Feature Article

— Malaria Infection Diagnostic Tests —
Predictive Flags in Hematology Analyzers and Quantification of Parasitized Red Blood Cells.

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We present here the advances that HORIBA Medical R&D implemented in its hematology analyzers to progress against malaria. On one hand we describe the utilization of the Microsemi CRP for the screening of novel malaria infections in the field thanks to the combination of platelet count and CRP measure. On the other hand we present the application of DNA staining technology integrated into an ABX Pentra 60 derivative system for the precise measure of parasitemia. Based on HORIBA Medical reagent specificity, the simultaneous use of two fluorochromes was adapted to direct dilution and automation. This analytic method allows enumeration of parasitized red blood cells.

Introduction

Malaria represents a global health problem, causing disease and death in several tropical and subtropical areas: there are still 106 countries where the disease is endemic according to the WHO 2011 Report.[1]
Malaria is a protozoan infection of erythrocytes caused by five species of the genus *Plasmodium* (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*). Two are most common. *Plasmodium falciparum*, present globally but mainly in Africa and Asia, is the most aggressive species. *Plasmodium vivax*, ranges widely throughout Asia, Africa, the Middle East, Oceania, the Americas and in Eastern Europe. The parasite is transmitted to human by the bite of infected *Anopheles* mosquitoes, but the infections can also occurs through the exposure to infected blood products (transfusion malaria) and by congenital transmission ,[2] (Figure 1).
The clinical symptoms are primarily due to schizont rupture and destruction of red blood cells; these allow the release of parasites and free hemoglobin into the bloodstream. Malaria can have gradual or a fulminant course with nonspecific symptoms that often remind of common viral infections: fever, chills, headaches, profuse perspiration. Other common and nonspecific symptoms include dizziness, malaise, myalgia, abdominal pain, nausea, vomiting, mild diarrhea, and dry cough. Alterations of hematological and biochemical parameters are well documented as well as inflammation and infection markers. However, as the clinical symptoms, laboratory findings are not really malaria specific and can be more or less predictive according to the context and if

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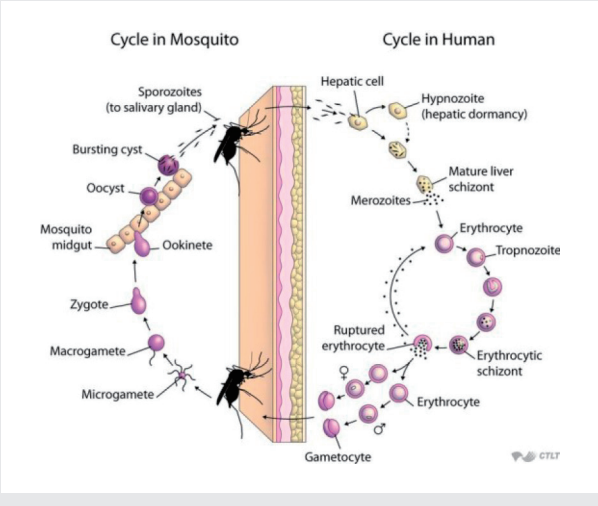

Figure 1 The Malaria Parasite Cycle in Mosquito and Human.
Johns Hopkins Bloomberg School of Public Health's OPENCOURSEWARE (OCW): http://ocw.jhsph.edu.

combined together.[3]

The gold standard for confirmation of malaria diagnosis remains light microscopy examination of thick or thin stained blood smear allowing identification and determination of parasitemia with a detection threshold of 4 to 100 parasites /µl. However, its accuracy depends on the quality of the reagents and microscope as well as the experience of the microscopist.[4] Rapid and accurate diagnosis of malaria is necessary to appropriate treatment and therapeutic follow-up of patients, thus preventing infection spread and drug resistance. A reliable detection method for malaria, incorporated into the routine Complete Blood Count (CBC), would be of great help to detect cases earlier and potentially reduce adverse outcomes related to malaria infection. In addition, these instruments would have the benefit of being already available even where diagnostic tools are limited.

We present here two different diagnostic approaches that HORIBA Medical developed to contribute to the diagnosis of malaria. In the first part of the article we present an application of current hematology analyzers for the screening of suspected patients. In the second part of the article we present a high sensitive method for detection and quantification of parasitized red blood cells.

Predictive Flags in Hematology Analyzers

Introduction and Purpose

Identification of peripheral blood biomarkers that could help identify malaria infection in the absence or with low patient peripheral parasitemia would be of great value since they would accelerate the process for definitive diagnosis, could minimize unnecessary drug treatment and thus improve patient outcomes. Following this reasoning, we wanted to verify the hematology analyzer performances in field conditions and to test if we could select some hematology parameters that would allow creating a flag to help the screening and diagnosis of malaria. Alterations of hematological and biochemical parameters are well documented in malaria. Thrombocytopenia is the most common laboratory abnormality, followed by hyper-bilirubinemia, anemia and elevated hepatic aminotransferase levels, whereas the leukocyte count may be normal or decreased.[3] Although anemia can be often found in malaria, it is not always a reliable marker. Indeed in countries where the social-economic situation is more difficult, the status of general health is also degraded and malnutrition and iron-deficiency are common. When malaria infection installs, anemia becomes more severe and consequently it becomes a good predictor for malaria. This applies to Africa, but for example it does not correspond to the reality of Vietnam. Here, the population has a better general health. Moreover, people consult the doctor as soon as fever arises and they do not wait to be in severe condition before asking medical help. Therefore the severe anemia caused by a concealed anemia and a subsequent malaria infection is less common, making this finding low sensitive for malaria screening and therefore useless. Inflammation and infection markers (erythro-sedimentation rate, C-Reactive Protein (CRP), procalcitonin are also commonly modified. However, as well as clinical symptoms, laboratory findings are not really malaria specific and can be more or less predictive according to the context and if combined together.[4], [6] In particular we decided to focus on combining hematology parameters and CRP to create a dedicated malaria alarm. For this purpose we planned a study in collaboration with the UMR-MD3 (Host-Parasite relationships, Pharmacology and Therapeutic), Aix-Marseille University- France.

Study Plan and Methods

A test campaign was conducted in endemic regions of Vietnam (Binh Phuoc in the South of Vietnam) for two months as part of a collaboration between the French and Vietnamese army health services. The Microsemi CRP and the ABX Pentra 60, both instruments from HORIBA Medical, were installed in a dispensary, a basic outpatient health facility providing primary healthcare services in a rural community. This location had double advantage (1) it offered a perfect setting to test the behavior of the hematology analyzers in rude utilization conditions for temperature and hygrometry and (2) it permitted

recruitment of blood samples directly in an endemic country at the time when propagation by the mosquitos is most frequent. A total of 119 whole blood specimens were collected from patients presenting with fever at the dispensary and after they had given their informed consent. All samples were tested in parallel on the ABX Pentra 60, on the Microsemi CRP and compared with the microscopic examination on thick blood film stained with RAL555 (RAL DIAGNOSTICS, France), a fast acting variation of May-Grünwald Giemsa Staining.

Results and Conclusions

The first step was to verify the proper functioning of the instruments in these adverse environmental conditions. In particular all the analytical alarm flags that were triggered in the ABX Pentra 60 were identified and analyzed:
NOISE (informing about the presence of noise in certain count channels): 6 times.
LMNE-(meaning that the two counts of white cells performed by two distinct channels are not identical/in agreement): 5
LMNE+ (as for the previous, meaning that the two counts of white cells performed by two distinct channels are not identical/in agreement: 7.
A total of 14 (11.7%) specimens triggered one or multiple flags. There were no flag related to incorrect working conditions, proving that the instrument was not disturbed by the environmental situation of high temperature and hygrometry. Indeed those alarms didn't appear in series but they were triggered from time to time without any pattern. We consider that all generated alarms were related to the peculiar features of the specimens and not to the instability of the instrument.
The criteria for the selection of patients were the presence of fever and the fact that he/she had spent sometime in the forest. Among these patients, 61 (51.3%) were diagnosed with uncomplicated malaria by microscope-based analysis that was used as reference diagnostic method.
The hematology parameters obtained for each sample with the ABX Pentra 60 and the Microsemi CRP were analyzed in order to extrapolate those that would be discriminative for malaria diagnosis.
The two analyzers showed comparable results (data not shown).
The machines were used in normal working conditions (standard pathology ranges). However for post-analytical data treatment we considered the following thresholds on relevant parameters:
Platelet absolute count. Alarm threshold < $150 \times 10^3/mm^3$;
Neutrophil absolute count. Alarm threshold > $7.5 \times 10^3/mm^3$ and < $2 \times 10^3/mm^3$;
Lymphocyte absolute count. Alarm threshold > $4 \times 10^3/mm^3$ and < $1 \times 10^3/mm^3$;
Monocyte absolute count. Alarm threshold > $1 \times 10^3/mm^3$ and < $0.2 \times 10^3/mm^3$;
Eosinophil absolute count. Alarm threshold > $0.5 \times 10^3/mm^3$;
Basophil absolute count. Alarm threshold > $0.2 \times 10^3/mm^3$;
Hemoglobin concentration. Alarm threshold > 17 g/dL and < 11.5 g/dL;
C-reactive protein concentration. Alarm threshold > 16 mg/L.

The relationship between parasitemia and each of the above parameters was established from the calculation of Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) (Figure 2).

Out of 119 samples, only 19 samples were out of normal range for monocyte count, 8 for eosinophil count and 3 for the basophil count. Therefore these three parameters were not taken into account as potential candidates. Also neutrophils, lymphocyte and monocyte showed low sensitivity and therefore they were discarded as well. On the other side the platelets and the CRP presented a good sensitivity, but the specificity was not satisfactory. In order to improve the specificity the PLT and CRP alarm have been combined (Figure 2). When PLT (< $150 \times 10^3/mm^3$) and CRP (> 16 mg/L) were both altered we obtained an alarm with 79.8% of sensitivity and 76.9% of specificity, with a positive predictive value of 82% and a negative predictive value of 73.2%. The thresholds were selected in order to optimize the identification of malaria cases. In particular for the CRP, the limit was chosen in order to have guiding criteria for diagnosis of malaria which needs a rapid confirmation by a specific test. Indeed, the treatment of malaria access is an emergency treatment. These results suggest that a dedicated flag incorporated in a hematology analyzer triggered when both the PLT and CRP are out of range would be a reliable screening method allowing decrease of the time-lapse between early symptoms and definitive diagnosis, thus potentially reducing adverse outcomes related to malaria infections. Further studies should be planned to confirm these encouraging preliminary results.

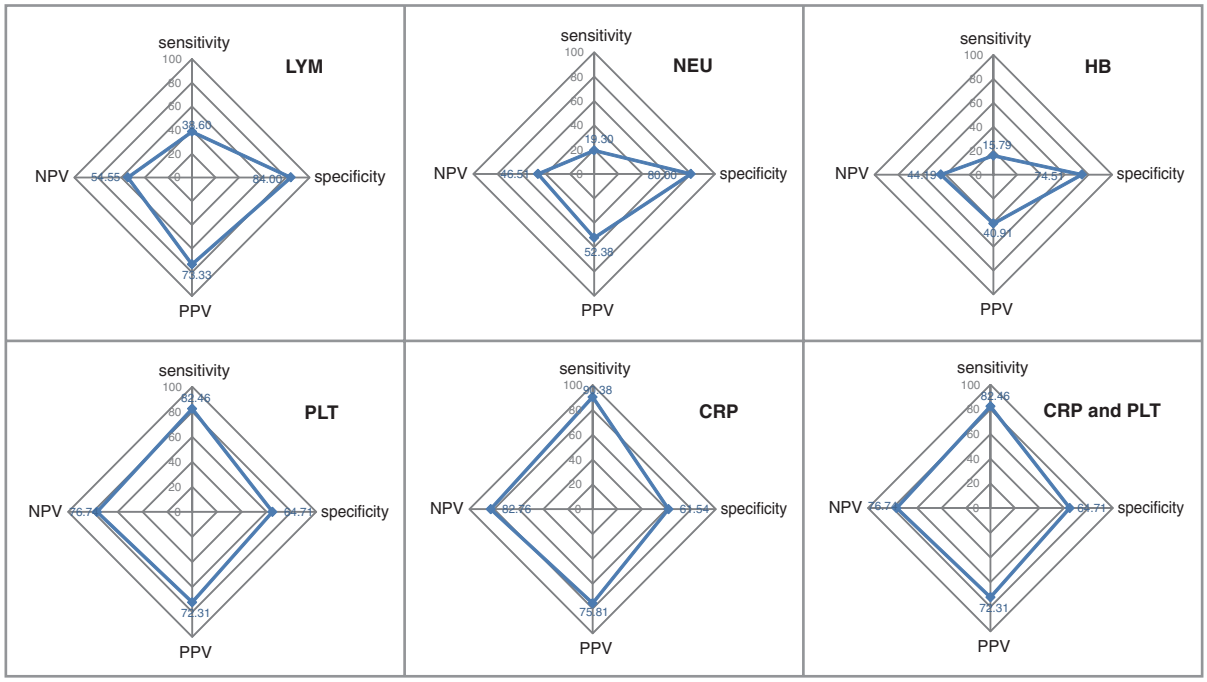

Figure 2 Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) calculated on single relevant parameters and in the combination of CRP and PLT.

Detection and Quantification of Parasitized Red Blood Cells.

Background

Flow cytometry has a high rate cell counting potential that is associated with low levels fluorescence detection system. This technology has been shown to be the most appropriate, robust and flexible to detect low concentration and size of parasitic forms in peripheral blood. Flow cytometry is a technology for individual, quantitative and qualitative characterization of particles suspended into a liquid and based upon the analysis of optical or other physical signal, emitted by a particle crossing the light beam of a laser. Particle characteristics such as structure, size and shape are given by the intrinsic optical properties of the particle. Identification of main cellular structures or functions needs specific chemical tags like fluorescent probes. Efficiency of flow cytometry for detection, characterization, and counting of malaria parasites, reported by Shapiro et al,[7] dates back to the 1970s. Acridine orange for example was used for staining in an apparatus with 488 nm laser sources, whereas Hoechst dyes,[8] which are more DNA-selective, were preferred where UV excitation was available. The study done by Grimberg et al.[9] described the results obtained by two fluorescent probes for the differentiation of parasitized red blood cells (Hoescht 33342) from other nucleic acid elements present in blood (Thiazole orange).

The fluorescent dye Thiazole Orange (TO) allows the suppression of the main interference susceptible to reduce infected blood cells determination. As TO is present into some HORIBA Medical reagents*, the hematology analyzer fluorescent technology already proper to HORIBA Medical could be integrated efficiently into a malaria diagnosis automatic procedure.

*(*ex.: Fluocyte ™ dedicated to reticulocyte determination of the HORIBA Medical Pentra DX120 Hematology system)*

Experiments

An experimental analytic device was developed for this application. It includes a specific optical bench (Figure 3) integrated into a HORIBA Medical ABX Pentra 60 analyzer. The preparation of the sample is direct and automatic, including dilution and treatment of the blood by fluorescent chemical reagents. The design incorporates a focus nozzle and three light sources. The first source, a Low Temporal Coherence (LED), sets the optical axis. Associated with this light source measurement is the measurement of the optical extinction. The detection is done in a light background. The other two sources, located at 90° to the optical axis of the source S1, are monochromatic (laser) at 375 nm and 488 nm respectively. They are associated with the FL1 and FL2 fluorescence measurements. A black background is used for detection. With such a device, four parameters are associated with

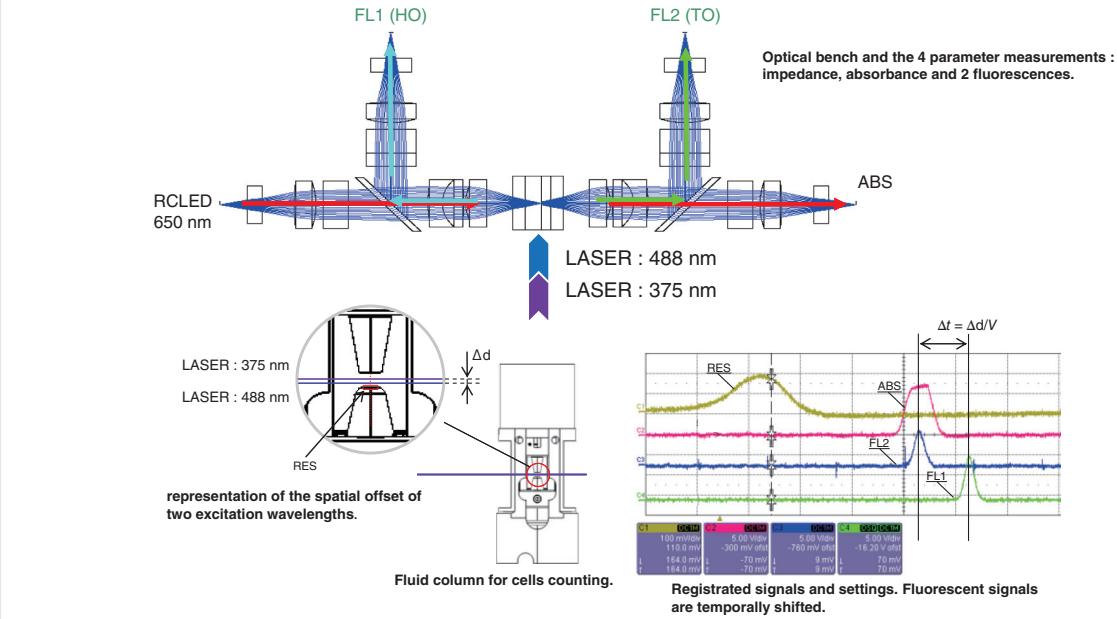

Figure 3 Optical Bench.

each red cell measured:

1- impedance measurement for a volumetric classification of each cell.
2- measure of the extinction at λ_{em}. 650 nm for cell refractive/diffractive effects.
3- measure of fluorescence FL1 at λ_{em}. 480 nm for the DNA parasite determination within red blood cells by the specific dye - Hoescht 33342 .
4- measure of fluorescence FL2 at λ_{em}. 530 nm for determination of reticulocytes (young red blood cells) by RNA content measurement.

Experiments were carried out with cultured infected red blood cells and human whole blood infected samples. Culture cells and human samples are provided by the research unit "Host-Parasite relationships, Pharmacology and Therapeutic" (UMR-MD3-Marseille).[10] The material used for analysis was a flow cytometer LSR type (from Becton Dikinson) equipped with argon laser (20 mW) and UV laser (Helium-Cadmium 8mW) and the prototype previously described. The flow cytometry method was optimized and adapted to the automatic analyzer constraints. Based on HORIBA Medical reagent specificity, the simultaneous use of the two fluorochromes TO and Hoescht-33342 (HO) was adapted to direct dilution and automation. Moreover, the initial incubation time of 75 minutes was drastically decreased to 30

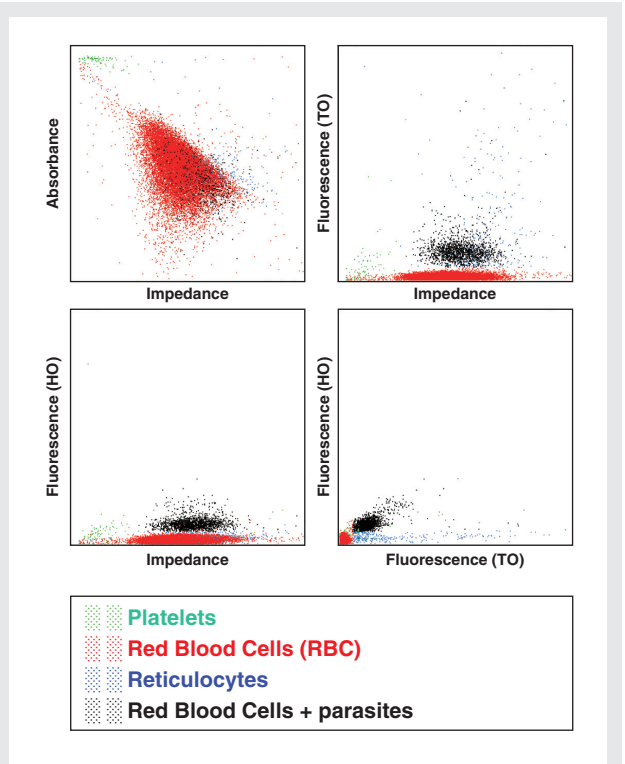

Figure 4 Determination of Parasitic DNA into Red Blood Cells by Four Parameter Analysis.
The 4 parameters are absorbance, impedance and two.

seconds, mainly thanks to the permeabilization control of parasite and red blood cell membranes. This analytic method allows the identification and quantification of parasitized red blood cells (Figure 4).

TO fluorescence (λ_{em} 530 nm) allows specific identification of RNA present in immature red blood cells (Reticulocytes). HO fluorescence (λ_{em}. 480 nm) allows specific identification of DNA present in parasites.

The results of the developed parasite staining methodology are compared to the flow-cytometric reference method (Grimberg et al.), as shown in Figure 5. The percentage of parasitized red blood cells is equivalently determinated with both methods ($r^2 = 0.99$, no bias). A detection threshold of 0.008% infected erythrocytes for this set of analysis has been demonstrated.
The determination of the parasitemia obtained with the prototype is presented in Figure 6. The automatic analysis results are compared to the results of the gold standard method, the microscopic smear examination. A good correlation was obtained between test and reference results with 28 infected human blood samples.

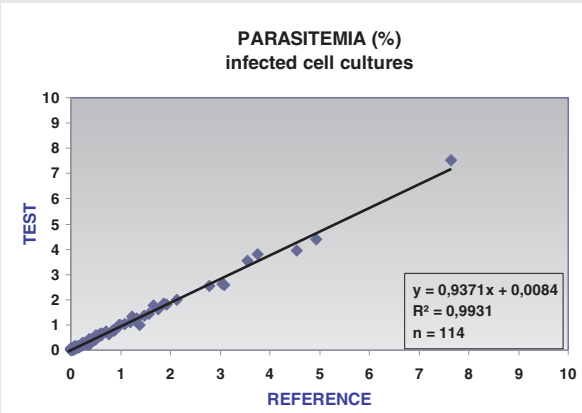

Figure 5 Accuracy Evaluation of the Fluorescent Method.
Samples of cultured infected red blood cells (n=114) were analysed by flow cytometry; the prototype method (TEST) and the flow cytometry reference method are compared.

Conclusion

The goal of this project was to define a strategy to upgrade the current instrument and obtain a dedicated device with good detection specificity of infected red blood cells to diagnose malaria. The fluorescent method presented here based on nucleic acid differentiation (DNA versus DNA/RNA) is rapid (30 sec./sample) and sensitive (~ 0.01% infected erythrocytes). In association with some CBC parameters, a specific and reliable diagnostic tool could be proposed and could participate to the malaria medical efforts.

Perspectives

The implementation of an automatic flag, in the Microsemi CRP, based on Platelets count and C-reactive protein level, would contribute to the improvement of detection capacity at local level, in agreement with the needs emphasized by the recent literature. Indeed this compact hematology analyzer provides first-line tests generally prescribed for patients presenting with fever. This makes the Microsemi CRP an affordable and robust device for malaria screening able to reach isolated and poor areas. The second solution, presented above, is the logic complement to the process of malaria infection diagnosis. This module, derived from the ABX Pentra 60, is highly sensitive but user-friendly as a routine hematology analyzer. It has the advantage of providing quantitative and reproducible results without the need of a well-trained technologist for microscopic examination and definitive malaria diagnosis and follow-up.
The solutions presented here are in line with the WHO recommendations that highlight the importance of using locally appropriate intervention for malaria prevention and case management to reduce adverse outcomes related to this infection.

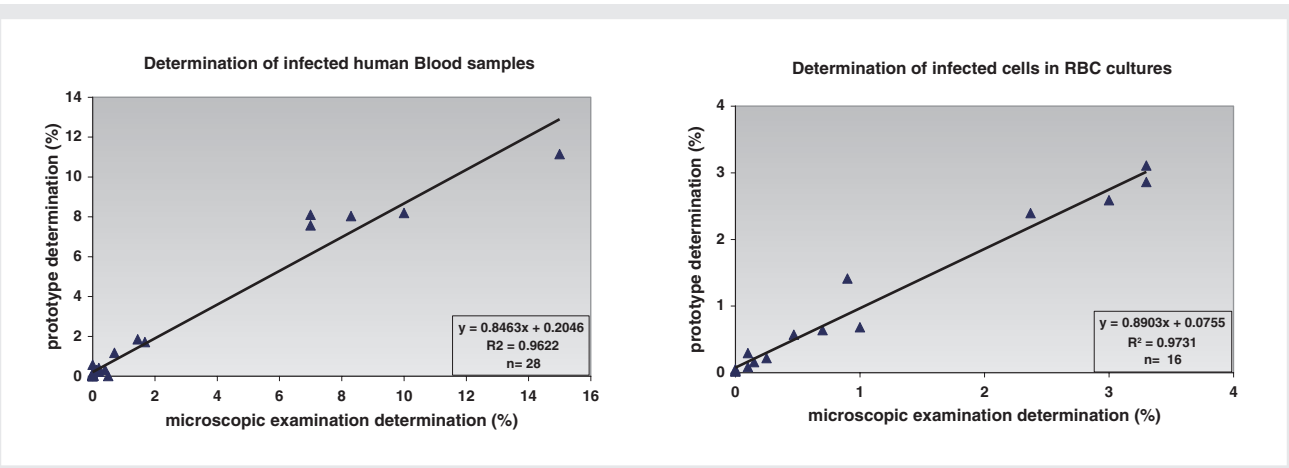

Figure 6 Determination of infected red blood cells (%) with the prototyped analytical device.
(left) Samples of malaria infected human whole blood - (right) Samples of malaria infected cultures of red blood cells.

References

[1] World malaria report: 2011. World Health Organization 2011. http://www.who.int/malaria/world_malaria_report_2011/9789241564403_eng.pdf

[2] Crawley J, Chu C, Mtove G, Nosten F. *Lancet*. 2010 Apr 24; 375(9724):1468-81.

[3] Trampuz A, Jereb M, Muzlovic I, Prabhu RM. *Crit Care*. 2003 Aug;7(4):315-23.

[4] Campuzano-Zuluaga et al. Malaria Journal 2010, 9:346.

[5] Schwake L, Streit JP, Edler L, Encke J, Stremmel W, Junghanss T. *Crit Care*. 2008; 12(1):R22.

[6] Conroy AL, Liles WC, Molyneux ME, Rogerson SJ, Kain KC. *PLoS One*. 2011; 6(12):e28540.

[7] Shapiro H.M., Mandy F. Cytometry Part A. 71A: 643_645, 2007.

[8] Howard RJ, Battye FL, Mitchell GF. *J Histochem Cytochem* 1979; 27 : 803–813.

[9] Grimberg BT, Erickson JJ, Sramkoski RM, Jacobberger JW., et al. *Cytometry A*. 2008; 73(6):546-54.

[10] Parzy D., Sinou V. Malaria research at the IMTSSA le Pharo. *Med. Trop*. 2001; 61(1):11-4.

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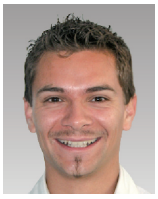

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