from roof like surfaces. [3,5]. Air conditioned water is water formed from the cold coils of the air conditioner through squeezing and

condensation of hot air in the room [4,6].

The different water types have different pH and different chemical elements in them, which may exert significant effect on the morphology of the blood cells when used in leishman staining. However, the effects of these water samples in leishman stain have not been extensively studied, hence, the reason for the study.

Materials and Methods

Study Design

This was a comparative and analytical study that was carried out for four months, between March to July, 2021 at University of Calabar Teaching Hospital Calabar.

Study Area

The study was carried out in Calabar Metropolis, Cross River State, Nigeria specifically at Haematology Laboratory, University of Calabar Teaching Hospital Calabar.

Sample collection

1. Water samples from the respective sources were collected and stored in 4 litres Jerry can and labeled accordingly as Tap water (bore hole), Tap water (water Board), Fridge water, Rain water, Air conditioner water and Distilled water.
2. About 2ml of blood was collected from patients with different pathological conditions (Sickle cell anaemia) and apparently healthy Individual which was used to prepare blood films.

Water Analysis

Water from different sources; Tap water(bore hole), Tap water(water Board), Rain water, Fridge water, Air conditioned water and Distilled water was collected and put inside a clean container and sent to CROSS RIVER STATE WATERBOARD LTD. for analysis. The different water sources were blindly labelled 1, 2, 3, 4, 5, and 6 for identification.

Making of blood film

1. A microscope slide was cleaned with a cotton wool to remove debris and make it grease free.
2. A small drop of well mixed blood sample was placed at the centre 2cm of the length of the slide.
3. A clean smooth edged spreader was used; the spreader is held at angle of about 45^0 and is drawn back to touch the drop of blood; blood is allowed to spread to the edge of the spreader.
4. The spreader was pushed gently and quickly forward, making a film in the process; the edge of the spreader is wiped clean.
5. The blood film was allowed to dry completely; and slide labelled appropriately.

Leishman staining

1. The air dried thin film was placed on a staining rack with smear surface upwards.
2. The entire area of the smear was flooded with Leishman's stain.
3. The flooded slide was allowed to stand for 2 minutes. To ensure cells are fixed properly by methanol
4. The stain was double diluted with standard buffer of pH 6.8 and also water from other sources as buffer differently.
5. The slide was blown gently to enhance mixture of stain with buffer; a metallic sheen is formed over the surface of the fluid and allowed to stand for 10 minutes.
6. The stain was rinsed with tap water.
7. The reverse side of the slide was carefully wiped with a soft absorbent tissue.
8. Slide was kept on a slide rack and allowed to air dry.

Constituents of phosphate buffer: 28.39g of disodium hydrogen phosphate dissolved in one liter of distilled water. The pH range is checked using a pH meter.

Microscopy

The stained film was air dried and viewed under the microscope using x10 and x100 oil immersion lens. The film was photomicrographed using photomicrograph microscope and the films were compared.

Results

Appearance of stained blood films

A well stained thin blood film appeared salmon pink.
Nucleus of the cell appeared purple-violet.
Eosinophil granules appeared orange-red.
Neutrophil granules appeared mauve purple.
Basophil granules appeared dark blue.
Red blood cells appeared Salmon pink.
Platelets appeared small with violet granules.
Lymphocytes and monocytes appeared pale blue

PLATE 1 shows the film of an apparently healthy individual stained with leishman stain using phosphate buffer and viewed with x400 magnification. It shows well stained erythrocytes €, eosinophil (EO), neutrophils (N), basophils (B), monocytes (M) and lymphocytes (L).

PLATE 2 shows well-stained erythrocyte €, eosinophil (EO), neutrophil (N), and platelets(P) stained with Leishman stain using deionized water as buffer and viewed with x400 magnification. The morphology of the cells are well defined without any distortion and presence of crenated red cell.

PLATE 3 shows well-stained erythrocyte €, Lymphocyte (L), and platelets (P) stained with Leishman stain using tap water from borehole as buffer and viewed with x400 magnification. There is no distortion in the morphology of the cells.

PLATE 4 shows well-stained erythrocyte € with faint staining of monocyte (M) and platelet (P) stained with Leishman stain using tap water from waterboard as buffer and viewed with x400 magnification. In this plate, central pallor is not well defined and the faintly stained monocyte could be as a result of the amount of chlorine present in the water used as buffer.

PLATE 5 shows well-stained erythrocyte €, lymphocyte (L) and platelets (P) stained using Leishman stain using rainwater as buffer and viewed with x400 magnification.

PLATE 6 shows well-stained erythrocyte €, neutrophil (N), and platelet (P) stained using Leishman stain using fridge water as buffer and viewed with x400 magnification. The erythrocytes appear crenated and not clearly defined.

PLATE 7 shows erythrocyte € with fluorescence at the center, well-stained neutrophil (N), and faintly-stained platelets (P) stained using Leishman stain using distilled water as buffer and viewed with x400 magnification. The erythrocytes are not clearly defined and they appear crenated.

PLATE 8 shows moderately-stained erythrocyte €, lymphocyte (L), and platelet (P) stained using Leishman stain using air conditioned water as buffer viewed with x400 magnification. The erythrocytes show crenated cells and are not well defined.

PLATE 9 shows a sickle cell film stained with leishman stain using phosphate buffer and viewed with x400 magnification. It shows well stained erythrocytes and sickle red cells.

PLATE 10 shows poorly-stained erythrocyte €, well-stained lymphocyte (L), and moderately-stained platelet (P) stained using Leishman stain using deionized water as buffer viewed with x400 magnification. The red cell morphology is distorted and appear like crystals.

PLATE 11 shows poorly-stained erythrocyte €, moderately-stained neutrophil (N), and poorly-stained platelet (P) stained using Leishman stain using tap water from borehole as buffer and viewed with x400 magnification. The morphology of the red cells are not well defined.

PLATE 12 shows poorly-stained erythrocyte €, lymphocyte (L), and platelet (P) with numerous artefacts (A) in the background stained using Leishman stain using tap water from waterboard as buffer and viewed with x400 magnification. The red cells are not exactly well defined which could be as a result of the amount of chlorine present in the water.

PLATE 13 shows poorly-stained erythrocyte €, neutrophil (N), and platelet (P) with numerous artefacts (A) in the background stained using Leishman stain using rainwater as buffer and viewed with x400 magnification.

PLATE 14 shows poorly-stained erythrocyte € and neutrophils (N) with numerous artefacts (A) in the background stained using Leishman stain using fridge water as buffer and viewed with x400 magnification.

PLATE 15 shows poorly-stained erythrocyte €, lymphocytes (L) and neutrophil (N) with numerous artefacts (A) in the background stained using Leishman stain using distilled water as buffer and viewed with x400 magnification.

PLATE 16 shows poorly-stained erythrocyte € and lymphocytes (L) with numerous artefacts (A) in the background stained using Leishman stain using air conditioned water and viewed with x400 magnification.

Table 1 and 2 shows the result of water analysis. The table shows that different water sources has different Ph and different chemical constituents.

Discussion

Phosphate buffer acts as mordant during leishman staining by enhancing staining reaction [1]. It intensifies the stain and helps to attach the dyes methylene blue and eosin to the blood cells.

This study has shown that other water samples as buffer indeed interferes with staining results. Deionized water used as buffer was able to stain the red cells, white cells and platelets very well in a normal film as shown in plate 2, it could not stain the red cells and platelets properly but could barely stain the white cells well in sickle cell film as shown in plate 10.

Tap water from borehole used as buffer was able to stain the red cells, white cells and platelets in a normal film as shown in plate 3, it could not stain the red cells and platelets properly but moderately stained the white blood cells in a sickle cell film as shown in plate 11 whereas Tap water from waterboard used as buffer was able to stain the red cells and platelets well but was not able to stain the white blood cells properly in normal film as shown in plate 4, in sickle cell film, the red cells, white cell and platelets appeared poorly stained as shown in plate 12. The difference in staining properties that were observed in tap, deionized and waterboard water may be attributed to chlorine which is present in waterboard water but absent in both tap and deionized water.

However, Rainwater used as buffer as shown in plate 5 was able to stain the white cells, platelets and red cells very well in a normal film but in plate 13, it poorly stained the red cells, white cells and platelets in sickle cell film. Whereas Fridge water used as buffer stained the white cells and platelets very well, red cells appearing crenated as shown in plate 6 in normal film but in sickle cell film, it stained the red cells, white cells and platelets very poorly as shown in plate 14. The crenation of red cells observed in fridge water could be as a result of the slight acidic pH of the water.

Furthermore, distilled water used as buffer stained white cells well, platelets

appeared faintly stained and it stained the red cells poorly with the cells appearing crenated and with fluorescence at the center as shown in plate 7 in normal film while in sickle cell film, it stained very poorly the red cells, white cells and platelets as shown in plate 15.

On the other hand, air conditioned water used as buffer, moderately stained the red cells, white cells and platelets as shown in plate 8 in normal film but in sickle cell film, the red cells, white cells and platelets are poorly stained as shown in plate 16. The water sources used as buffer could not stain the cells properly because of its inability to intensify and fix the dyes to the blood cells. Another reason the water sources were not able to stain the blood cells well could be because of their varying pH. In normal blood film, the study shows that some of the water samples such as deionized water, tap water without chlorine could be used as substitutes for phosphate buffer in a situation where it is not available or in areas where it is not easily accessible. In diseased film, the study has shown that it is best to use the recommended phosphate buffer during leishman staining for the production of better results.

TABLE1: COMPONENTS OF DIFFERENT WATER SAMPLES ANALYSED

S/N	PARAMETERS	Units	NIS Guidelines	1 (Underground Borehole)	2 (Urban Board Water)	3 (Rain Water)	4 (Fridge Drain Water)	5 (Distilled Water)	6 (AC Water)	7 (Deionized Water)
1	Aluminum	Mg/L	0.02	0.00	0.11	0.00	0.12	0.00	0.01	0.00
2	Ammonia NH_3-N	Mg/L	0.05	0.12	0.16	0.22	1.16	0.21	0.22	
3	Appearance	-	Clear	-	-	-	-	-	-	-
4	Calcium Ca	Mg/L	50	24.0	55.0	10.1	9.80	8.10	10.0	0.00
5	Chlorine Cl	Mg/L	100	0.251	2.162	0.112	0.241	0.112	0.121	0.00
6	Coliforms (Feacal) counts/100ml	Cfu	0	6	13	105	300	113	84	-
7	Coliforms (Total) counts/100ml	Cfu	0	2	1	59	320	74	61	-
8	Colour	Hu	3.0	1.62	1.68	0.92	4.21	0.81	0.85	0.00
9	Copper Cu	Mg/L	1.0	0.12	0.11	0.11	0.15	0.16	0.15	0.00
10	Dissolved Oxygen	Mg/L	34	2.44	2.31	3.20	3.11	3.20	3..20	0.02
11	Electrical coductivity	µS/cm	1000	148.0	504.0	6.0	88.0	24.0	24.0	0.00
12	Fluoride F	Mg/L	1.0	0.54	0.44	0.46	0.56	0.54	0.55	0.00
13	Hardness (Total) as $CaCO_3$	Mg/L	100	34.1	68.4	17.1	17.1	12.1	17.1	-
14	Temperature	^{0}C	Ambient	28.0	28.0	28.0	28.0	28.0	28.0	28.0
15	pH	-	6.5-8.5	3.6	3.9	5.6	5.7	6.2	6.1	6.7
16	Phosphate	Mg/L	200	6.25	2.61	6.55	2.18	1.82	1.92	0.00
17	Total Alkalinity as $CaCO_3$	Mg/L	250	8.69	8.16	9.60	9.62	12.0	12.21	0.00
18	Turbidity	NTU	5	104	4.16	2.61	17.3	1.88	1.47	0.01

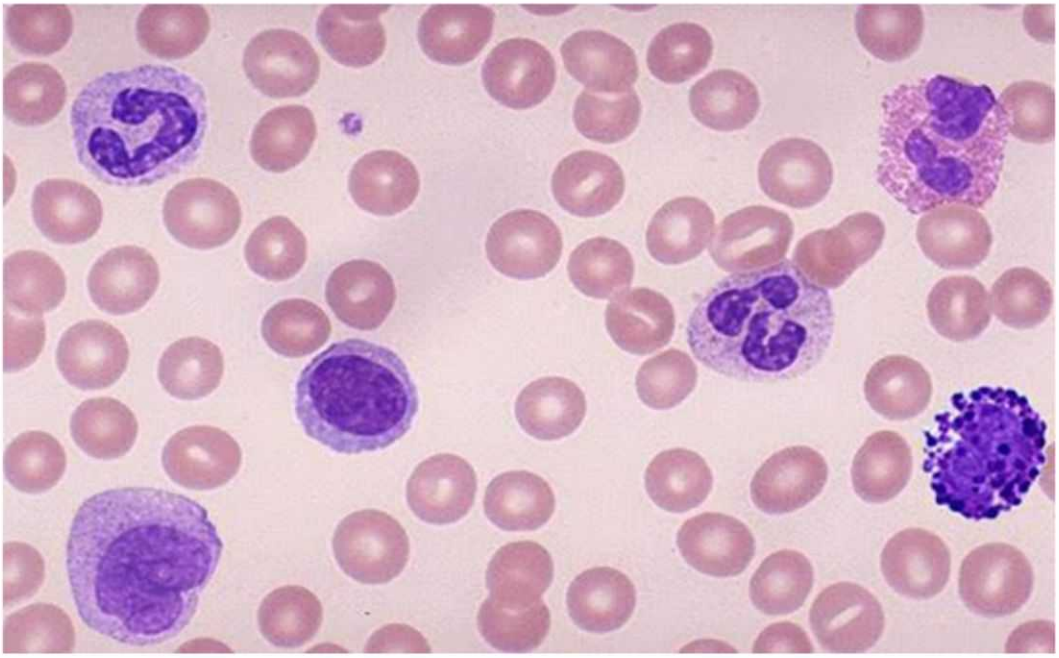

Plate 1- showing well-stained erythrocyte €, eosinophil (EO), neutrophil (N), monocytes (M), basophil (B), Lymphocytes (L) and platelets (P) using Phosphate buffer. Leishman x400 magnification

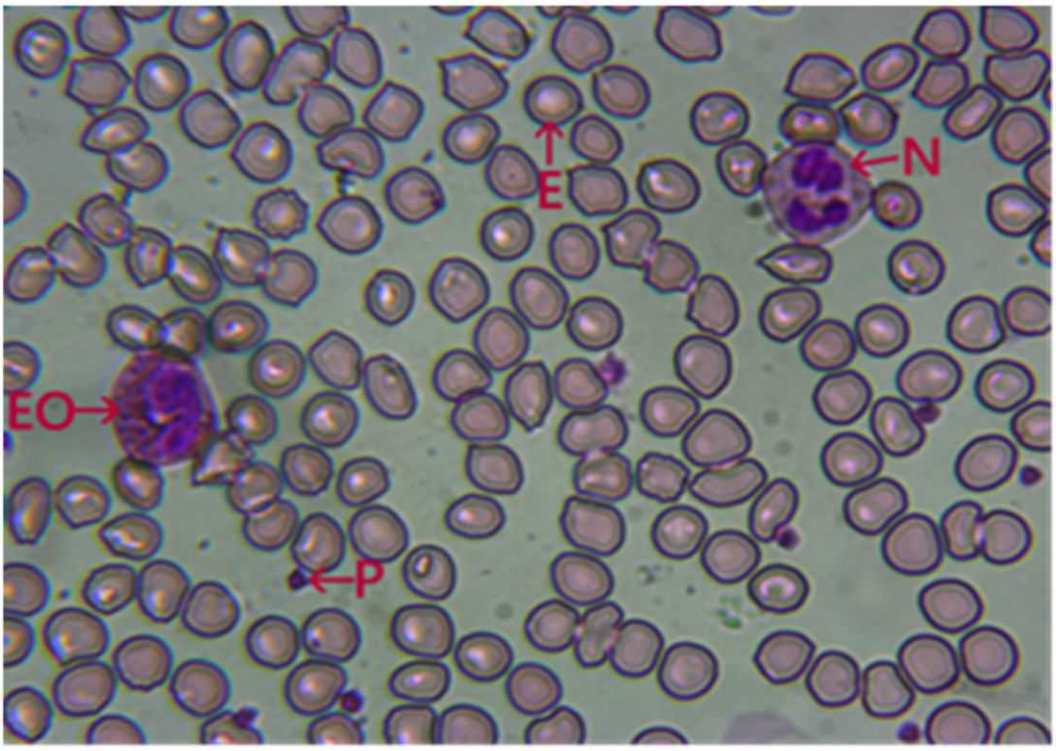

Plate 2- showing well-stained erythrocyte €, eosinophil (EO), neutrophil (N), and platelets(P) using deionized water as buffer. Leishman x400 magnification

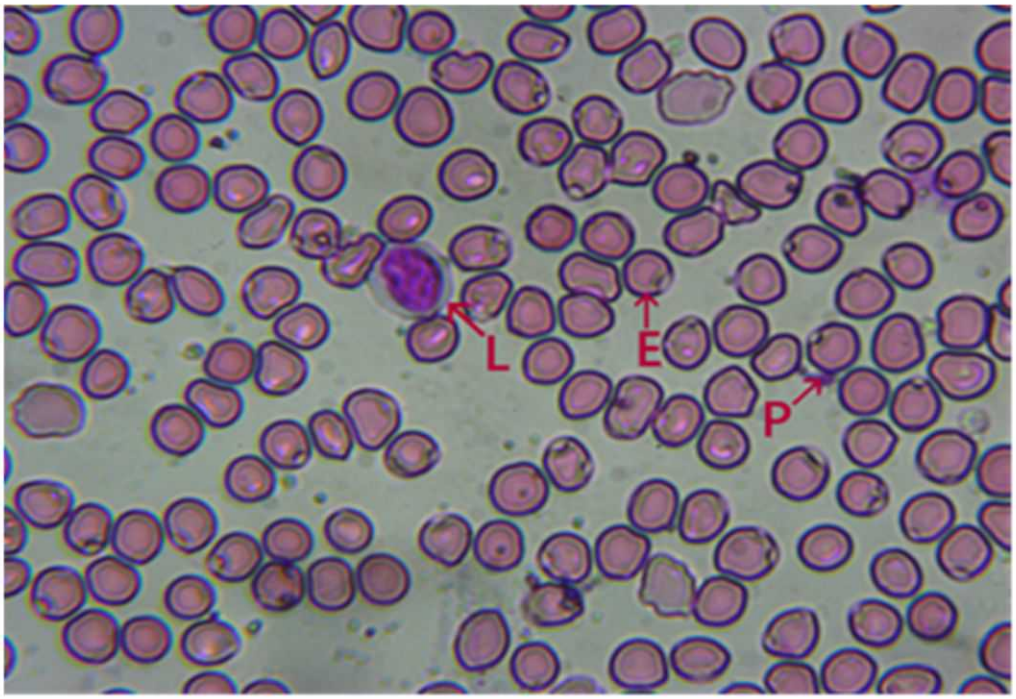

Plate 3- showing well-stained erythrocyte €, Lymphocyte (L), and platelets (P) using tapwater gotten from underground borehole as buffer. Leishman x400 magnification

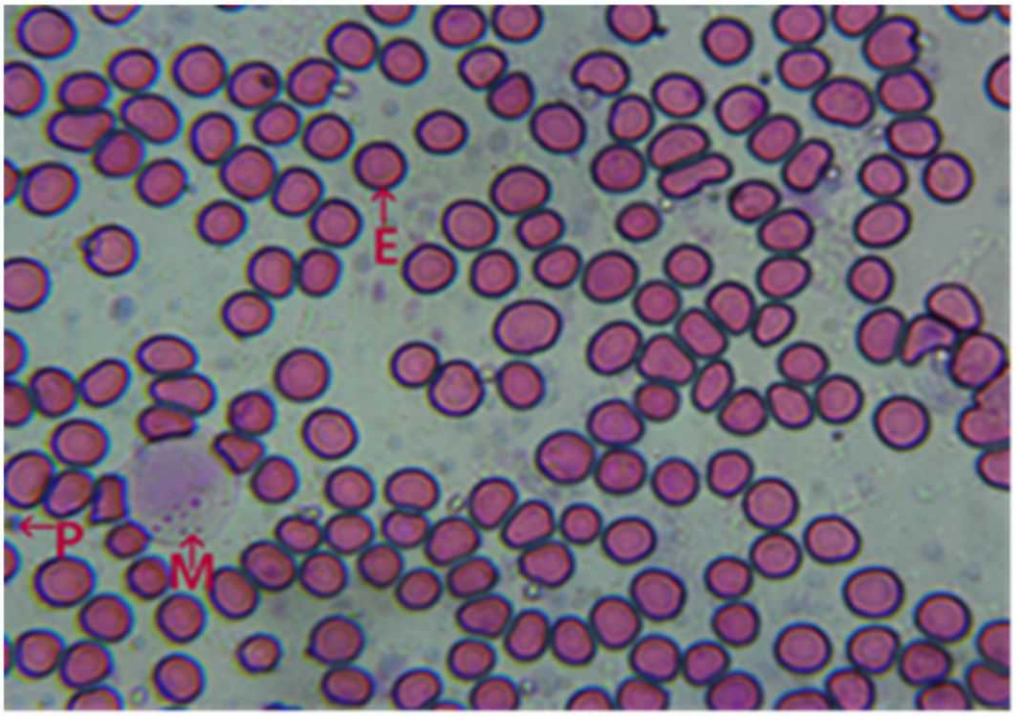

Plate 4- showing well-stained erythrocyte€ with faint staining of monocyte (M) and platelet (P) using tapwater gotten from waterboard as buffer. Leishman x400magnification

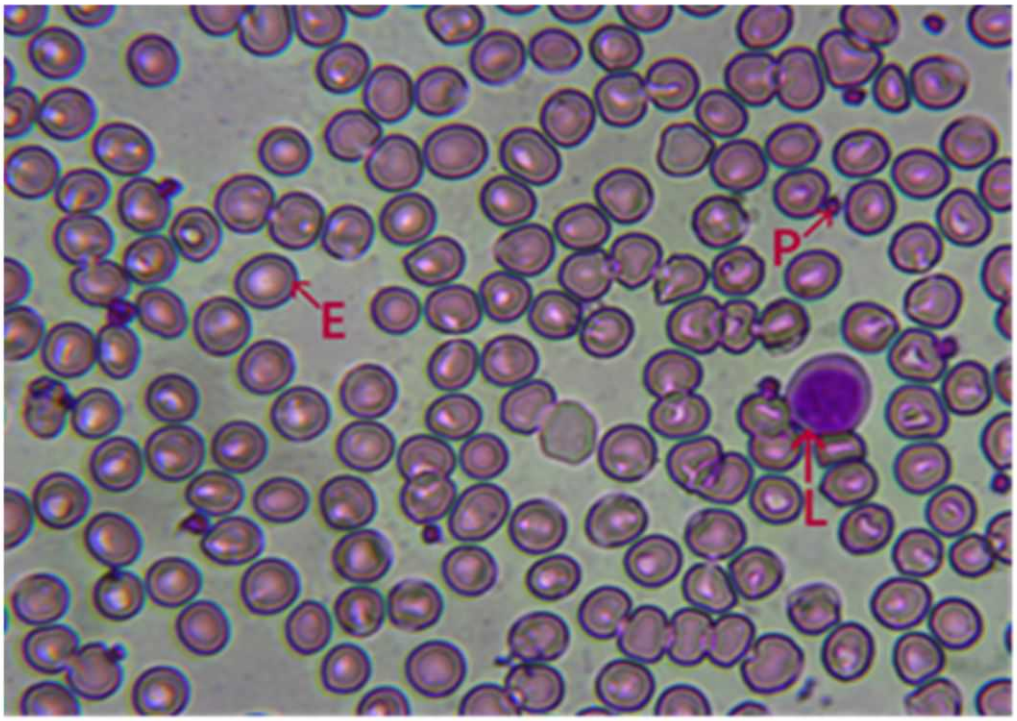

Plate 5- showing well-stained erythrocyte €, lymphocyte (L) and platelets (P) using rainwater as buffer. Leishman x400 magnification

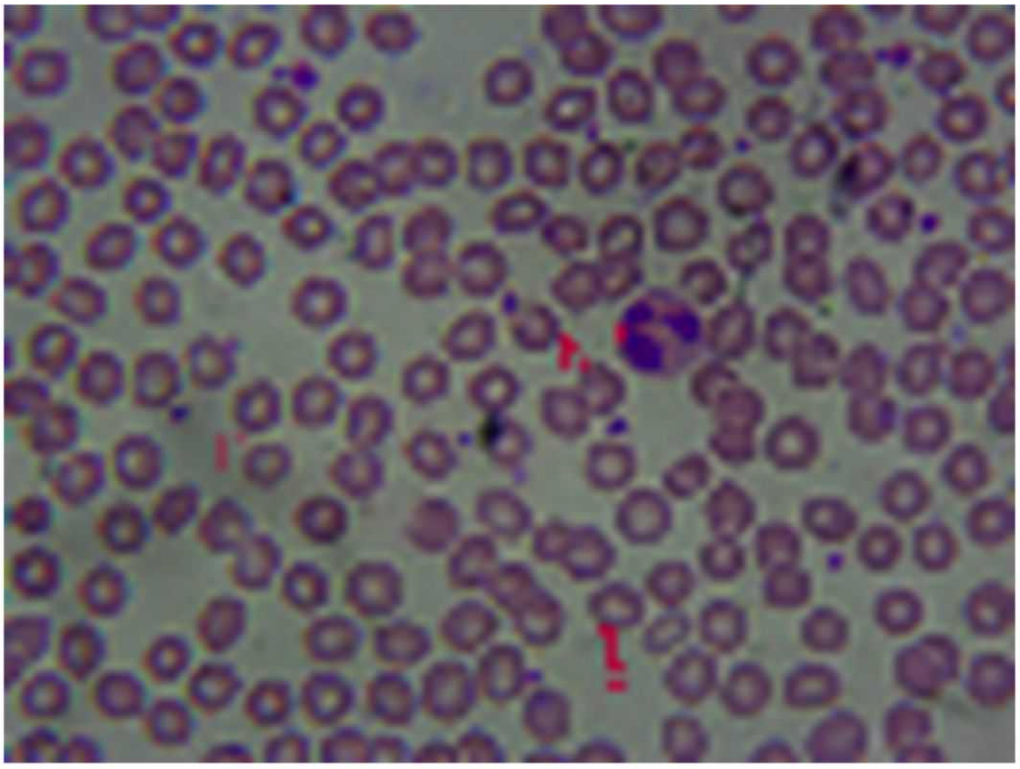

Plate 6-showing well-stained erythrocyte €, neutrophil (N), and platelet (P) using fridge drain water as buffer. Leishman x400 magnification

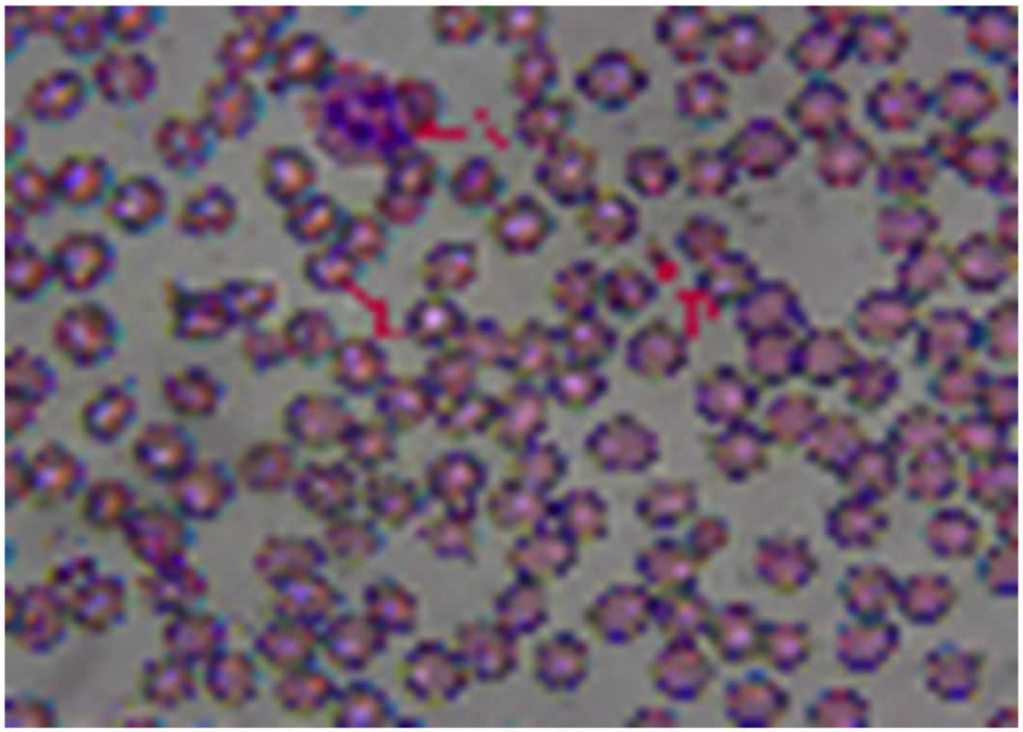

Plate 7-showing erythrocyte € with fluorescence at the center, well-stained neutrophil (N), and faintly-stained platelets (P) using distilled water as buffer. Leishman x400 magnification

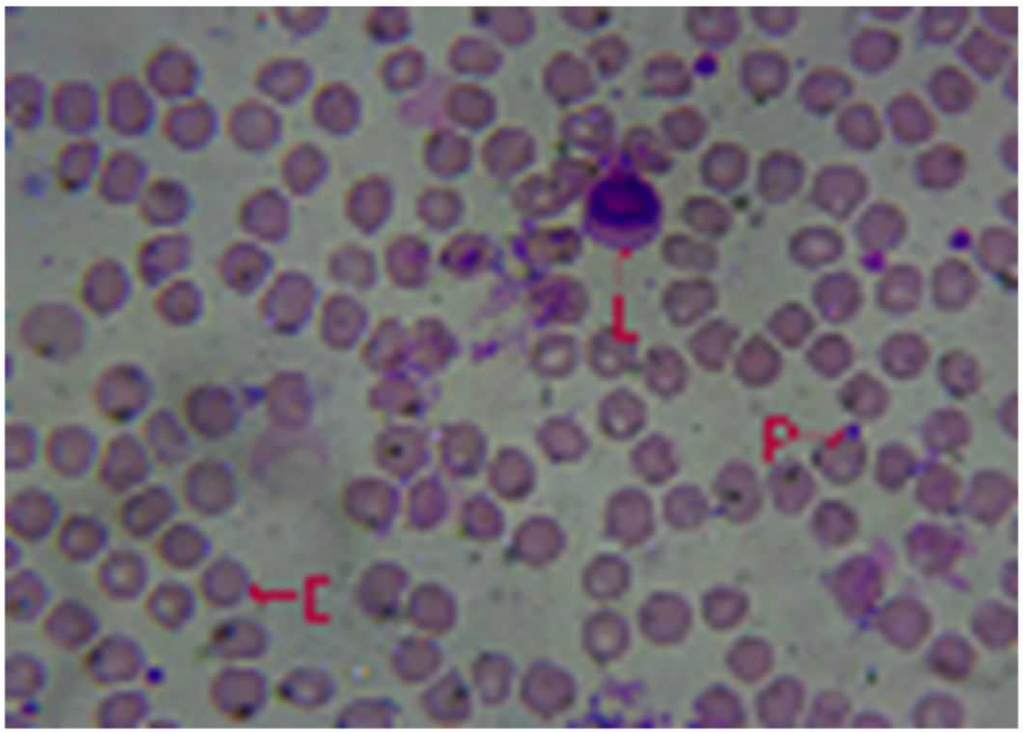

Plate 8-showing moderately-stainederythrocyte €, lymphocyte (L), and platelet (P) using air conditioned water as buffer Leishman x400 magnification

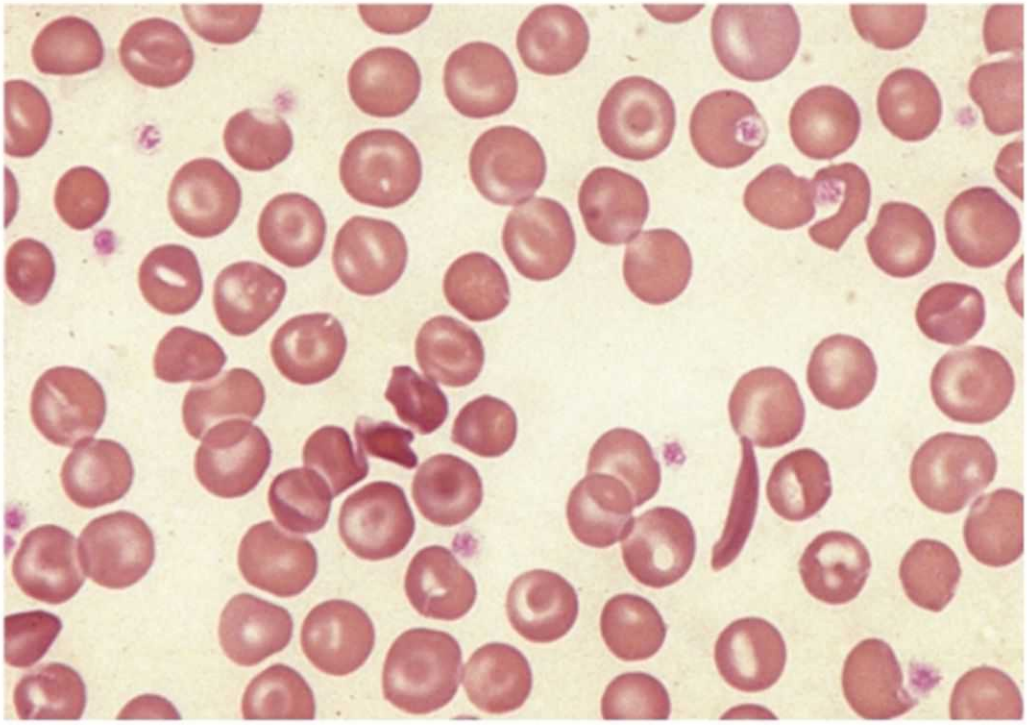

Plate 9- showing well stained erythrocytes including a sickled red cell and platelets (P) using phosphate buffer

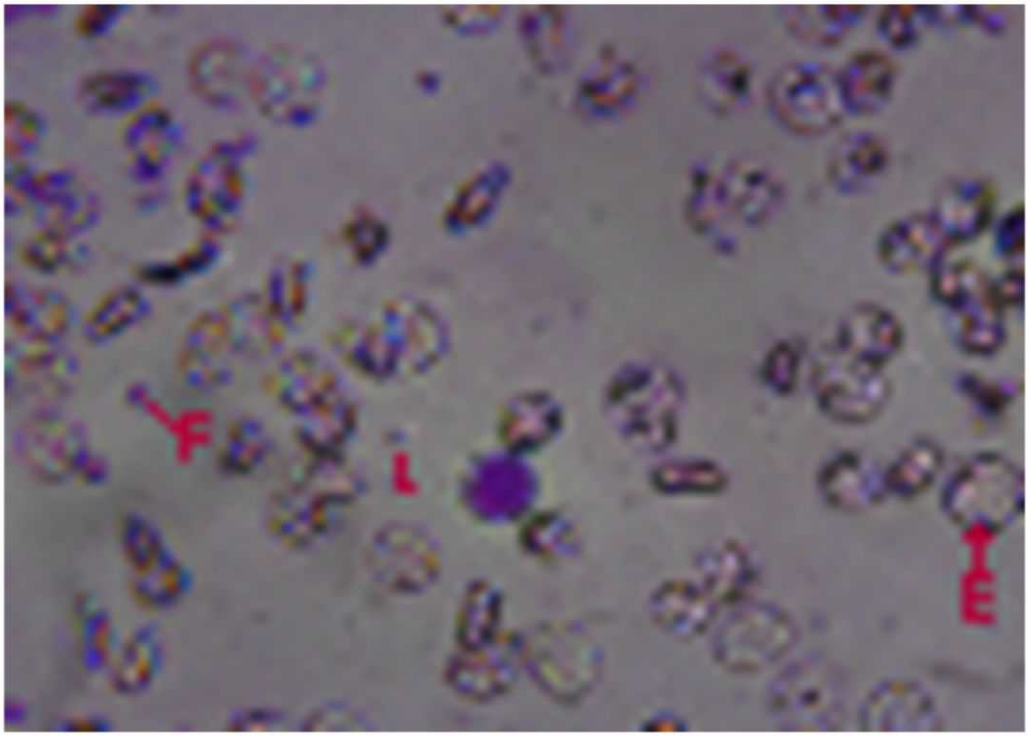

Plate 10-showing poorly-stained erythrocyte €, well-stained lymphocyte (L), and moderately-stained platelet (P) using deionized water as buffer. Leishman x400 magnification

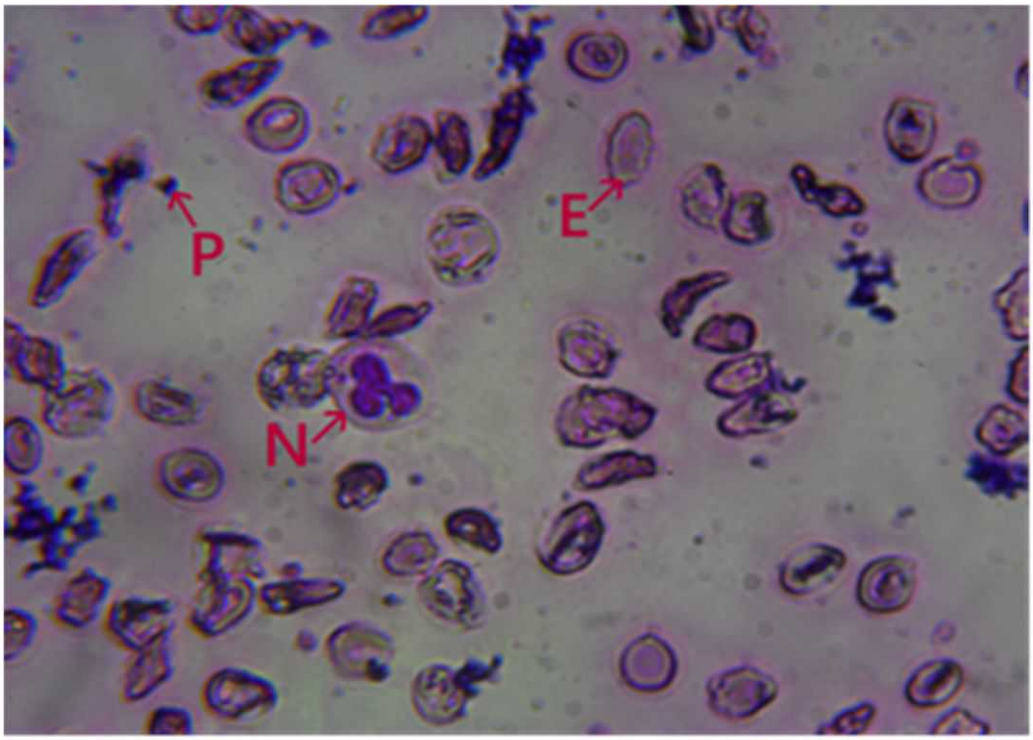

Plate 11-showing poorly-stained erythrocyte €, moderately-stained neutrophil (N), and poorly-stained platelet (P) using tap water from borehole as buffer. Leishman x400 magnification

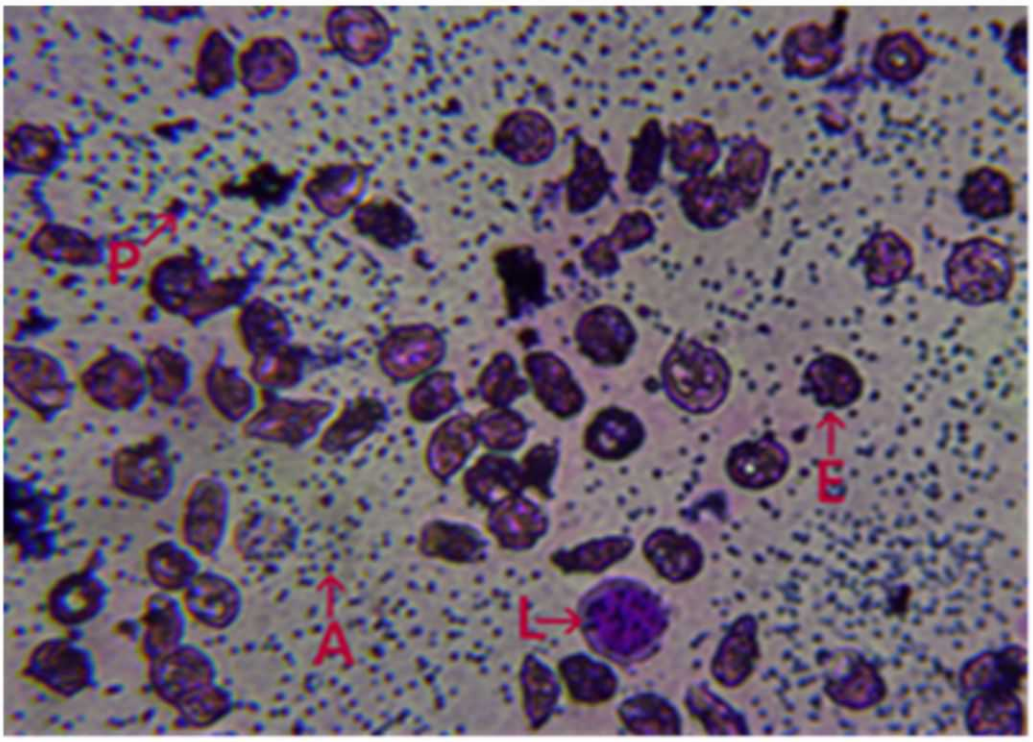

Plate 12- showing poorly-stained erythrocyte €, lymphocyte (L), and platelet (P) with numerous artefacts (A) in the background using tap water from water board as buffer. Leishman x400 magnification.

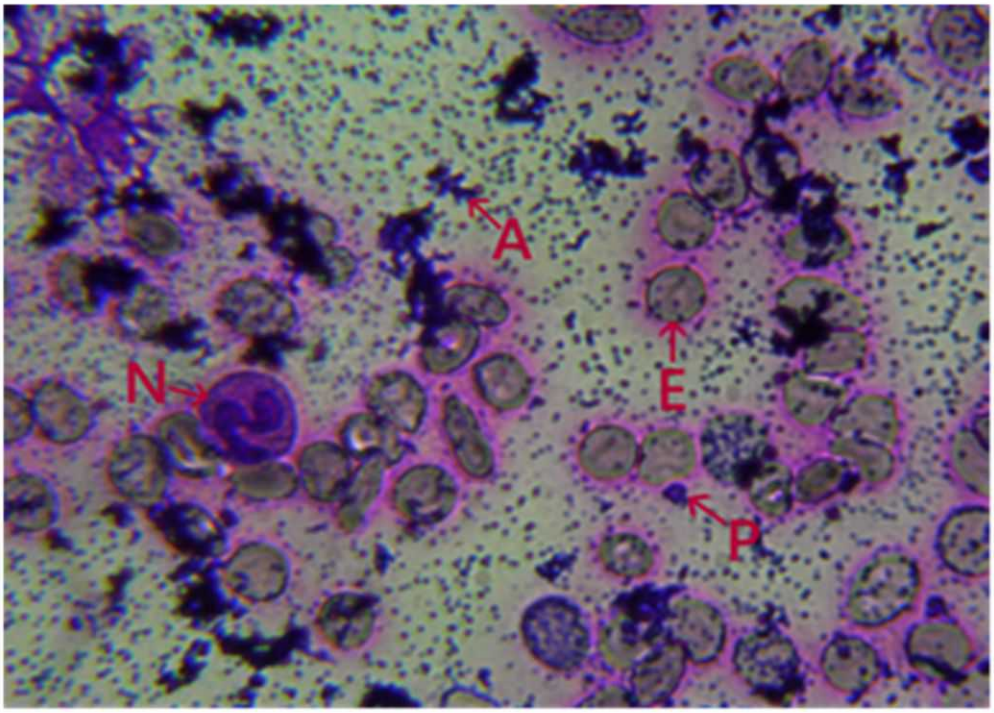

Plate 13-showing poorly-stained erythrocyte €, neutrophil (N), and platelet (P) with numerous artefacts (A) in the background using rainwater as buffer. Leishman x400 magnification.

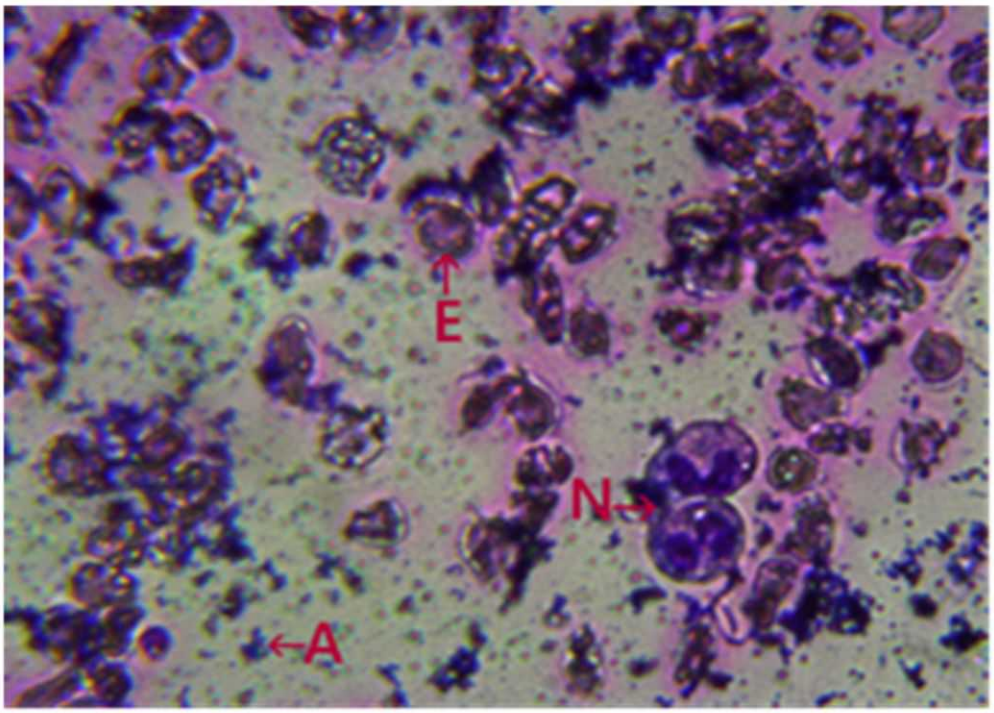

Plate 14-showing poorly-stained erythrocyte € and neutrophils (N) with numerous artefacts (A) in the background using fridge water as buffer. Leishman x400 magnification.

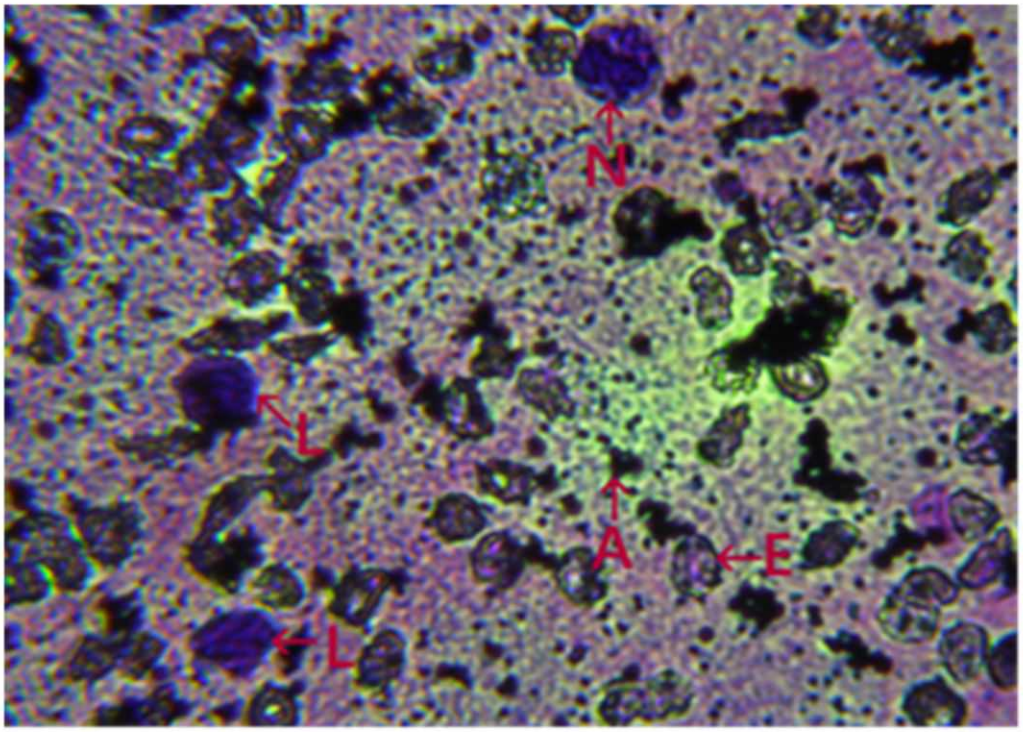

Plate 15-showing poorly-stained erythrocyte €, lymphocytes (L) and neutrophil (N) with numerous artefacts (A) in the background using distilled water as buffer. Leishman x400 magnification.

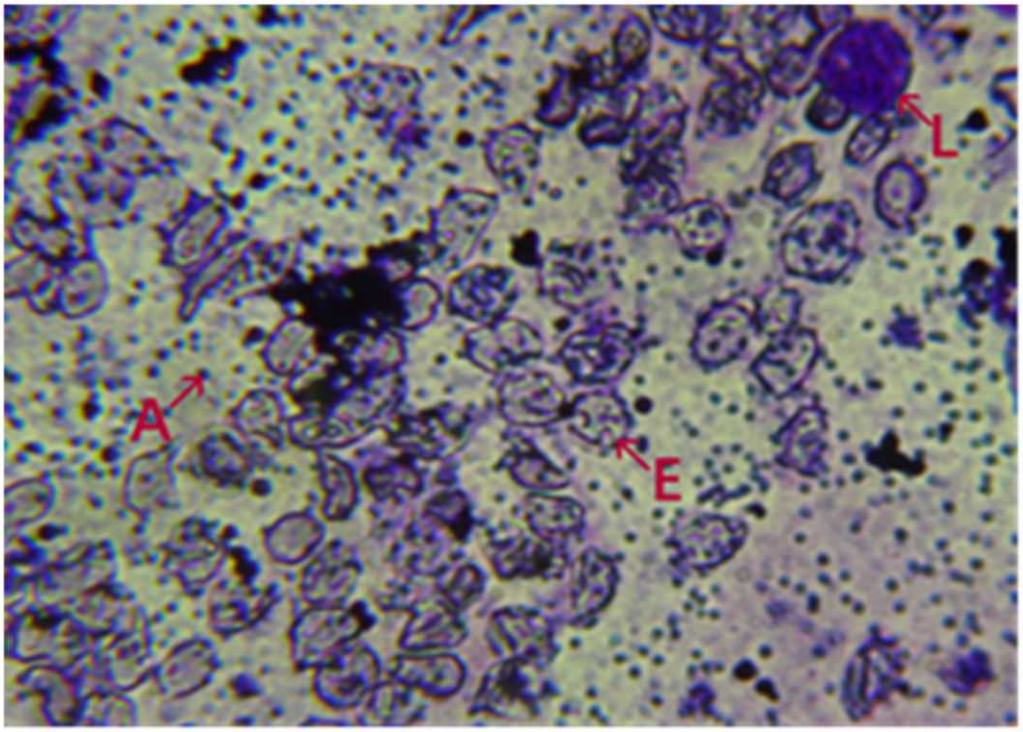

Plate 16-showing poorly-stained erythrocyte € and lymphocytes (L) with numerous artefacts (A) in the background using air conditioned water as buffer. Leishman x400 magnification

Conclusion

This study has emphasized that the type of buffer used during staining interferes with the staining procedure and affects the quality of slides produced. The study further shows that deionized and tap water from borehole could be used as substitute buffer for leishman staining in cases where the recommended buffer is not available because they are devoid of minerals such as chlorine in them.

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