AN EVALUATION OF THE SYSMEX XN-30 HAEMATOLOGY ANALYSER IN THE DETECTION AND QUANTITATION OF MALARIA PARASITAEMIA

By

Evashin Pillay (MBBCh)

Submitted in partial fulfillment of the requirements for the degree of Master of Medicine (M. Med) in Haematology, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg

2019

DECLARATION OF ORIGINALITY

This study represents the original work by the author and has not been submitted in any form to another university. Contributions by collaborators and co-authors have been outlined and duly acknowledged in the text.

The research described in this report was conducted during December 2016 and June 2017 under the supervision of Professor Thérèsa L. Coetzer in the Department of Molecular Medicine and Haematology, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg.

Evashin Pillay

Student number: 0705399E

Date: 08 AUGUST 2019

DECLARATION OF CO-AUTHORSHIP

The following publication is being submitted by **Evashin Pillay** as part of a research report for the degree of Master of Medicine (M. Med) in Haematology at the University of the Witwatersrand:

Pillay E, Khodaiji S, Bezuidenhout BC, Litshie M, Coetzer TL. Evaluation of automated malaria diagnosis using the Sysmex XN-30 analyser in a clinical setting. Malar J. 2019; 18:15.
Available online: https://malariajournal.biomedcentral.com/articles/10.1186/s12936-019-2655-8

Authors' contributions to the publication:

Evashin Pillay

Applied for and obtained the ethics clearance certificate for the XN-30 study.

Co-compiled the approved study protocol for the XN-30 study.

Conducted the XN-30 study which entailed the following:

- Receiving, screening and processing all samples at Wits medical school.
- Performing daily quality control procedures and trouble-shooting the XN-30 analyser.
- Planning of experiments, sample preparation and analyses by rapid diagnostic tests, peripheral blood smears and dried blood spots.
- Performing microscopy, including manual parasite enumeration for each sample, and assessment of *P. falciparum* / non-*P. falciparum* species.

Compiled and organised the data spreadsheets and co-evaluated the data.

Compiled the figures and tables including those in text and listed in the additional files.

Drafted and structured the manuscript.

Completed the submission of the manuscript to *Malaria Journal.*

Shanaz Khodaiji

Conducted the prototype study in India.

Belinda C. Bezuidenhout

Conducted the prototype study in the Republic of South Africa.

Monwabisi Litshie

Provided technical and logistical assistance for the prototype and XN-30 studies in the Republic of South Africa.

Performed routine microscopy, including manual parasite enumeration for each sample, for the prototype and XN-30 studies in the Republic of South Africa.

Thérèsa L. Coetzer

Supervised the prototype and XN-30 studies conducted in the Republic of South Africa.

Co-compiled the approved study protocols.

Co-performed microscopy.

Co-evaluated the data.

Critically appraised the manuscript.

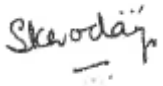
AUTHOR DECLARATION

Evashin Pillay[1]

Date: 29 April 2019

Shanaz Khodaiji[2]

Date: 26 April 2019



Belinda C. Bezuidenhout[1]

Date: 30 April 2019

Monwabisi Litshie[3]

Date: 30 April 2019

Thérèsa L. Coetzer[1]

Date: 29 April 2019

AUTHOR DETAILS

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2. Hematology Department, P. D. Hinduja National Hospital & Medical Research Centre, Mumbai, India.

3. Department of Microbiology, Chris Hani Baragwanath Academic Hospital, National Health Laboratory Service, Johannesburg, South Africa.

DEDICATION

This study has taken me on an unforgettable journey through the intricacies of scientific research. In the beginning I found myself extremely overwhelmed having to manage routine work requirements and coordinate my research project, but I remained focussed and developed key time management strategies to cope and successfully complete what I started. As the project grew, so did I, continuously finding myself engrossed in the various aspects of the study, particularly with the intelligent engineering concepts of the XN-30. Along the way I acquired valuable haematology laboratory skills including peripheral blood smear preparation; processing and interpretation of rapid diagnostic tests for malaria infection; malaria parasite detection, enumeration and species classification using microscopy; and operation of automated Sysmex haematology analysers including sample processing, daily quality control measures and troubleshooting. I recall having to write the literature review, a task which I found initially quite challenging; but with the correct guidance I was able to overcome this and many other challenges including drafting the study protocol; applying for medical ethics clearance; compiling and analysing data; submitting abstracts to conferences; preparing for and executing presentations; and finally drafting the manuscript. Fortunately, I have also had the opportunity to travel as a result of this project, enabling me to interact with researchers from various countries, experience first world diagnostic and therapeutic options for haematology patients and most importantly, make new friends. Overall, it has been a career and life-changing experience which I thoroughly enjoyed and for which I am very grateful.

This research report is dedicated to:

- The hard-working scientists of the world who continuously endeavour to drive innovation and technology for the greater good of humankind. Your relentless perseverance is remarkable and so encouraging.
- True mentors who dedicate themselves unconditionally to facilitate the best possible guidance and outcome for their mentees. Of you, there are truly very few.
- My family including my wife, parents and sister. Your ongoing support and encouragement mean everything to me, thank you for always having my back.

CONFERENCE PRESENTATIONS

International

Poster **(APPENDIX 4, page 32)**

Name: American Society of Hematology (ASH), 59th Annual Meeting and Exposition

Date: Saturday, 9 December 2017

Venue: Georgia World Congress Center, Atlanta, Georgia, USA

Session: 901. Health Services Research: Non-Malignant Conditions

Title: A New Era in Malaria Diagnosis and Surveillance Using an Automated Analyzer

Authors: Evashin Pillay, Monwabisi Litshie, and Thérèsa Louise Coetzer

Abstract Achievement Award - issued by the American Society of Hematology (ASH)

Value: US $500

Oral

Name: International Federation of Clinical Chemistry and Laboratory Medicine, 2017 Worldlab Conference

Date: Monday, 23 October 2017

Venue: International Conference Centre (ICC), Durban, Kwazulu-Natal, South Africa

Session: Sysmex Seminar

Title: Novel Flow Cytometric Approach to Automated Malaria Diagnostics: Performance Evaluation of the Sysmex XN-30 Analyser

Authors: Evashin Pillay and Thérèsa Louise Coetzer

Local

Oral

Name: University of the Witwatersrand Faculty of Health Sciences Research Day & Postgraduate Expo

Date: Monday, 2nd July 2018

Venue: University of the Witwatersrand, Medical School Campus, Johannesburg, South Africa

Session: Infectious Diseases

Title: A Novel Tool for Malaria Diagnostics

Authors: Evashin Pillay and Thérèsa Louise Coetzer

Best Student Oral Presentation Award - issued by the Wits Health Sciences Research Office (HSRO)

Value: R5 000

Oral

Name: South African Medical Research Council, 3rd Malaria Research Conference

Date: Wednesday, 28 November 2017

Venue: National Health Laboratory Service Head Office, Johannesburg, South Africa

Session: Surveillance

Title: Automated Diagnosis of Malaria: A Key Component in Eliminating the Disease?

Authors: Evashin Pillay and Thérèsa Louise Coetzer

PUBLICATIONS

International Peer-reviewed Journals

Title: Evaluation of automated malaria diagnosis using the Sysmex XN-30 analyser in a clinical setting

Authors: Evashin Pillay, Shanaz Khodaiji, Belinda C. Bezuidenhout, Monwabisi Litshie and Thérèsa L. Coetzer

Citation: Malar J. 2019; 18:15

Impact factor: 3.017

Manuscript published online in *Malaria Journal* on 22 January 2019 by Biomed Central (BMC)

Available online: https://malariajournal.biomedcentral.com/articles/10.1186/s12936-019-2655-8

Title: A New Era in Malaria Diagnosis and Surveillance Using an Automated Analyzer

Authors: Evashin Pillay, Monwabisi Litshie, and Thérèsa Louise Coetzer

Citation: Blood 2017; 130:2097

Impact factor: 15.132

Poster abstract published online in *Blood Journal* on 7 December 2017 by the American Society of Hematology (ASH) **(APPENDIX 5, page 33)**

Available online: http://www.bloodjournal.org/content/130/Suppl_1/2097

ACKNOWLEDGEMENTS

Several people have assisted in various ways toward the completion of this project. I am grateful to those who offered encouragement or who contributed in effort and time.

- First and foremost, to my best friend and wife Presantha Govender who has forever believed in my capabilities and supported me throughout my undergraduate and postgraduate studies. To you I am eternally grateful.
- To my supervisor, Prof. Thérèsa L. Coetzer, thank you for affording me the opportunity to be a part of this research study. Your ongoing encouragement, experience in the field, technical expertise and eternal optimism are unrivaled. Your constructive criticism during the preparation of the publication, this research report and the various other academic presentations is greatly appreciated. I have grown personally, academically and professionally under your guidance and mentorship, and for this I am eternally grateful.
- To the phenomenal team from Sysmex, thank you for affording me the opportunity to be a part of this remarkable study which has taught me so much. I thank Dr. Marion Münster, Dr. Jarob Saker, Ndwakulu Nemuthengame and Tiaan van den Berg for all their input and technical support.
- Lastly, I would like to thank the following individuals also who contributed to the study:
  Prof. Jeannette Wadula and Prof. Adriano Duse for providing access to residual blood samples.
  Prof. John Frean and Bhavani Moodley for expert advice in identifying *P. ovale* within samples.
  Desiree du Plessis who performed the PCR assays for the XN-30 study.
  The medical technologists in the routine NHLS laboratories at Charlotte Maxeke Johannesburg and Chris Hani Baragwanath Academic Hospitals.

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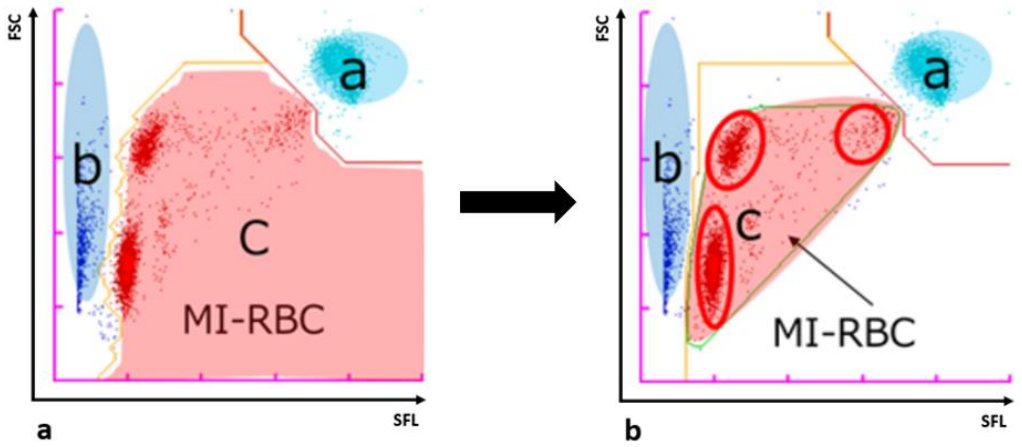
ADDITIONAL FIGURES FOR PUBLISHED MANUSCRIPT

Additional file 1: Fig. S1



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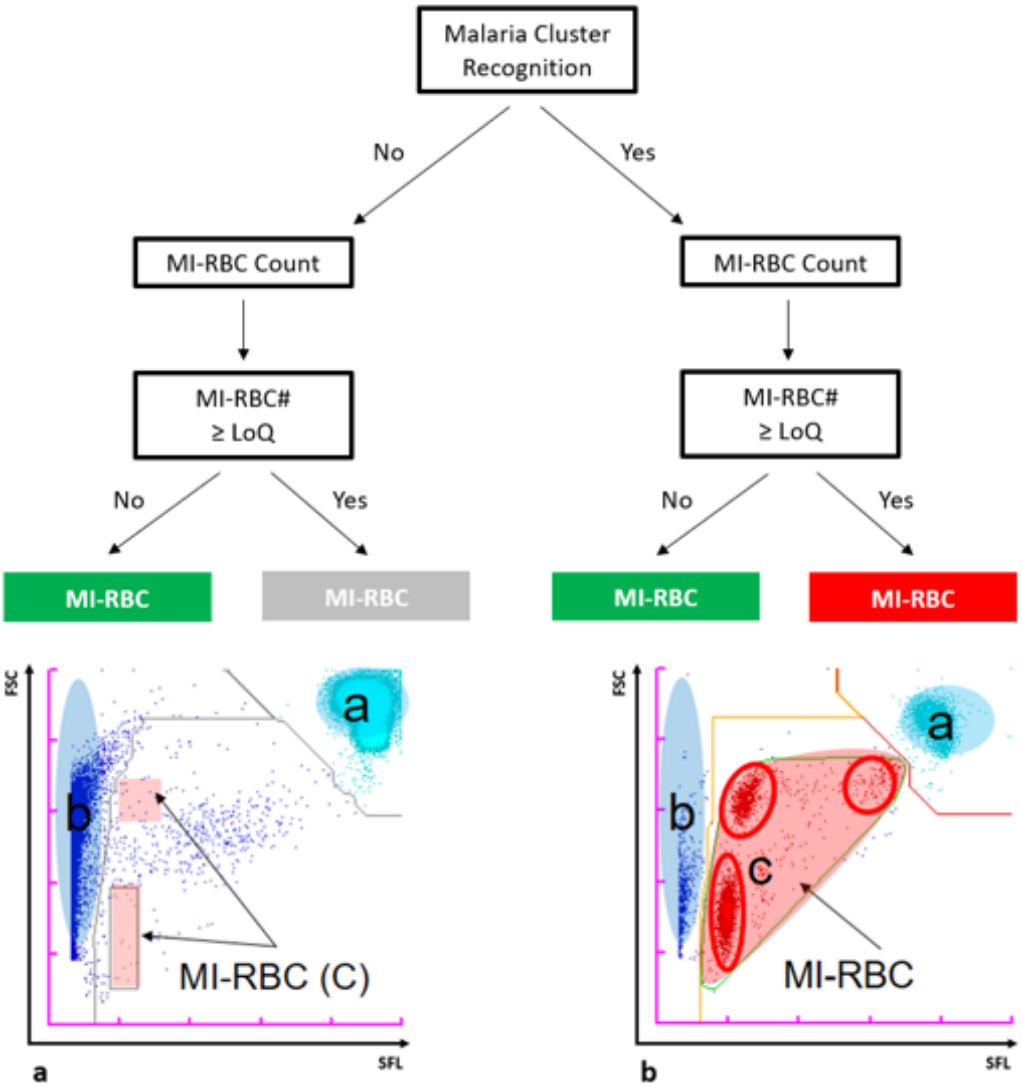

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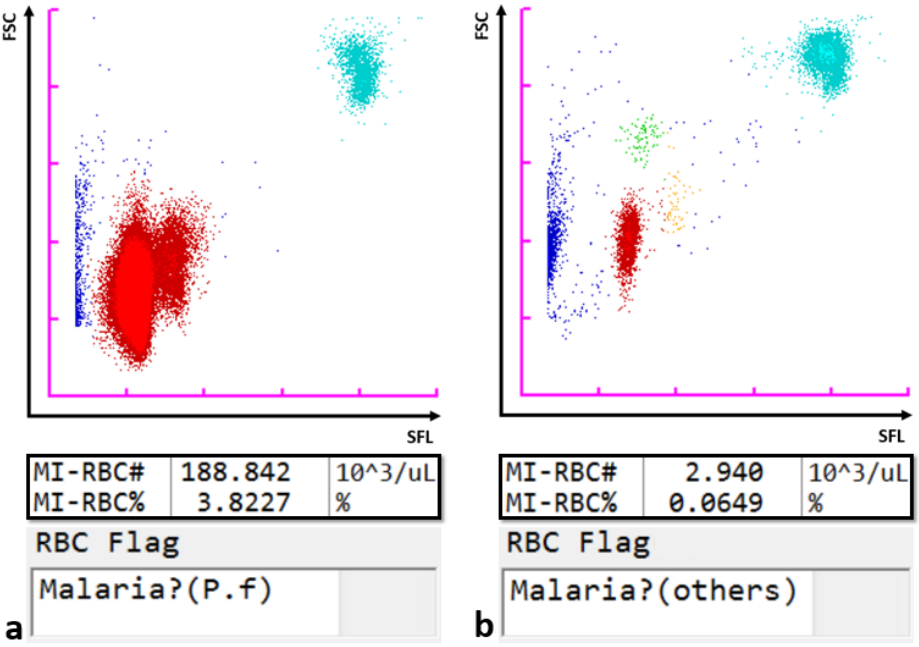

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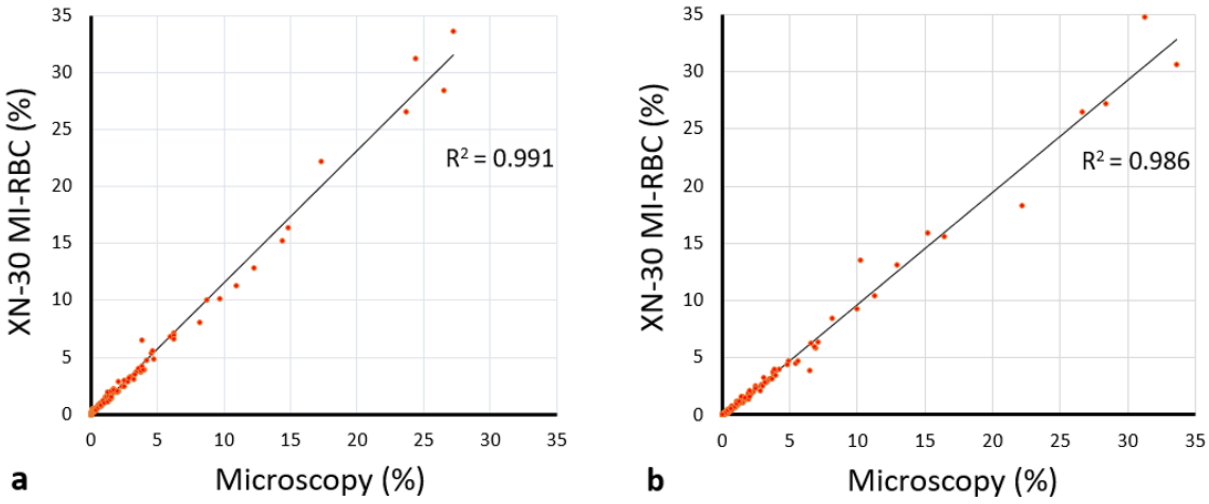

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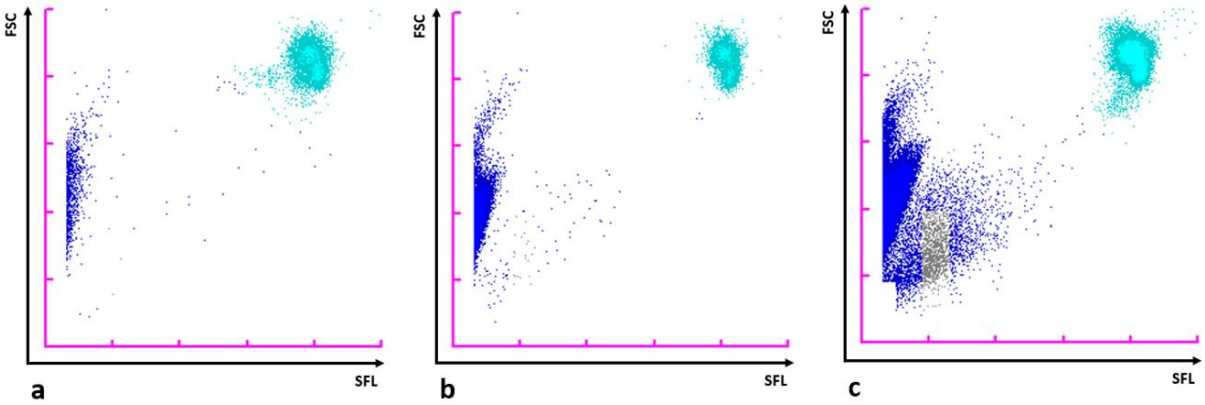

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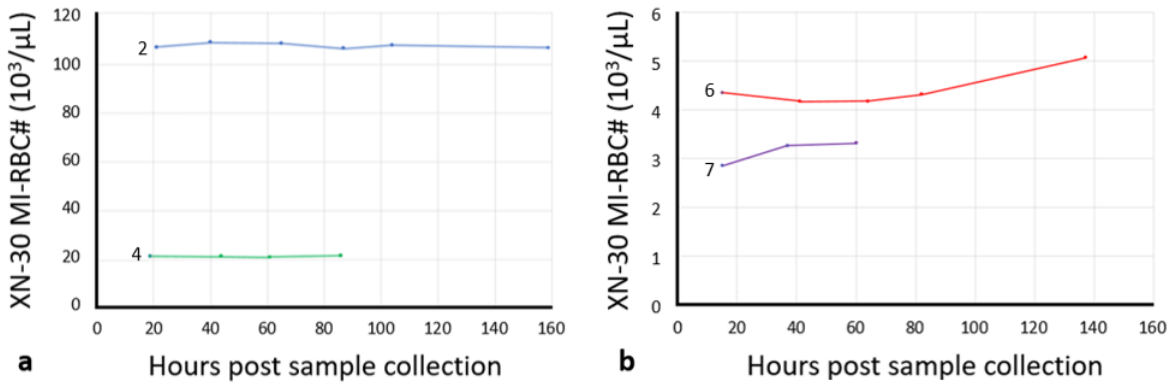

ADDITIONAL TABLES FOR PUBLISHED MANUSCRIPT

Additional file 2: Table S3

Whole blood mode (WB)	RBC (x 10^6/µL)	Mean MI-RBC% (%)	Mean MI-RBC# (x 10^3/µL)	CV (%) MI-RBC#	Low malaria mode (LM)	RBC (x 10^6/µL)	Mean MI-RBC% (%)	Mean MI-RBC# (x 10^3/µL)	CV (%) MI-RBC#	Pre-dilute mode (PD)	RBC (x 10^6/µL)	Mean MI-RBC% (%)	Mean MI-RBC# (x 10^3/µL)	CV (%) MI-RBC#
	1.89	0.064	1.201	3.5%		1.89	0.074	1.400	4.6%		1.80	0.071	1.284	6.7%
	3.01	0.149	4.485	1.5%		3.03	0.136	4.128	1.5%		2.90	0.136	3.931	3.6%
	4.52	0.170	7.680	1.0%		4.43	0.172	7.596	1.2%		4.13	0.165	6.826	2.4%
	4.95	0.199	9.850	0.9%		4.95	0.199	9.846	0.4%		4.48	0.198	8.870	0.9%
	4.21	0.537	22.586	1.1%		4.17	0.531	22.140	0.6%		3.67	0.516	18.963	0.7%
	4.60	0.552	25.409	0.7%		4.60	0.549	25.271	0.9%		4.24	0.545	23.093	1.3%
	4.89	0.983	48.089	0.8%		4.93	0.970	47.783	0.8%		4.52	0.962	43.531	1.1%
	3.77	1.535	57.820	0.8%		3.77	1.540	58.055	0.4%		3.51	1.509	53.008	0.6%
	4.57	1.287	58.790	1.2%		4.54	1.275	57.910	2.4%		4.23	1.243	52.618	0.6%
	2.00	26.892	538.620	0.8%		2.02	26.420	533.392	0.5%		1.98	27.151	537.028	0.4%
	Average CV = 1.2% Range (0.8% - 3.5%)					**Average CV = 1.3% Range (0.5% - 4.6%)**					**Average CV = 1.8% Range (0.4% - 6.7%)**			

*** Additional file 2: Table S1 and Table S2 - available electronically and online:**

https://malariajournal.biomedcentral.com/articles/10.1186/s12936-019-2655-8

APPENDIX 1: APPROVED STUDY PROTOCOL

1. Background literature analysis

Malaria continues to be one of the most important parasitic diseases internationally, with approximately 3.2 billion people in 97 countries at risk of infection (Hemingway *et al*., 2016). Worldwide in 2015, there were approximately 200 million cases of malaria with a total mortality of approximately 400 000 (WHO, 2016). Notably, 90% of all deaths due to the disease occurs in central and southern Africa and mainly affects young children and pregnant women (Cholewiński *et al*., 2015).

To effectively combat the global burden of malaria, early and accurate diagnosis of the disease is important so that specific treatment can be initiated thereby preventing complications and reducing mortality. Unfortunately, clinical diagnosis is not accurate enough even with a travel history as the presenting clinical signs and symptoms of malaria are often non-specific and can mimic other tropical infections (Chotivanich *et al*., 2007). According to the World Health Organisation (WHO), malaria diagnosis based solely on clinical features has a very low specificity and often leads to overtreatment and irrational use of antimalarial drugs (WHO, 2015a).

Current recommendations by the WHO for malaria diagnosis are a parasitological test for confirmation of disease to avoid presumptive therapy and minimise exposure to anti-malarial drugs thereby minimising the development of drug resistance. The two methods routinely used for parasitological confirmation are microscopy and immunochromatographic rapid diagnostic tests (RDTs) (WHO, 2015a).

For more than a century microscopy has been the preferred diagnostic test for malaria (Murphy *et al*., 2013). Thorough review of a well prepared and well stained peripheral blood smear by a well-trained morphologist remains the gold standard for malaria parasite detection (WHO, 2000). Not only does it represent a highly sensitive and specific method for diagnosis, but it also allows quantitation of malaria parasitaemia as well as species classification and stage differentiation (Abba *et al*., 2014; WHO, 2015a). However, it requires ongoing training and supervision to ensure competence of skilled laboratory staff in malaria diagnosis (WHO, 2015a). In addition, the outcome of assessment depends on many variables including well prepared peripheral smears, working microscopes and good morphological technique. Further challenges include that it is labour-intensive and time-consuming (WHO, 2000).

Aside from microscopy, the other recommended parasitological test which has gained much interest in the last decade is the RDT. Like the home-pregnancy test and urine dipstick, this technology employs the use of monoclonal antibodies on a test strip directed against parasite-specific antigens in infected blood (Murphy *et al*., 2013; Odaga *et al*., 2014). These commercially available tests are also able to detect different malaria species and can be employed if quality-assured microscopy is not available (WHO, 2015a). Although they may serve as a great complement or alternative to microscopy, there are also some notable concerns regarding these tests. Firstly, they are not quantitative, thereby not allowing for prognostic significance of infection as well as monitoring of parasite clearance as a measure of treatment efficacy. In addition, they may not allow a health professional to distinguish a new infection from a recently treated one (WHO, 2015a). They are also relatively expensive, have a limited shelf life and are quite sensitive to high temperatures (Murphy *et al*., 2013; Abba *et al*., 2014). Lastly, they have been shown to generate false-positive results following cross-reactivity with antigens such as rheumatoid factor (Wongsrichanalai *et al*., 2007).

As the field of medical diagnostics continues to evolve, there is also a constant search for alternate methods to detect and quantify malaria (Sunilkumar and Naik, 2016). To reduce analytical time and improve accuracy, automation of the process involving malaria diagnosis is highly desirable (Kudella *et al*., 2016). Automated analysers can offer fast, sensitive and cost-effective assessment of all suspected malaria cases (Singh *et al*., 2015). Some studies have highlighted specific patterns that were recognised during routine full blood count (FBC) analysis of malaria-infected blood samples by automated haematology analysers (Mendelow *et al*., 1999; Mohapatra *et al*., 2011; Khattak *et al*., 2011; Singh *et al*., 2015; Sunilkumar and Naik, 2016). As these analysers are based on the principle of flow cytometry, it has been noted that various abnormal scattergrams are generated in the presence of malaria infection as white blood cells scatter light differently after they have phagocytosed the parasite and contain the hemozoin pigment (Mohapatra *et al*., 2011). Although quite remarkable, these distinct scattergrams may only suggest the diagnosis and confirmation by microscopic assessment of a peripheral blood smear will always be required (Singh *et al*., 2015).

Something that may prove more definitive for malaria detection with modern haematology analysers is their potential ability to detect the actual malaria parasite within a red blood cell and quantify percentage parasitaemia. If the analyser can then be optimised to provide a flag indicating the presence of malaria-infected red blood cells (MI-RBCs), it could be utilised as a

valuable diagnostic method and become part of a routine parameter in sample analysis (Singh *et al*., 2015). Sysmex Corporation (Japan) have designed an automated haematology analyser (XN-30) which can detect MI-RBCs and report them as a percentage parasitaemia as well as an absolute number of MI-RBCs per microlitre of blood. The automated ability of this instrument to detect the actual malaria parasite and not any by-product (antigens or phagocytic cells which have ingested the parasite) could significantly improve malaria diagnostics and monitoring of response to therapy.

2. Working hypothesis

The detection and quantitation of malaria parasitaemia using flow cytometry methodology of an automated haematology analyser is superior to microscopy (current gold standard).

3. Study objectives

a) To evaluate the performance of malaria parasite detection and quantitation using the Sysmex XN-30 haematology analyser (hereafter referred to as the "XN-30") compared to microscopic determination, RDT, and where necessary PCR.

b) To assess carryover, repeatability and lower limit of detection.

c) To determine the impact of sample age on positive malaria classification.

d) To determine the possible factors that may interfere with detection of malaria parasites.

4. Methods

Inclusion criteria

Peripheral blood samples submitted to the NHLS laboratories at CHBAH and CMJAH with suspicion of malaria infection will be analysed. However, only samples which have been processed within 24 hours of collection from the patient will be included in the data analysis. An estimated total sample number of approximately 600 (minimum 200 malaria-positive samples) will be processed.

Study design

Objective a) To evaluate the performance of the XN-30 for malaria detection and quantitation:

1) XN-30 automated sample analysis

All samples will be measured on the analyser in 3 different standard / pre-selected modes:

a) Whole blood (WB): Each sample will be analysed as is and the amount of blood aspirated by the analyser will be the same as for routine specimen analysis i.e. 60 µL.

b) Low malaria (LM): Each sample will be analysed as is, however, the amount of blood aspirated by the analyser and the time taken to analyse each sample will be more than that which is utilised for routine specimen analysis.

c) Pre-dilute (PD): Each sample will be diluted in a 1:7 ratio using a standardised diluent (Cellpack DCL - a standard reagent used by the XN-30 for processing each sample).

For each of these modes, the instrument will provide an FBC, an estimate of the number of MI-RBCs per microlitre and the percentage parasitaemia. The LM and PD modes will be performed to further assess the accuracy of the instrument in malaria parasite detection and quantitation.

2) RDT

The RDT result from the primary laboratory (ICT Malaria cassette test for qualitative detection of *Plasmodium falciparum*) will be recorded and all samples will be independently re-tested at the PMRU lab using a separate RDT (ICT Malaria dual test) and will be documented as positive or negative.

3) Peripheral blood smear preparation

Four thin peripheral blood smears will be made for each sample of which two will be stained using a conventional malaria stain (Rapidiff stain kit) and two will be kept unstained.

4) Microscopic evaluation

Independent microscopic smear review will be conducted by Dr. Evashin Pillay and Prof. Thérèsa Coetzer. This evaluation will be performed according to the current WHO guidelines (WHO, 2015b). In this regard, the following applies:

- For samples with a parasite count less than 0.1% (~ 5000 parasites/µL), 10 000 RBCs will be counted.
- For samples with a parasite count greater than or equal to 0.1%, 5000 RBCs will be counted.
- If no parasites are visualised after 10 000 RBCs have been counted, a sample will be deemed microscopy negative.
- The *Plasmodium* species and presence of gametocytes will be documented.

5) Qualitative PCR

Qualitative PCR will be performed according to a standardised PMRU protocol (adapted from Hang *et al.,* 1995) if there is a discrepancy between the result of the XN-30 and/or microscopy/ RDT as outlined in the table below. Briefly, whole blood will be lysed and DNA extracted with phenol/chloroform. A 206 bp rifin *P. falciparum* gene fragment will be amplified and the product

analysed by agarose gel electrophoresis. The PCR products will be visualised on a Syngene Geldoc system. The PCR result will be deemed to be correct.

RDT	Microscopy	XN-30	PCR required
POS	POS	NEG	YES
POS	NEG	NEG	YES, only if it is a new patient and not one who has been successfully treated, but the antigen is still present
NEG	POS	NEG	YES
NEG	NEG	POS	YES
POS	NEG	POS	YES

Objective b) To assess carryover, repeatability and limits of blank, detection and quantitation

1) Carryover

This will be assessed on the XN-30 in all modes (WB, LM and PD). High and low target value (HTV, LTV) samples will be selected according to the following:

- HTV: MI-RBCs > 200 000/µL, equivalent to > 4% parasitaemia

- LTV1: Malaria negative sample

- LTV2: Cellpack DCL

Procedure:

The HTV sample will be measured three times consecutively followed by three consecutive measurements of the LTV1 sample. This sequence will be repeated three more times. The above process will then be performed using LTV2 instead of LTV1.

2) Repeatability

This will be assessed on the XN-30 in all modes (WB, LM and PD). Ten samples with at least one of the following percentage parasitaemia levels: high (5-10%), mid (~1%) and low (~0.1%), will be selected.

Procedure:

Each sample will be analysed ten times consecutively in all modes. In the event there isn't a sufficient volume of residual blood for a particular specimen, then separate samples will be utilised provided they have similar percentage parasitaemia.

3) Limits of blank, detection and quantitation

These will be assessed on the XN-30 in all modes (WB, LM and PD).

Limit of Blank (LoB)

Peripheral blood samples (n = 75) submitted in EDTA specimen tubes for routine FBC analysis without clinical suspicion of malaria will be utilised. These samples will be randomly selected from the routine haematology bench in the NHLS laboratory at CMJAH. In all cases, absence of malaria will be confirmed by RDT and peripheral smear review.

Procedure:

Fifteen samples will be analysed once in all 3 modes according to the following:

a) On the day that the sample was obtained and within 24 hours of collection from the patient, for a total of five days.

b) The LoB will then be calculated using data obtained from the above measurements.

c) Data generated with an "abnormal flag" will be excluded.

Limit of Detection (LoD)

Procedure:

Four malaria-positive samples, as determined by the XN-30, RDT and microscopic examination will be utilised as follows:

- The percentage MI-RBCs will be determined for each sample using the WB mode of the XN-30. Each sample will be typed for the ABO blood group using a standard kit and then diluted using ABO compatible malaria-negative blood (obtained from volunteers within the department and tested for blood grouping) to generate three standards with the following dilutions: WB: 30/µL; LM: 20/µL; PD: 40/µL.
- The samples prepared above will be analysed ten times consecutively on the respective modes (WB, LM, PD) giving a total of 40 measurements per mode of analysis.
- The LoD will then be calculated using data obtained from the above measurements.

Limit of Quantitation (LoQ)

Procedure:

Three malaria-positive samples, as determined by the XN-30, RDT and microscopic examination will be utilised as follows:

a) The percentage MI-RBCs will be determined for each sample using the WB mode of the XN-30. Each sample will then be diluted using ABO compatible malaria-negative blood in order to generate the following dilutions: 10, 20, 30, 40, 60 and 100/µL.

b) The samples prepared above will be analysed ten times consecutively on the respective modes (WB, LM, PD).

c) The LoQ will then be determined using data obtained from the above measurements.

Objective c) To determine the impact of sample age on positive malaria classification

This will be assessed on the XN-30 in 2 modes (WB, LM). Ten malaria-positive samples will be collected and stored at 4°C and room temperature (18 - 25°C). These samples will be selected from routine specimens received for this study. Each mode of measurement will be assessed using five malaria-positive samples as follows:

Procedure:

a) A minimum volume of 3.5ml residual blood per sample will be required. Each sample will then be divided into 1.5ml and 2ml aliquots for the 4°C and room temperature storage stability studies respectively and as outlined in the table below.

	Sample 1 (volume in ml)		**Sample 2 (volume in ml)**	
Time (hours)	**Whole Blood Mode**		**Low Malaria Mode**	
	4°C	**18 – 25 °C**	**4°C**	**18 – 25 °C**
2		0.268		0.268
4		0.268		0.268
8	0.268	0.268	0.268	0.268
24	0.268	0.268	0.268	0.268
48	0.268	0.268	0.268	0.268
72	0.268	0.268	0.268	0.268
Dead volume	0.300	0.300	0.300	0.300
Total	1.372	1.908	1.372	1.908
	~1.5	~2.0	~1.5	~2.0
Total volume	~3.5		~3.5	

b) The aliquots stored at 4°C will be tested at 8h, 24h, 48h and 72h, whereas the aliquots stored at room temperature will be tested at 2h, 4h, 8h, 24h, 48h and 72h. In the event of delays because of specimen demand for further investigations as well as transport/logistic constraints, it may not be possible to obtain the 2h and 4h measurement time points. In this regard, the aim will be to process samples at least at the 8h time point.

c) Each sample will be measured three times.

Objective d) To determine the possible factors that may interfere with malaria classification:

Peripheral blood samples submitted in EDTA specimen tubes for routine FBC without clinical suspicion of malaria will be utilised. These samples will be randomly screened at the haematology bench in the NHLS laboratory at CMJAH. Residual blood of those which meet the following criteria shall be analysed in WB and LM modes:

a) Isolated anemia with a haemoglobin level less than 8g/dL (n = 20).

b) Isolated thrombocytopenia with a platelet count less than 100 x 10^9/L (n = 20).

c) Raised reticulocyte counts greater than 100 000/µl (n = 20).

d) Haemoglobinopathies including sickle cell disease and the thalassaemias (n = 20).

5) Consent

Patient consent is not required as this project will use residual blood samples. In addition, patient identification will not be included and results will not be utilised in clinical patient management.

6) Data handling and record keeping

For each sample, the data collected will include the following:

- Patient age, gender and HIV status (if known).

- Date and time of sample collection.

- Date and time of sample analysis.

- The FBC, corresponding scattergrams / graphs, the number of MI-RBCs per microlitre and percentage parasitaemia as measured by the XN-30.

- The RDT result obtained at the primary laboratory as well as at PMRU.

- Microscopic malaria parasite counts, malaria species and presence of gametocytes.

- The PCR results.

All data generated will be stored in a standardised Microsoft Excel spreadsheet.

7) Data analysis

All results obtained from the XN-30 will be compared to the results of the corresponding RDTs, microscopic assessments and PCR. Correlations, sensitivity, specificity, negative predictive values and positive predictive values will be calculated according to different commercial programs such as Medicalc.

8) Quality control

In order to ensure adequate functionality of the XN-30, standardised quality control material provided by Sysmex will be utilised, and quality assurance assessments will be performed on a daily basis according to the appropriate user manual and training. Only after the instrument has passed the above assessments shall the samples in question be processed for analysis. Maintenance of the instrument as well as replacement of reagents will be performed as required when flagged by the analyser, and will be in accordance with the appropriate personnel training. Reagents which have passed the expiry date shall be returned to Sysmex and will not be utilised for the purposes of this study. In the event of a non-correctable technical or software error, sample analysis will be temporarily halted and engineers shall be contacted for assistance. Once corrected, the study will resume.

9) Funding

Sysmex will carry the costs of personnel training, the analysers, reagents, special kits, laboratory consumables and equipment maintenance.

10) Ethics

The Human Research Ethics Committee of WITS has granted approval for the study under the numbers M140995 and M160549. Additional ethics approval under these numbers has been requested for the purposes of a Master of Medicine (M. Med) degree and is currently pending.

11) Study period

Laboratory orientation and training took place in December 2016. Sample collection of residual blood began in January 2017 and will continue until the end of June 2017. An estimated total of 6 months will be required to collect and process an estimated total sample number of approximately 600 (minimum 200 malaria-positive samples). The data will be analysed between July and October 2017. The research report and manuscript(s) for publication will be written and finalised by March 2018.

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APPENDIX 2: ETHICS CLEARANCE CERTIFICATE

R14/49 «Tit init name»

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M1704132

NAME: (Principal Investigator)	Dr Evashin Pillay and Dr Marion Munster
DEPARTMENT:	Molecular Medicine and Haematology Plasmodium Molecular Research Unit University of the Witwatersrand
PROJECT TITLE:	An Evaluation of the Sysmex XN-30 Haematology Analyzer in the.Detection and Quantitation of Malaria Parasitaemia
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	Sub-Study (M140995 and M160549)
SUPERVISOR:	Prof Theresa Coetzer
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	24/05/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. in this case, the study was initially review in April and will therefore be due in the month of April each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 05/06/2017

APPENDIX 3: PLAGIARISM REPORT

Turnitin Originality Report

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Evashin Pillay

Date: 04 May 2019

Thérèsa L. Coetzer[1]

Date: 04 May 2019

APPENDIX 4: ASH POSTER

A New Era in Malaria Diagnosis and Surveillance Using an Automated Analyzer

Evashin Pillay[1], Monwabisi Litshie[2] and Thérèsa Louise Coetzer[1]

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Department of Microbiology, Chris Hani Baragwanath Academic Hospital, National Health Laboratory Service, Johannesburg, South Africa

59th ASH® Annual Meeting and Exposition
Atlanta, Georgia · December 9 – 12, 2017

BACKGROUND

Malaria continues to be one of the most important parasitic diseases with ~3.2 billion people in 97 countries at risk of infection. According to the World Health Organisation (WHO) malaria report there were ~212 million cases globally in 2015, with a mortality of ~400 000. To effectively combat the burden of disease, **early and accurate diagnosis of malaria is important** so that specific treatment can be initiated thereby preventing complications and reducing mortality. Unfortunately, clinical diagnosis alone is not adequate even with a travel history, as the presenting clinical symptoms and signs can mimic other infections and are often non-specific. Notably, malaria diagnosis based solely on clinical features often leads to overtreatment and irrational use of antimalarial drugs. Therefore, current **recommendations by the WHO** for parasitological confirmation are either **microscopy or immunochromatographic rapid diagnostic tests (RDTs)**. Despite being the gold standard diagnostic test of malaria for more than a century, microscopy is prone to a few drawbacks as are the immunochromatographic RDTs (table 1).

Table 1: Drawbacks of microscopy and RDTs in malaria diagnosis

Microscopy	RDTs
Subjective: variability of results among different microscopists	**Not quantitative:** cannot monitor parasite clearance; no prognostic significance
Human error: poor quality peripheral blood smears; suboptimal maintenance of microscopes	**False negatives:** some *P. falciparum* parasites lack an epitope for a common target antigen used in RDTs (histidine-rich protein 2 – HRP2)
Highly skilled technical process: requires an expert microscopist and ongoing refresher training	**False positives:** treated patients with persistent circulating parasite antigens; cross-reactivity with non-malarial antigens
Time consuming: low density cases	**Difficulty interpreting results:** weak positives

To reduce analytical time and improve accuracy, **automation of malaria diagnosis** is highly desirable. To this end, pertinent studies have identified various atypical scattergrams which are generated during routine CBC analysis of malaria-infected blood samples by automated hematology analyzers. Such scattergrams depict white blood cells containing phagocytosed hemozoin pigment and can suggest a diagnosis of malaria, but confirmation by microscopy will still be mandatory. What may prove more definitive for malaria diagnosis by automated analyzers is their ability to **detect the physical malaria parasite within a red blood cell and quantify parasitemia**. Sysmex Corporation (Japan) have designed such an analyzer, the XN-30, which can detect **malaria-infected red blood cells (MI-RBCs)** and report them as an **absolute number** per microliter of blood **(MI-RBC#)** together with a corresponding **percentage parasitemia (MI-RBC%)** for each sample that is analyzed.

OBJECTIVES

- To evaluate the performance of the XN-30.
- To compare the analytical findings of the XN-30 to microscopy, RDT and where necessary, polymerase chain reaction (PCR).

SYSMEX XN-30 TECHNOLOGY

Once an EDTA-anticoagulated whole blood sample is introduced into the XN-30, it then automatically labels intracellular malaria parasites (MI-RBCs) with a proprietary reagent. Using **fluorescence flow cytometry (405nm blue laser)**, the labeled sample is then introduced into a sheath flow detector where forward scattered light and side fluorescence light are measured. A **unique scattergram comprising different cell clusters**, most importantly those signifying **parasitized red blood cells**, is then generated and computed by the instrument (figure 1).

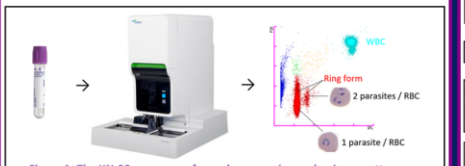


Figure 1: The XN-30 sequence of sample processing and unique scattergram.

In addition to the unique scattergram, the XN-30 also provides a CBC and corresponding MI-RBC parameters (MI-RBC# and MI-RBC%; figure 2).

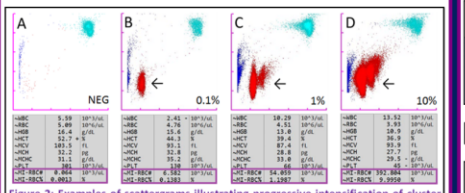


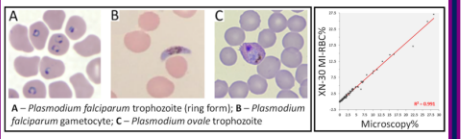
Figure 2: Examples of scattergrams illustrating progressive intensification of cluster signals with sequential increases in parasitemia (A: Negative; B: 0.1%; C: 1%; D: 10%).

EVALUATION

- Conducted in an academic laboratory at **WITS University**, South Africa.
- A total of **189 residual peripheral blood EDTA samples from suspected malaria cases** presenting to tertiary level hospitals in Johannesburg.
- All samples were processed within 24 hours of collection from patients.
- Analyzed by the XN-30 in each of 3 different, pre-selected modes:
  - Whole blood (**WB**)
  - Low malaria (**LM**: 3-fold increase in sample volume analyzed)
  - Pre-diluted (**PD**: 1:7 dilution)
- Peripheral blood smears for microscopic correlation, RDTs for antigenic correlation and dried blood spots for PCR (when discrepant results were obtained) were also prepared.

RESULTS

Excellent correlation of XN-30 MI-RBC% compared to Microscopy



A – *Plasmodium falciparum* trophozoite (ring form); **B** – *Plasmodium falciparum* gametocyte; **C** – *Plasmodium ovale* trophozoite

Detection Limits & Precision

MI-RBC# [cells/µL]	WB	LM	PD
Limit of Blank **(LOB)**	13	12	16
Limit of Detection **(LOD)**	22	20	36
Limit of Quantitation **(LOQ)**	22	20	36

	WB	LM	PD
Precision	1.7%	1.7%	5.5%

Interference Factors

Malaria negative samples with the following:

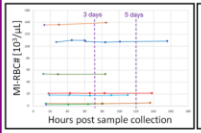
Anemia → Hemoglobin < 8g/dL
Thrombocytopenia → Platelet count < 100 x 10^9/L
Reticulocytosis → Reticulocyte count > 100000/µL
Hemoglobinopathies → Thalassemia, Sickle Cell D_s

These assays demonstrated **no false positive** results

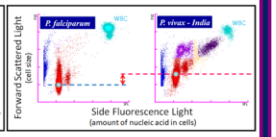
Sensitivity and Specificity

		XN-30 MI-RBC Present: WB / LM / PD			
		Positive	Negative	Total	
Reference (microscopy ± PCR)	Positive	122	1	123	Sensitivity 99.2%
	Negative	0	66	66	Specificity 100%
	Total	122	67	189	

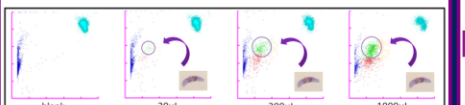
Stable detection at room T°



Species differentiation on scattergrams



Detection of gametocytes, the transmissible form of the parasite



Spiking of malaria negative blood with increasing concentrations of purified *P. falciparum* gametocytes

ADVANTAGES AND IMPACT

- User-friendly, fully automated analytical process
- Daily quality control measures ensure reliability of results
- Rapid sample analysis with results computed in ~1 minute
- Direct detection and quantitation of parasites
- Differentiation of *Plasmodium* species
- CBC and MI-RBC parameters allow baseline cytopenias and parasitemia levels to be documented at presentation:
  - Enables therapeutic monitoring
  - Facilitates early indication of drug resistance
- Gametocyte detection:
  - Identify asymptomatic carriers / parasite reservoirs
  - → treat to disrupt parasite life-cycle & block transmission to vector = a key component of elimination
- Detection of unsuspected cases:
  - Low index of suspicion for malaria
  - CBC requested as part of a routine diagnostic blood panel
  - → countries in pre-elimination phase
  - → infected tourists returning home to non-endemic countries
- Suitable for finger prick and heel stick samples in PD mode:
  - Infants, population studies and mass screening campaigns
  - Clinical trials → efficacy of vaccines or new drugs

CONCLUSION

- **The XN-30 is a revolutionary analyzer** with proven analytical performance and the potential to serve as an **alternative to routine microscopic and antigenic malaria diagnostic methods.**

- The automated detection of the actual parasite and not any by-product holds **immense promise to positively influence malaria diagnostics** and **accelerate global initiatives toward elimination of the disease.**

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THE AUTHORS OFFER THANKS TO THE FOLLOWING PEOPLE AND ORGANISATIONS FOR THEIR INVOLVEMENT

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- WITS University: Faculty Research Committee.
- National Institute for Communicable Diseases (NICD): The Department of Parasitology; Prof. John Frean and staff.
- Sysmex Corporation: Dr. Marion Münster, Mr. Ndwakhulu Nemuthengame and Mr. Tiaan van den Berg.

APPENDIX 5: PUBLISHED ABSTRACT

A New Era in Malaria Diagnosis and Surveillance Using an Automated Analyzer

Evashin Pillay, Monwabisi Litshie, and Theresa Louise Coetzer

Blood 2017 130:2097;

Available online: http://www.bloodjournal.org/content/130/Suppl_1/2097

Malaria remains a global health threat with 91 countries still endemic for the disease. Despite a recent substantial decline in morbidity and mortality, the magnitude of disease burden is still enormous as indicated by the World Health Organisation (WHO) report of an estimated 212 million new cases and approximately 429 000 deaths in 2015. Sub-Saharan Africa bears the brunt of the disease with 92% of all deaths. *Plasmodium falciparum* is the most lethal of the 5 parasite species that infect humans, and causes 99% of all deaths. In patients infected with *P. falciparum*, parasitemia can dramatically escalate every 48 hours or less and can be rapidly fatal if left untreated. Accurate, timely diagnosis is thus a critical first step in the management of malaria and the WHO recommends that all suspected cases must have diagnostic confirmation either by microscopy or rapid diagnostic testing (RDT), prior to initiation of artemisinin combination therapy. Microscopic assessment of peripheral blood is the current gold standard but is subjective and requires a high level of skill, whereas RDTs indirectly detect parasite antigen and can potentially give false positive/negative results. Other methods include PCR and the detection of monocytes with ingested hemozoin, which is a surrogate marker for malaria parasites.

This study describes novel technology in the form of an automated Sysmex XN-30 analyzer, which utilizes a blue laser and flow cytometry to detect and count malaria-infected red blood cells. To evaluate the performance of the XN-30, 189 residual EDTA blood samples from suspected malaria

cases referred for routine diagnosis by microscopy and RDT, were analyzed within 24 hours of collection. A total of 127 *P. falciparum* positive samples and 62 negative samples were processed over a 6-month period. Discrepancies between the analyzer and microscopy/RDT were resolved by PCR using dried blood spots prepared at the time of sample reception. The analyzer showed excellent specificity (98.5%) and sensitivity (98.4%). Measurements were reproducible with a precision of 1.24% and showed no carryover between samples. The percentage parasitemia that was accurately detected ranged from 0.003 to 27%. Positive samples were stored at room temperature and 4°C up to 7 days and daily measurements indicated that the parasite count was stable. To exclude interference by non-malaria factors, separate malaria negative samples (20 per index) with low hemoglobin, high reticulocyte count, thrombocytopenia and hemoglobinopathies (thalassemia, sickle cell anemia) were analyzed, but these had no effect on the malaria gates. Other *Plasmodium* species were also detected, including *P. vivax, P. ovale* and *P. malariae.*

The direct detection and quantitation of *Plasmodium* parasites with the XN-30 holds great promise and has many advantages over microscopy/RDT/PCR. It is automated, easy to use and provides rapid, robust and objective diagnosis of malaria, which will ensure prompt treatment and thus undoubtedly improve the quality of healthcare and alleviate the disease burden in endemic countries. Since the XN-30 provides a CBC with each analysis, it may also serve as a valuable diagnostic aid in detecting unsuspected cases with fever of unknown origin. This scenario is becoming more common as international travel to endemic countries increases and tourists often present with symptoms when returning to their home country, where clinicians may not have a high index of suspicion for malaria, but would request a CBC as part of a diagnostic workup. The decline in parasitemia can be monitored by the XN-30 and if the clinical response to therapy is delayed, this may serve as an early warning sign of drug resistance. This will yield valuable data on the spread of artemisinin resistance, which is alarmingly prevalent in the Greater Mekong subregion. The analyzer can differentiate gametocytes making it useful for detection of asymptomatic carriers who can be treated to prevent the transmission of parasites to the mosquito vector and thus protect communities. This is a vital step in disrupting the lifecycle of the parasite and is in line with global initiatives to eliminate malaria. It can also process finger prick samples making it suitable for infants and population studies, as well as monitoring efficacy of vaccines or new drugs in clinical trials. The XN-30 is currently for research use only but further development and clinical studies are in progress.