

Evaluation of Two New Fixatives for Peripheral Malaria Smear

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ABSTRACT

Introduction: Malaria is still a leading killer disease affecting millions of people worldwide, particularly in the tropical and subtropical areas. Till now, the cost-effective, gold standard test for diagnosing malaria with best sensitivity and specificity is stained peripheral smear for different stages of *Plasmodium* spp., even after invention of newer tests like Quantitative buffy coat and PCR. Staining involves use of absolute methanol as fixative. Using methanol is often very dangerous because its systemic absorption can lead to serious toxicities in humans like ocular damage and metabolic acidosis. Also it can be costly to use. Hence we evaluated three new fixatives, namely 70% ethanol, absolute (95%) ethanol and mixture of acetic acid (20%) and formalin (10%) as fixative for staining peripheral blood smear for malaria.

Material and methods: We prepared four thin and four thick smears from each patient's blood after obtaining written informed consent from them, fixed them with absolute methanol, 70% ethanol, 95% ethanol and acetic acid-formaldehyde mixture, dried the smears, stained them with dilute Giemsa stain and observed the dried, stained smears microscopically under oil immersion.

Results: The alcoholic fixatives, 70% ethanol and 95% ethanol were found to be good substitutes for methanol, and 70% ethanol even gave better results than methanol after staining as seen by different observers. Acetic acid-formaldehyde mixture was not found to be a good fixative for this purpose.

Conclusion: Ethanol at both concentrations (70% and 95%) was a good fixative and can substitute methanol for the purpose of observing stained peripheral blood smear for malaria.

Keywords: Malaria, Fixative, Methanol, Smear

INTRODUCTION

Malaria is a leading killer infectious disease with about 200 million new cases and 0.4 million deaths worldwide per year recorded in the year 2015 as per records of the World Health Organisation¹. It is caused by *Plasmodium* spp., namely *P. vivax*, *P. falciparum*, *P. malariae* and *P. ovale* and a fifth new species, *P. knowlesi*². Cases are mostly reported from tropics and subtropical areas of the world but are also found in other areas². Diagnosis is achieved best by staining stained peripheral smear, the stain used being Giemsa stain and methanol is used as fixative for this purpose³. However, methanol is toxic to man, and can produce effects like metabolic acidosis and ocular damage following period of latency⁴. Moreover methanol is expensive and not suitable for routine use in busy labs in developing countries⁵. So keeping these things in mind, we tried to assess the efficacy of 3 new fixatives (70% ethanol, 95% ethanol, and mixture of 10% Formalin and 20% acetic acid in water, for malaria smear staining using Giemsa stain, and comparing it individually

with methanol.

MATERIAL AND METHODS

This was a lab based observational study, carried out in the department of Microbiology of the institute from July 2017 to December 2017. Ethical clearance was obtained before the study from institute, and informed written consent was taken from patients. Routinely venous blood samples (1 ml) were received in the lab in EDTA vials. Tongue-shaped peripheral blood smear was prepared in glass slides that were made grease-free by dipping overnight in absolute methanol. Smear was dried, fixed with (a) methanol, (b) 70% ethanol, (c) 95% ethanol and (d) 10% Formalin and 20% acetic acid in water, for 5 minutes. So there were 8 slides prepared from blood of one patient (4 thick smears and 4 thin smears). After this, thin smears were stained using a 1 in 10 dilution of Giemsa stain in Phosphate buffered saline, pH 7.2, prepared in-house by mixing 3.8 Grams of Giemsa powder (Thomas Baker Chemicals private limited, Mumbai, India), 500 ml glycerol (Khona IINC, Thane, Maharashtra, India) and 500 ml methanol (Himedia, Mumbai, India) and ripened in dark for 1 week. Thick smears were dried and 2 ml of distilled water was added over the slides for dehemoglobinisation. After 10 minutes, water was decanted and slide were again fixed with the fixatives mentioned above and stained likewise. After drying slides, they were observed microscopically under 100X objective by cedar wood oil immersion for malaria parasites. Methanol fixed smear was considered as Gold standard and other fixative-treated smears were compared with it.

RESULTS

Fifty four (54) blood samples were tested in this period. Out of these, only 2 came out to be positive for malaria parasite (One for *P. vivax* and one for *P. falciparum*). They were positive using the first three fixatives (methanol, 70% ethanol, 95% ethanol) but not the fourth (10% Formalin and 20% acetic acid in water). Alcohol (70%) and 95% ethanol were found to be equally good as compared to methanol for this. Morphology of parasite was well appreciable using both these two new fixatives. In all positive cases using the first two new fixatives, parasite trophozoite nucleus appeared

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red and cytoplasm blue. In fact, the clarity of stained smear was qualitatively best viewable using 70% ethanol and equally good as compared to methanol using 95% ethanol, as tested by 3 different observers for each stained smear where staining was done using the methanol and the first two new fixatives. Even for smears negative for malaria parasite, staining of RBC and WBC was best with 70% ethanol followed by methanol and 95% ethanol. The mixture of 10% formalin and 20% acetic acid was not a good fixative as per our study, since all RBCs were damaged on using it a fixative and nothing was visible post staining. Even positive smears tested using other fixatives, gave negative results when fixed using the mixture (fourth fixative).

DISCUSSION

A large number of cases of malaria are diagnosed each year in our country, and rapid and early diagnosis is essential to reduce mortality and also disease transmission in the community⁶. Staining of blood films and subsequent microscopy remains the gold standard test for detecting malaria in the laboratory accurately, even after 100 years of its discovery⁷. Our study found out, for the first time, the utility of three new fixatives for malaria smear staining. This is important since methanol is toxic and costly as mentioned above. Also, methanol has doubtful toxic effect on HIV virus, and thus testing for malaria in known HIV virus positive patient can be very dangerous⁸. Humans normally ingest or inhale small amounts of methanol, and about 0.5 gram of Methanol per day is the upper safe limit for humans for consumption⁹. There are reports that methanol can cause blindness and even death if swallowed accidentally in any quantity¹⁰. Though methanol is needed for preparing Giemsa stain and making it grease-free, still by substituting it with other chemicals for fixing smear, its toxicity can somewhat be mitigated. This finding can be applied for other tests also where blood or even bone marrow smear staining is required, like for LD-bodies in visceral leishmaniasis or in babesiosis or filariasis. One drawback of our study was the small sample size and the lesser number of positive smears. Nevertheless, these things have not been tested earlier and further such studies are needed. As far as we know, this is the first type of such a study at least in this part of the country. These findings should show the way for further research in this area of medical microbiology.

CONCLUSION

Ethanol in 70% concentration and also in 95% concentration can be suitable substitutes of methanol for fixing peripheral blood smear before staining for malaria parasite.

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