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A modified Method of Preparing Thick Blood Films for the Examination of Malaria Parasites among Patients in Kosti City, White Nile State, Sudan

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Abstract:

Malaria parasites can be diagnosed with the direct examination of blood; this can be done best by examining thick blood films but it have many problems during the preparation and staining or in storage due to haemoglobin in red cell. This study aimed to assess the use of 1%acetic acid and buffer solution in dehaemoglobinization of thick blood film on morphological features of the parasites and to determine the problems happen during the long storage of thick blood film. The thick film firstly fixed in absolute methanol and let to dry and then laid in 1% glacial acetic acid or buffer water to remove haemoglobin then washed and let to dry and stained with Giemsa's staining method. 180 (90%) of the treated thick films were retained their distinct ring shape in the first week in all thick smear treated with both techniques. The cytoplasm of parasites begun to loss its ring shape after two weeks in 70 (35%) of blood smears treated with buffer

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solution and in 42 (21%) of that treated with 1% acetic acid technique. After three weeks 52 (26%) of that treated with 1% acetic acid technique and 90 (45%) of blood smears treated with buffer solution were affected. The white blood cells and platelet were well preserved. The 1% acetic acid method was found to be more reliable than buffer solution method since it retains the morphological details of parasites and both techniques were efficient in treating the smear before storage.

Key words: Malaria parasites, Thick blood film, Morphological distortion, Platelet, Dehaemoglobinization, Wash off, Glacial acetic acid, Buffer solution.

INTRODUCTION:

Malaria remains a major global health problem. The infection is the most important parasitic disease globally. It was infected human for over 50,000 year and *Plasmodium* may have been a human pathogen for the entire history of the species (Joy, *et- al* 2003, Farnetast, *et- al* 2014). In human, malaria is caused by *P. falciparum*, *P. vivax*, *P .ovale* and *P .malariae* (Singh, *et- al* 2004, Mueller, *et- al* 2007). The fatality rates of malaria can exceed 20% even with intensive care and treatment (Kain, *et- al* 1998). In Sudan, it leads to 7.5 million cases and 35000 deaths every year. Children under 5years of age and the male have the highest incidence and mortality than female (NMCP, 2004).

Early diagnosis and appropriate treatment can be life-saving. The careful examination of a well-prepared and well-stained blood film currently remains the "gold standard" for malaria diagnosis (WHO, 2010). It is relatively inexpensive, sensitive to a threshold of 5-50 parasites/µl and able to characterize the infecting species and their relative densities. It depends on the visualization of parasites by light microscopy of Giemsa's- stained thick and thin blood smears (Tham, *et-*

*al*1999, Playford and Walker, 2002, WHO, 2009). However, it is labour intensive and requires personnel who are well trained in the morphological difference of the *Plasmodium* species for successful diagnosis (Warhust and William, 1996, Castell and Carosi, 1997).

Since its introduction 1903 thick smear allows identification of the plasmodial parasite and its stages, it consists of a thick layer of dehemoglobinized red blood cells. The blood elements including parasites, if any are more concentrated than in an equal area of a thin smear. Thus, thick smears allow a more efficient detection of parasites (Ebrahim, *et- al* 2013, Norgan, *et- al* 2013). The correct thickness of thick smear is the one through which new print is clearly visible. It must be dried for thirty minutes and not fixed with alcohol, this allows the red cells to hemolyse and only leukocytes and malaria parasite are seen. The advantages of thick smear are that it use for rapid detection of parasite since the amount of blood examined in five minutes require a full hour with a thin smear beside it use for large scale investigation such as field work also it increase the number of positive finding so it preferable to a routine examination. The disadvantages are that it requires a more accurate technique for staining than the thin smear and morphology of the parasite is not clear (Ichhpujani and Bhatia, 2002). The sensitivity of malaria thick blood films can vary greatly with preparation methods, experience and skill of the microscopist and time spent examining the film. In non ideal field lab conditions, the estimated sensitivity is only 100-500 parasites/μL which is comparable to the antigen detection methods under similar conditions (Moody, 2002).

Blood smears should be prepared as soon as possible after collecting venous blood sample since the delay can result in changes in parasite morphology and staining characteristics

(Norgan, *et- al* 2013). Malaria parasite is distorted by slow drying of thick blood smear and dehaemoglobinization and there are some artifacts resembling the parasite such as platelets and other unexplainable materials originating either from the blood sample or Romanwesky stains used (Ichhpujani and Bhatia, 2002, Kakkilaya, 2003, Norgan, *et- al* 2013). Therefore, the smear must be fixed in absolute methanol and then dehaemoglobinized thereafter using 1% glacial acetic acid solution to minimize the distortion and to reduce the washing off (Alkhairy, 1992).

Buffer solution is also used in this process which contains a mixture of weak acid and its salt of strong base, or weak base and its salt with strong acid. Due to their composition, buffers are able to resist change in pH (Cheesbrough, 1987). These techniques should be done before the storage of the thick smear, since the removed of haemoglobin become increase difficult with time which lead to increase distortion of parasites (Henry, 2003). The quality of staining depends strongly on the pH of the working stain solution which ensures recognition of the specific features of malaria parasites visually.

The aim of this study is to assess the use of 1%acetic acid and buffer solution in dehaemoglobinization of thick blood smear on morphological features of the parasites and to determine the problems happen during the long storage of thick blood smear.

MATERIALS AND METHODS:

This work was conducted in the laboratory of Kosti hospital and laboratories of the clinics scatter around the hospital among the patients their blood smears showed positive results for malaria. Ethical approval was obtained from the Ethical Committee of

the Faculty of Medical Laboratory Sciences, El Imam El Mahdi University.

About 1ml of venous blood was collected from one hundred patients in EDTA blood container and mixed well. At least thirty films were made from each sample, one film was stained immediately to confirm the presence of the parasite and the remaining films were divided to two groups, group one was treated using 1% acetic acid and group two was treated by buffer solution and both were kept in boxes for further examination at interval every week to 6 weeks.

The thick smear was prepared as follow; small drop of blood was placed in the centre of clean slide and spread with corner of another slide to cover an area about 15-20 mm, after that the smear was allowed to dry for at least 30 minutes (Norgan, *et- al* 2013). The smear was firstly fixed in absolute methanol for 10 minutes and let to dry and then laid in 1% glacial acetic acid or buffer water for 5-10 minutes to remove haemoglobin (Alkhairy, 1992), then washed and let to dry and stained with Giemsa's staining technique for ten minutes and let to dry (Cheesbrough, 1999).

The smears were examined microscopically using 100X oil immersion lens and the parasite seen was evaluated for the following morphological details such as size, shape, intensity of cytoplasm and chromatin staining. The morphological details of other blood elements such as platelet and white blood cells were also evaluated. Three blinded reviewers independently estimated the severity of the defects happened to the film during the storage period which extended for 6 weeks keeping in mind the morphological appearance of parasite of similar species in thick blood smear as standard for comparison.

Statistical analysis:

Data were recorded and then analysed using statistical package of social science (SPSS version 16) program.

RESULTS:

Only one malaria parasite spices *P. falciparum* was detected among the one hundred blood samples evaluated. The size of the ring trophozoites were unaltered in both techniques in the thick smear examined immediately after it had been treated but in both their size was noticeably smaller in the subsequent examination during the period of examination.

Regarding the shape of parasites, the ring trophozoite retained their distinct ring shape in 180 (90%) of the entire thick smear treated with both techniques in the first week of evaluation, table 1.

Table 1: the number of parasites altered in thick films treated by both techniques in the first week.

Technique / Shape	1% acetic acid	Buffer solution
Altered	20 (10%)	20 (10%)
Unaltered	180 (90%)	180 (90%)
Total	200	200

With buffer solution the cytoplasm of parasites had lost its ring shape and become difficult to be differentiated from other mass of shapeless basophilic material. This has begun to happen after two weeks in 70 (35%) of blood smears treated with buffer solution and in 42 (21%) of that treated with 1% acetic acid technique, table 2.

Table 2: the number of parasites altered in thick films treated by both techniques after the two weeks.

Technique / Shape	1% acetic acid	Buffer solution
Altered	42 (21%)	70 (35%)
Unaltered	158 (79%)	130 (65%)
Total	200	200

After three weeks 52 (26%) of that treated with 1% acetic acid technique and 90 (45%) of blood smears treated with buffer solution were affected, table 3.

Table 3: The number of parasites altered in thick films treated by both techniques after the three week.

Technique / Shape	1% acetic acid	Buffer solution
Altered	52 (26%)	90 (45%)
Unaltered	148 (74%)	110 (45%)
Total	200	200

In the last week of evaluation, the cytoplasm was completely replaced by the basophilic materials in all blood films treated with buffer technique and becomes less distinct in that treated with 1% acetic acid technique. The chromatin dots remained unaltered. The white blood cells and platelet were well preserved; their cytoplasm and nuclei were distinct and well differentiated from each other. The quality of staining was good by 1%acetic acid technique and the number of washed off thick smear were two smears by both techniques.

DISCUSSIONS:

Since its introduction in 1903, thick smear allows identification of the plasmodial parasite and its stages; the blood elements including parasites are more concentrated than in an equal area of a thin smear. Thus, thick smears allow a more efficient

detection of parasites (Ebrahim, *et- al* 2013, Norgan, *et- al* 2013). Malaria parasite is distorted by slow drying of thick blood smear and dehaemoglobinization and some artefacts resembling the parasite originating either from the blood sample or Romanwesky stains used (Ichhpujani and Bhatia, 2002, Norgan, *et- al* 2013). To minimize these problems and to reduce the washing off, the smear was treated in 1% glacial acetic acid and buffer solutions before the thick smear have been storage since the removal of haemoglobin becomes increase difficult with time which leads to increase distortion of parasites and the quality of staining depends strongly on the pH of the working stain solution which ensures recognition of the specific features of malaria parasites, the buffer solution has ability to resist change in pH (Cheesbrough, 1999, Henry, 2003, NIAID, 2010). It was found that among 100 samples evaluated the size of the ring trophozoites was unaltered and an average of 90% of ring trophozoite retained their distinct ring shape. The same observation was reported by Alkhairy, (1992).

The majority of the trophozoites had lost its ring shape and become a mass of shapeless basophilic material with dot or dots. Also the artefacts of the Giemsa's stain are produced in the form of basophilic or reddish dots resembling the cytoplasm and chromatin of the ring stage of malaria; this make the differentiation between the distorted malaria parasites and the artefacts very difficult to identify the correct species even for experts specially when the parasites are very scanty. This happen more notably in buffer solution than in 1% glacial acetic acid technique. Therefore, it may be assumed that the 1% acetic acid technique is more reliable than buffer solution technique.

The white blood cells and platelet were well preserved; their cytoplasm and nuclei were distinct and well differentiated from each other aiding their correct identification. The amount of

artefacts originating from the stain solutions can be reduced by using coplin jar in staining process; in addition the fixing of the thick film reduced the washing off from the slide but this takes more time in preparing the smear than in conventional method as stated by Alkhairy, (1992).

Any techniques which can help to keep the morphology of malaria parasite in blood smear, it recommended to be used in treating thick blood smear before storage. It makes the detection and diagnosis of parasite easier and advances the prognosis of the disease.

CONCLUSION:

Morphological identification of malaria parasite in blood smear will continue play an important role in diagnosis of malaria in developing countries with poor funded health services. Treatment of thick blood smear when used 1% acetic acid is better than buffer solution since it retains the morphological details of parasites and both techniques were efficient in treating the smear before storage.

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