Annals of Medical Laboratory Science (2022) **2**(1): 35 - 41
https://www.annalsmls.org
https://doi.org/10.51374/annalsmls.2022.2.1.0056

Validation of automated malaria parasite diagnostic machines based on first principle: A pre-requisite for acceptable results and treatment monitoring in resource limited settings

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How to cite this paper: Peletiri, I.C., Osita, B.U., Okonkwor, C.E. and Fyaktu, E.J. (2022) Validation of automated malaria parasite diagnostic machines based on first principle: A pre-requisite for acceptable results and treatment monitoring in resource limited settings, *Annals of Medical Laboratory Science* **2**(1): 35 - 41

Received: October 11, 2021
Accepted: February 26, 2022
Published: March 30, 2022

ISSN No: 2805-4024

ABSTRACT

Background: Following the very recent introduction of automated malaria parasite diagnostic machines; the need to validate these high technology machines based on the first principle protocol in malaria parasite density determination for acceptable results and treatment monitoring cannot be over-emphasized. The aim of this review is to update Medical Laboratory Scientists, Medical Laboratory Technicians, and researchers alike on the first principle in the diagnosis of malaria using Giemsa stained thick and thin blood films and to build their capacity on how to validate any automated malaria parasite diagnostic machine.

Methods: The first principle protocol in malaria parasite density determination was used. With 8 µL of blood spread within 18 mm diameter of circle (thick film), the volume of blood in one thick film field (0.002 µL) is obtained; which when multiplied by a factor (500) gives 1 µL. The number of parasites seen per 100 thick film fields or average number per each thick film field multiplied by 500 gives the number of parasites / µL of blood.

Results: Malaria parasites counts of 5 – 50 parasites (1+), 50 – 500 parasites (2+), 500 – 5000 parasites (3+), and (4+) > 5000 parasites / µL of blood, and with the results obtained from the automated machine which when entered into a 2 x 2 table reveal the performance evaluation of automated machine.

Conclusion: With several results obtained, any automated malaria diagnostic machine can be validated for its ability to detect disease (sensitivity, specificity, positive and negative predictive values). Commencement of the use of automated malaria parasites diagnostic machines in parasitology laboratory should not lead to discontinuity in the use of thick and thin blood films in malaria diagnosis as it remains the gold standard in resource limited settings.
Annals of Medical Laboratory Science (2022) 2(1), 35 - 41

Keywords: Validation, automated, malaria, diagnostic machines, first principle

INTRODUCTION

Automation as an emerging trend in modern clinical laboratories impacts positively on service delivery to patients and on staff safety / protection (Archetti *et al*., 2017); with automation of tests procedures which began more than 50 years ago (Hawker *et al*., 2017). While automation in clinical chemistry and haematology has moved steadily and in tune with laboratory advancement drives, medical microbiology and parasitology laboratories have not benefitted much from these high technology protocols (Somagen, 2015).

Therefore, the very recent introduction of automated malaria parasite diagnostic machines to the parasitology laboratory is a welcome

https://doi.org/10.51374/annalsmls.2022.2.1.0056

development. However, there is the need to validate these high technology machines based on first principle in order that results emanating would be acceptable based on our understanding of the production capacity of the machine and so rely on such results for diagnosis and treatment monitoring.

In order to ensure effectiveness amongst practitioners and to also retain competence, it is necessary that good supervision and regular re-training takes place. Operational fatigue is also a limiting factor and it has been suggested that the examination of 50 thick films daily should be the absolute maximum for any microscopists, and that no more than 20 films should be examined without a break for at least 30 minutes of non-microscope activity (Hommel, 2002). In actual practice, about 30 blood films should be examined because both the thick and thin films are viewed for each patient. Standard practice requires that the thick film should be examined for at least 5 minutes (corresponding to approximately 100 microscopic fields under oil immersion). Meanwhile, the standard duration of examination of the thick film has been extended to 200 oil immersion fields by some authorities for at least 10 minutes before a negative result is being released (or issued out), as this may allow for the detection of very low parasitaemia, which would not be seen on the examination of 100 fields (Paniker and Ghosh, 2013; UKNEQAS Parasitology, 1986).

The aim of this review is to update Medical Laboratory Scientists (MLS) and certified Medical Laboratory Technicians (MLT) and researchers alike on the first (1st) principle in the diagnosis of malaria using Giemsa stained thick blood films; build the capacity of MLS and certified MLT on their ability to carry out (perform) malaria parasites count with Giemsa stained thick blood films; and to build capacity of MLS and researchers on how to validate any automated malaria parasite diagnostic machine.

MATERIALS AND METHODS

The preparation and staining of thick and thin blood films as reported by Peletiri and Ibecheozor (2013); while the First (1st) principle on malaria parasite quantification protocol was used. The first principle on malaria parasite quantification (parasite count) states that "*When a measured volume of blood (e.g., 8 µL) is spread within a defined diameter of the circle (thick film) (e.g., 18mm) under a magnification of 1000 (100x objective), the volume of blood in one thick film field (0.002 µL) is obtained; and with the number of parasites seen per 100 fields or average number per each field which when multiplied by a factor (500) will give malaria parasites quantification (count) per microlitre of blood*" (Peletiri, 2021). We simply modified the proposal by Greenwood and Armstrong (1991) to deduce the statement of this first principle.

Parasites are counted by estimating the parasite numbers / µL of blood from the thick film as reported by Peletiri and Ibecheozor, (2013). The factor of 500 was proposed by Greenwood and Armstrong (1991); they had calculated that 5 – 8 µL is the volume of blood required to make a satisfactory thick film and that the volume of blood in one thick film field (100x objective) of a well-prepared thick film is about 0.002 µL. To verify their proposal and arrival of 0.002 µL as stated, Peletiri *et al*., (2021) in their original article shared how they investigated and verified the proposal by Greenwood and Armstrong. For ease of access, we repeat it here as such:

Calculation of the diameter of the circle of thick blood film required will be equal to the diameter of field of view (dFOV, 0.178 mm) (Armstrong, 2012) multiplied by 100 fields (Cheesbrough, 2000; WHO, 2010). Therefore, dFOV multiplied by 100 fields = 0.178mm x 100 = 17.8mm = 18mm.

Estimation of volume of blood in one thick film field (100x objective). The volume of blood in one thick film (100x objective) of a well-prepared thick film will be equal to the diameter of the circle of thick blood film divided by the volume of blood used and further divided by the magnification.

Diameter of the circle of thick blood film = 18mm;
Volume of blood used = 8 µL;
Magnification of 100x objective = 1000
Formula: Diameter of the circle of thick blood film/volume of blood used/magnification
= 18/8/1000;

https://doi.org/10.51374/annalsmls.2022.2.1.0056

= 2.25/1000;
= 0.00225;
= 0.002 µL

With this outcome, we can create a table (Table 1) for the required diameter of the circle of thick blood film prepared from other volumes of blood used (e.g., 5, 6 & 7 µL). However, it should be noted that only with the use of 8 µL spread within 18 mm thick film ensures coverage of 100 fields.

Malaria parasites counts are measured as per microlitre (1 µL) of blood. Therefore, to convert 0.002 µL to one microlitre (1 µL) is simply by dividing 1 µL with 0.002 µL, which gives 500. Therefore, the number of parasites seen and counted per 100 thick films or the average number per thick film field multiplied by 500 gives the number of parasites / µL of blood. Greenwood and Armstrong (1991) found the use of 500 as a factor to be more accurate and quicker than counting the parasites against 100 white blood cells in a thick film using the WHO method as was used by Molineaux *et al*., (1980), and Greenwood and Armstrong (1991) or against 200 or 500 WBCs and multiply by 800 as a factor (WHO, 2010). The counting of malaria parasites with Giemsa-stained thick blood film and reporting system should be done as reported by Peletiri *et al*., (2021) in their original article on "Paradigm shift in malaria parasite density determination to First principle protocol".

RESULTS

There is the need to update our reporting format to meet requirement for personalized medical care (individual patient care) as reported by Peletiri *et al*., (2021).

Validation of Automated Malaria Parasite diagnostic machines

The validation of automated malaria parasite diagnostic machines would involve the following steps; proper study of the manufacturer's instruction manual insert; performance evaluation; and verification of manufacturer's performance claims. Prior to testing patients' samples, it is important to evaluate the performance of new equipment to ensure it is working correctly with respect to accuracy and precision (WHO, 2011). Also, laboratory instruments need to be evaluated for the ability to detect disease (sensitivity, specificity, positive and negative predictive values) and to determine normal or reportable ranges (WHO, 2011). Therefore, with the accurate manual parasite count values obtained, the process of performance evaluation (sensitivity, specificity, positive and negative predictive values) can then proceed.

Sensitivity and specificity: These are two important measures of test function used to report the performance of diagnostic tests when the true disease state is known (Katz *et al*., 2014). Sensitivity refers to the ability of a test to detect a disease when present; while specificity refers to the ability of a test to indicate nondisease when no disease is present (Katz *et al*., 2014). To calculate these measures, the data concerning the subjects studied and the test results can be put in a 2 x 2 table (Table 2).

Predictive values: Sensitivity and specificity are helpful but do not directly answer two important clinical questions: 1) If a patient's test is positive, what is the probability that the person has the

Table 1: Volume of blood used and required diameter of thick film to get 0.002 µL per volume of blood in one thick blood

Volume of blood (µl)	Diameter of thick blood film (mm)	Value of (b/a)	Volume of blood in one thick film field = Value (c)/magnification of 100X objective (1000) = (µl)
a	b	c	
5	12	12/5 = 2.4	2.40/1000 = 0.002
6	14	14/6 = 2.33	2.33/1000 = 0.002
7	16	16/7 = 2.28	2.28/1000 = 0.002
8	18	18/8 = 2.25	2.25/1000 = 0.002

Table 2: Performance evaluation 2 x 2 table

Test Results (thick film)	**Automated Machine**		
	Positive	Negative	Total
Positive	a	b	a + b
Negative	c	d	c + d
Total	a + c	b + d	a + b + c + d

Formulas:
a / (a + c) = Sensitivity
d / (b + d) = Specificity
b / (b + d) = False-positive error rate (alpha error rate, type I error rate)
c / (a + c) = False-negative error rate (beta error rate, type II error rate)
a / (a + b) = Positive predictive value (PPV)
d / (c + d) = Negative predictive value (NPV) (Katz *et al.,* 2014).

disease under investigation? 2) If the result is negative, what is the probability that the person does not have the disease? These questions, which are influenced by sensitivity, specificity, and prevalence, can be answered by doing a horizontal analysis, rather than a vertical analysis. Predictive values can either be positive or negative. Positive predictive value (PPV) is the proportion of subjects with positive test results who actually have the disease; while negative predictive value (NPV) is the proportion of subjects with negative test results who are truly free of the disease (Katz *et al.*, 2014).

Verification of Manufacturer's performance claims:
Medical laboratory directors need to ensure they verify the manufacturer's claims, and demonstrate they can get the same results using the kits or equipment in their laboratory, with their personnel. Investigate the machine to either rule-in or rule-out the manufacture's claims as to what the machine can actually do; by carrying out the actual validation protocol while utilizing the formula below to determine sample size for validation.

Determination of Sample Size for Validation:
The sample size for validation can be determined using the formula developed by Cochran (1977) to calculate representative sample for proportion as:

$$n_o = \frac{z^2pq}{e^2}$$

Where, n_o – is the sample size; z - is the selected critical value of desired confidence level at 95 % (standard value of 1.96), representing a level (likelihood) of error of 5 %; p – is the estimated proportion of an attribute that is present in the population (estimated prevalence of malaria in the study area: 32.9 % = 0.329, Peletiri & Ibecheozor, 2013, Abuja, Nigeria); q – is (1 - p); e – is the desired level of precision (margin of error at 5 %; standard value of 0.05).

Therefore:
$$n_o = \frac{z^2pq}{e^2}$$
$$= \frac{z^2p\,(1\text{-}p)}{e^2}$$
$$= \frac{(1.96)^2 \times 0.329\,(1 - 0.329)}{(0.05)^2}$$
$$n_o = 339.24$$

Therefore, 340 samples can be used for validation of any automated malaria parasite diagnostic machine. This 340 should include all categories of results obtainable with thick blood films (Negative, 1+ (5 – 50 parasites), 2+ (50 – 500 parasites), 3+ (500 – 5000 parasites), and 4+ (>5000 parasites); each result category will be 68 samples each.

For example, sample for use in the validation protocol should be as such:
Negative Samples - Sixty-eight different samples;
1+(5 – 50 parasites / µL of blood) - 68 different samples
Samples 1 – 10: 5 – 9 parasites / µL of blood
11 – 20: 10 – 14 parasites / µL of blood
21 – 30: 15 – 19 parasites / µL of blood
31 – 40: 20 – 29 parasites / µL of blood
41 – 50: 30 – 39 parasites / µL of blood
51 – 60: 40 – 44 parasites / µL of blood
61 – 68: 45 – 50 parasites / µL of blood
Also for 2+ (50 – 500 parasites / µL of blood) - 68 different samples; 3+ (500 – 5000 parasites / µL of blood) - 68 different samples; and 4+ (> 5000 parasites / µL of blood) - 68 different samples.

DISCUSSION
The importance of validation in quality control measures cannot be overemphasized. Validation is the act or process of making valid; producing the

desired result; verifiably correct as claimed by the manufacturer in this context. Literature search revealed pronounced diversity and in some cases usage of wrong techniques for assessing the sensitivity of microscopy thick blood films. In 2007, Mens *et al*., reported the sensitivity or lower limit of detection by microscopy of thick blood film as 5 – 10 parasites / µL of blood. In 2013, Peletiri and Ibecheozor reported the sensitivity or lower limit of detection by microscopy of thick blood film as 5 parasites / µL of blood. Both Mens *et al*., (2007) and Peletiri & Ibecheaozor (2013) followed the first principle protocol in their methodology.

However, Eshel *et al*., (2013) reported 300 parasites / µL of blood as lower limit of detection by microscopy. In this particular study, parasite count was done with thin blood films. In 2017, Mukry *et al*., reported 386 parasites / µL of blood. Milne and co-authors used thin blood films in their study in 1994 and reported 500 parasites / µL of blood as lower limit of detection with the microscope. Though, we prepare both thick and thin blood films on the same slide for diagnosing malaria infection; the thick film provides the sensitivity (it is more than 15 times sensitive than the thin film), whereas the thin film provides the specificity and confirming which species of *Plasmodium* is involved (Hommel, 2002). In her submission, Cheesbrough, (2000) stated that a thick film is about 30 times more sensitive than a thin film.

In examining thick film, WHO, (2010) stated that, Giemsa microscopy is extremely sensitive, and that an experienced examiner can detect malaria parasites at densities of 5-10 per microlitre of blood. This is a statement of fact! As stated above, researchers that used thick blood films in their studies (Mens *et al*., 2007; Peletiri and Ibecheozor, 2013) reported 5 – 10 parasites / µL of blood as the lower limit of detection; as against the 300 – 500 parasites / µL of blood lower limit of detection reported by those that used thin blood films in their studies (Eshel *et al*., 2013; Milne *et al*., 1994; Mukry *et al*., 2017).

In order to actualize the desire for accurate diagnosis of malaria parasite infection and treatment monitoring in resource limited settings; we recommend the following: 1) The use of automatic pipette to deliver the appropriate (desired) volume (e. g., 8 µL of blood) in the preparation of thick blood films with the corresponding diameter of the circle of thick film (18 mm) (Peletiri *et al*., 2021). 2) Regular training and re-training of Medical Laboratory Scientists and certified Medical Laboratory Technicians to retain competence. 3) Commencement of malaria parasite density determination (parasite count) nation-wide for personalized diagnosis and effective treatment monitoring using the 'First principle protocol' (Peletiri *et al*., 2021). 4) With the availability of funds, institutions can key into the diagnostic world of automation in parasitology laboratory – a welcome development. 5) Appropriate sample size based on malaria prevalence rate in your locality should be calculated and used for the validation of automated malaria diagnostic machines; 340 is the required sample size in our environment. 6) Finally, as you commence automation in your parasitology laboratory, we advise that the manual thick and thin blood film method should be done in parallel until such a time when you would have been satisfied with your own assessment of the capacity of the automated machine. Don't forget, one may run out of reagents used for the automated machine, therefore, the use of thick and thin blood films in malaria diagnosis should never be discontinued with; for it remains the gold standard in resource limited settings.

CONCLUSION

The volume of blood used and the size of thick blood film prepared for diagnosing malaria parasites is of utmost importance in the validation process of any automated malaria parasite diagnostic machine based on the first principle protocol. Therefore, following the sequence of activities judiciously, a well-trained trained Medical Laboratory Scientist, certified Medical Laboratory Technician or researcher will have developed the capacity to report the sensitivity of thick blood films as five (5) parasites / µL of blood. With this capacity being built up, the eventual validation of

https://doi.org/10.51374/annalsmls.2022.2.1.0056

any automated malaria parasite diagnostic machine can be effectively and efficiently carried out.

COMPETING INTEREST

Authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

We wish to appreciate Mr. N. K. O. Ibecheozor (M. Sc; FMLSCN), retired Assistant Director, Medical Microbiology & Parasitology Laboratories, National Hospital, Abuja for his leadership role in directing our activities in the initial work on malaria research at National Hospital, Abuja, Nigeria. We equally wish to acknowledge Dr. J. O. Ajobiewe (PhD; FMLSCN; FWAPCMLS), Deputy Director, Medical Microbiology & Parasitology Laboratories, National Hospital, Abuja, Nigeria for his understanding and support during the presentation of this manuscript at the department. We extend our profound acknowledgement to all other Medical Laboratory Scientists that were present during the departmental presentation for their questions, criticisms, comments and issues raised; we say a big thank you to all.

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