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Quality control of blood smears prepared on transparent methanol: an alternative method for the microscopic diagnosis of malaria

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Abstract
Background: Malaria is one of the leading causes of infant mortality in the Democratic Republic of Congo. A retrospective study examining 100 negative blood smear slides after methanol fixation was conducted at the National Malaria Control Program (NMCP) at Kinshasa. The objective of the study was to control the quality of Giemsa-stained negative slides fixation methods on the sensitivity and specificity of the two diagnostic methods.
Methods: The methanol solution was used prior to Giemsa staining to fix the smear preparations. Thick and thin blood smears were prepared using the conventional method on slides. Staining was performed using Giemsa staining for thick and thin smears.
Results: Of the 100 slides received for reading for malaria parasitic stage; 100 slides had been reported negative. Laboratory diagnosis by microscopic examination after methanol fixation of blood smears showed that 60 slides of the 100 slides were positive. Microscopic images of parasites and blood cells in a thick non-methanolic blood film were prepared on glass slides and fixed with methanol. The absence of methanol fixation showed a lower apparent prevalence than that fixed with methanol fixation. Previous information on the sensitivity and specificity of the diagnostic methods was available on malaria in the Democratic Republic of the Congo.
Conclusion: The preparation of thin and thick blood smears in the methanol binding makes it possible to identify the false negatives of certain slides for the microscopic diagnosis of malaria. It is reasonable to predict the applicability of methanol in relevant situations such as the training of qualified professionals for the microscopic diagnosis of malaria and the preparation of positive samples for the assessment of skills (quality control) of professionals and services involved in the diagnosis of malaria.

Keywords: Malaria, microscopy, blood smears, methanol

Introduction
Background
Accurate and up-to-date information on the number and trends of malaria-related cases and deaths is essential for monitoring the progress of malaria control and elimination efforts [1]. In countries with poor surveillance systems, such as those in sub-Saharan Africa, this information can only be obtained by modeling the relationship between parasite prevalence and case incidence, or mortality [2]. The estimates are intended to fill the gaps in the reported data [3]; but because they are based on relationships between uncertain variables and on data that can be measured imprecisely, estimates generally involve considerable uncertainty [4].
The current re-emergence of malaria in developing countries makes this condition still one of the major problems of public health [5]. The quality control of the blades must be a priority. With regard to the magnitudes of malaria, fixation by methanol is more critical for the identification of parasites [6]. It is an essential step in parasite research. Its contribution must be based on a guarantee of quality because the presence of a parasite does not necessarily exclude the presence of malaria [7, 8]. This case may be due to an error of slides not being fixed in methanol leading to false negatives [9].
The main strategy for malaria control consists of early diagnosis and appropriate treatment. Delayed diagnosis or treatment contributes to the rapid spread of malaria and an increased risk of severe and fatal malaria cases [10, 11]. The risk of succumbing to malaria in endemic areas

of this country, where malaria naturally exists, is 100 times higher for people visiting the Democratic Republic of Congo [12]. This is mainly due to misdiagnosis and delays in the treatment of malaria, due to the inexperience of professionals in these endemic areas as to the correct microscopic diagnosis of this infection. It is not uncommon for the disease to be diagnosed after death [13]. Microscopic analysis of a strongly colored blood smear remains the most appropriate method for the diagnosis of malaria. It is a simple, fast and inexpensive technique that can correctly diagnose the parasitic species of malaria and determine the parasite density. However, the lack of qualified professionals to correctly diagnose malaria and the lack of quality control in the laboratory diagnostic process have been identified as the main reasons for the lack of success of the current DR Congo control strategy [14].

To train professionals in obtaining a malaria laboratory diagnosis, educational institutions and malaria epidemiological surveillance services throughout the country, practical courses and training in microscopic diagnosis of *Plasmodium* spp. are being conducted. This practical teaching is essential for the training of students in biology, pharmacy and biomedicine and the evaluation of the expertise of technical professionals associated with the diagnosis of malaria [15]. Microscopy training requires readily available and high-quality educational resources. Blood smears, including those positive for malaria, have been the most widely used educational resource in these classes. However, because of the initial inexperience of the students, the slides are not well fixed and the reading of the blood smears poses many problems, which requires fixation in the methanol. Besides, positive blood smears and negative reports where smear-positive providers are found in malaria-endemic areas have been reported [16].

To minimize this problem, based on a transparent fixation blood smear preparation method, this study has been developed in order to implement easy resources available for the microscopic diagnosis of malaria. Fixation in methanol is a method used for visibility of malaria to the naked eye. We thus carried out a quality control study of the microscopic examination of the thick film fixed by the methanol and not fixed after staining of Giemsa by the reading of the slides.

Materials and methods

This prospective study consists of quality control of microscopically negative trophozoite slides. This has been done to improve the quality and morphology of the malaria parasite. This study was conducted at the National Malaria Control Program Research Laboratory at Kinshasa Gombe from November 2018 to April 2019.

To normalize the preparation and staining of blood smears in the fixation of methanol, blood from members of people living in the city of Kinshasa who presented with symptoms of malaria were used. Then, to analyze the quality of these blood smears for the diagnosis of malaria, blood samples were obtained from 100 patients with malaria. The participation of people in this study was voluntary and full consent was obtained.

A drop of blood was placed on the previously cleaned labeled blade near its frosted end. Another slide was brought at an angle of 30 to 45 ° to the drop. The drop was allowed to spread along the line of contact of the two blades. The upper slide (spreader) was quickly pushed toward the unfrosted end of the lower slide while making sure that smears had a right feather edge. This was achieved by using the correct amount of blood and the propagation technique; allowing the thin smears to dry. (They dry much faster than thick smears and are less subject to detachment as they will be repaired). The smears were repaired by immersing them in absolute methanol.

The slides were placed individually on the staining rack, making sure that they were not touching each other. Giemsa was poured gently onto the slides until they were totally covered. Each slide required approximately 3 ml of Giemsa. Care was taken to avoid pouring the Giemsa directly onto thick films. The slides were left on the Giemsa solution; 10–15 min with 10% Giemsa solution. The slides were then flooded gently with buffered water to float off the iridescent "scum" on the surface of the Giemsa. Water buffered to 7.2 pH was poured onto the slides from the thin film end to avoid undue disturbance and washing-off of the thick films. The slides were removed one by one and the thick film placed downwards, in a drying rack to drain and dry, making sure that the thick film did not touch the edge of the rack.

Results

Among 100 slides that had been received in reading for a malaria syndrome; 100 slides had been reported as negative for malaria. Laboratory diagnosis by microscopic examination after methanol fixation of the blood smears showed that 60 slides of the 100 slides were positive for trophozoites. Thus 60 slides left a presence of trophozoites with negative microscopy.

Two types of staining of blood smear slides were tested to fix smears. Only methanol binding gave a satisfactory result because of its fast drying and the absence of interference in the staining or structure of the blood smear. The other reacted with the immersion oil and damaged the blood smear.

On methanol fixation, thin and thick blood smears were prepared (Figures A and B). Microscopic examination (x100) of thin and thick blood films prepared on glass slides and methanol fixation yielded high quality images for parasites and blood cells. Smears mounted on methanol fixation appeared on a light background and with minimal dye precipitation. It was possible to clearly identify the main morphological features of *Plasmodium*, including chromatin (stained red), Schüffner granules of *Plasmodium falciparum* (pink) and small cytoplasmic granules of neutrophils. Other features, such as platelets (bright pink), leukocyte nuclei (dark), remaining blood cells (light blue) and parasite chromatin (red) confirmed the quality of malaria diagnostic equipment (Figures A and B). After fixation with methanol, there was no change in the quality of the image. Moreover, when the slides were fixed with methanol and examined again under a microscope, the image remained unchanged.

Table 1 shows the results of the tests obtained according to the possible combinations of tests. The proportion of slide readings that were negative for both methods was non-methanol fixation: 3.7% (95% CI, 3.0-4.5%), were positive for both methods and the proportion of the reading of slides that had positive results in both methods was 91% (95% CI 90-92%) after methanol binding. It also shows the apparent prevalence of malaria by the diagnostic method. No-fixation with methanol showed an apparent prevalence lower than that observed for methanol fixation. The prevalence of non-binding to apparent methanol found by optical microscopy was systematically lower than that fixed to methanol based on diagnostic tests.

Table 2 shows the apparent prevalence of malaria based on

the no-fixation method and the fixation method. The results show that, the apparent prevalence was higher when the fixation method was used.

Table 3 on the other hand shows the resulting distributions for the sensitivity and method of diagnostic test and light microscopy. It displays prior information on the sensitivity and specificity of the diagnostic methods and light microscopy that was available on malaria from the Democratic Republic of the Congo.

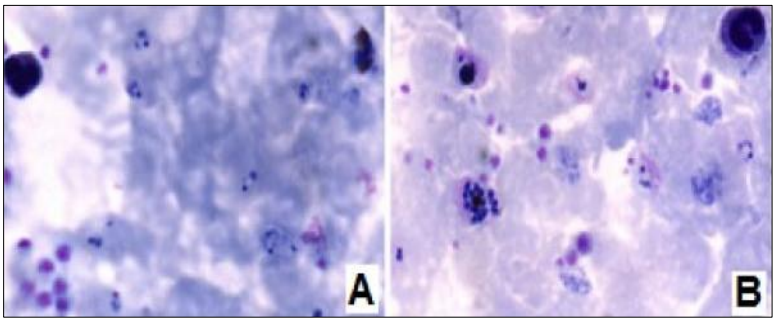

Fig 1: Microscopic images of parasites and blood cells in non-methanol thick blood smear prepared on glass slides (A) and fixed with methanol (B).

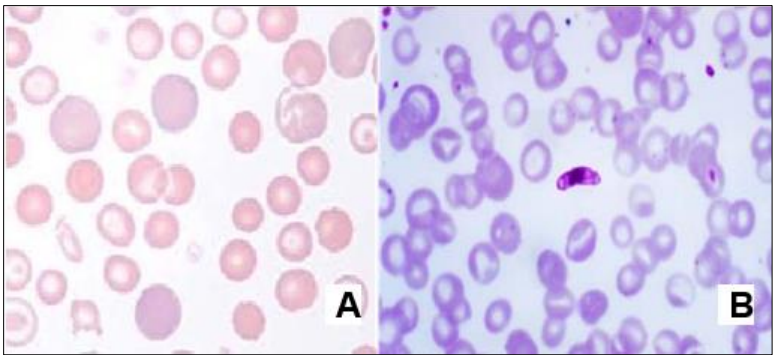

Fig 2: Microscopic negative images of parasites and blood cells in a thick blood smear without methanol prepared on glass slides (A). The same negative becomes positive after fixation with methanol (B)

Table 1: Number of slides based on the results of the two diagnostic methods for non-fixation (n = 100) and fixation (n =100).

Methods	Number of slides				
No fixation	**Fixation**	**Posit/N.F**	**Posit/F**	**Neg/N.F**	**Neg/F**
1	1	94	96	68	98
0	1	55	86	48	87
1	0	44	93	37	83
0	0	22	68	42	79

Table 2: Positive samples (x) and apparent prevalence (AP, %) with 95% exact confidence interval for malaria by diagnostic method

Methods	Positives (n=100)	Negatives (n=100)
	x *AP (95%CI)*	x *AP(95% CI)*
No-fixation	16 3.1 (2.1-3.1)	96 19 (18-20.2)
Fixation	99 30 (28-30)	20 4.4 (3.6-5.3)

Table 3: Prior information on sensitivity and specificity of different diagnostic methods for malaria

Methods	Sensibility	Specificity
No-fixation	Beta (7, 6) 0, 54(0, 31-0, 76)	Beta (6, 7) 0, 73 (0, 43-0, 79)
Fixation	Beta (34, 16) 0, 94(0, 87-1, 00)	Beta (26, 74) 0, 78 (0, 63-0, 89)

Discussion

In this study, the methodology for the preparation of methanol-fixed thin and thick blood smears for the microscopic diagnosis of malaria has been described.

The preparation of blood films on glass slides for the diagnosis of malaria was initially presented at the Congress of the Brazilian society in 1975. In the first experiment, the author proposed the use of the transparent material used in the overhead projectors without describing the technical processes, standardization or evaluation of this new smear preparation method [19]. Subsequently, an attempt to replicate this method was described in a monograph reinforcing the conclusions described above; this suggests that fixation with methanol prior to Giemsa staining is sufficient to support for blood smears used in microscopic examination of malaria [20].

The alternative method for preparing blood smears for methanol fixation has proved easy to implement and has not required additional expense because methanol is inexpensive

and easy to find in stores. In addition, the methanol binding of blood smears did not jeopardize the microscopic image quality of the various stages of the malaria parasite or blood cells. Even with a bluish tint at the bottom of the blood films mounted on methanol binding, the morphological and staining characteristics of parasites and blood cells were not impaired; this allowed for their easy identification (Fig. A and B). The parasitaemia recorded by the three microscopists, who analyzed the two blood smears (no-fixation and fixation), was very consistent and showed that this alternative method does not cause loss of parasites during editing and staining (Fig. A_1 and B_2). Differences in precision between diagnostic methods can lead to considerable variation in malaria prevalence estimates when the sensitivity and specificity of the methods are not taken into account [21]. As the current study shows, the apparent prevalence of malaria in each method varied considerably depending on the method used. For example, in setting methanol, diagnostic methods revealed a 39% prevalence of malaria, while non-microscopic fixation of parasites in blood smears resulted in a much lower 10% prevalence of malaria.

In the Democratic Republic of the Congo, the diagnosis of methanol fixation in the thick smear showed satisfactory sensitivities and specificities, while optical microscopy had different sensitivities but satisfactory specificities. Indeed, the estimated sensitivity of microscopy ranged from 60 to 90% in the Democratic Republic of Congo. In addition to the differences between the observed numbers of positive and negative cases, this variability may also be the result of differences in expert opinion. Indeed, the experts estimated that the results of the methanol-binding microscopy showed more false negatives with average sensitivities of less than 50%. Nevertheless, sensitivity analyses of both methods showed that the true prevalence estimates in methanol binding methods were robust against the non-methanol binding method.

The main limitations of the present study may be related to the unique sensitivity and specificity for each diagnostic method of the thick smear. A negative correlation of non-binding to methanol intensifies the low parasite density [22]. Fixation methanol thus influences the detection and sensitivity of trophozoite under light microscopy. This spatial heterogeneity in the performance of methanol-binding diagnostic methods may be taken into account in the approach presented here, but more detailed information from malaria experts will be needed regarding the relationship between the performances of the two methods of diagnosis.

Abbreviations

D.R. Congo: The Democratic Republic of the Congo
Posit/N.F: Positive no-fixation
Posit/F: Positive fixation
Neg/N.F: Negative no-fixation
Neg/F: Negative fixation

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Ethical approval

This work was carried out with the approval of the ethical committee of China, Tongji Medical College of Huazhong University of Science and Technology (HUST); and the Ministry of Health of the Democratic Republic of the Congo.

Conflict of interest statement

There was no conflict of interest

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Author contributions

JML contributed to conception and design of the study. JML and OAN performed the assays and the statistical analysis. JML, OAN, and MRC wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

Consent for publication

Not applicable.
We declare that we have no conflict of interest.

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