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TMek: A lab-on-chip diagnostic test for the malaria disease

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Summary. — The search for new rapid diagnostic tests is a priority in the fight against malaria, one of the most common life-threatening infectious diseases. In this paper, we present an easy to operate lab-on-chip platform that allows a pan-plasmodic, rapid and quantitative illness detection. This innovative system, named TMek, is based on the paramagnetic behavior of infected cells with respect to other blood components, thus allowing for a magnetophoretic separation in a high magnetic field gradient. The capability of our test to perform this selective detection has been tested by means of capture experiments on treated bovine red blood cells and suspensions of synthetic hemozoin crystals. Preliminary tests on patients with malaria were successfully done at the Sacco Hospital in Milano and the possibility to perform a follow up of the treatment has emerged. A study on the discrimination of the infection stages has been done on cultured red blood cells at Istituto Superiore di Sanitá in Roma. In April 2019, a preclinical validation has been carried out at Hôpital Saint Luc of Mbalmayo, in Cameroon, on 75 suspected malaria patients. Even though the number of samples was limited, this preliminary study indicates the on-field operability of TMek as a rapid diagnostic test for malaria.

1. – Introduction

Nowadays, according to the World Health Organization (WHO), malaria, caused by *Plasmodium* parasites transmitted to people through the bites of Anopheles mosquitoes, is one of the most common life-threatening infectious diseases and a global public health challenge. In 2019, around 229 million cases were registered with an estimated 409000 deaths worldwide. The majority of these, around 93%, occurred in the sub-Saharan Africa where the population exposed to the highest risk of death is composed by children

under 5 years of age, that are more susceptible to infection and illness because of their underdeveloped immune system, and by pregnant women since placental sequestration of the parasite can lead to maternal anaemia and to an increasing risk of death before and after childbirth [1]. It follows that great efforts are needed in prevention, aiming at avoiding the mosquito bites, and treatment, whose efficacy strongly depends on a correct usage in order to avoid drug resistance. Conventional methods for malaria diagnosis are not suitable for an accurate early stage and highly sensitive on-field detection of the infection and for a wide screening of the population. Optical microscopy examination, the time-honored Gold Standard for malaria, asks for an expert operator able to distinguish infected red blood cells (i-RBCs) in a blood smear using a good microscope. It is then an operator-dependent method, requiring about 60 minutes and a laboratory setting [2]. Rapid Diagnostic Tests (RDTs) based on immuno-assay lateral-flow devices require just 15–30 minutes but are not quantitative and suffer from a large number of false negative/positive results, especially in endemic zones [3]. Tests based on polymerase chain reaction (PCR) can detect extremely low parasite concentrations, but the on-field compatible version known as LAMP is not quantitative, has a high cost and long operation times (about 60 minutes) which limit the wide spreading of this technology [4]. To fill the gap between the current technology and the need for a cheap, fast and highly sensitive diagnostic tool suitable for wide screening in a low-resource setting, the World Health Organization itself strongly recommends the development of novel RTDs with the same sensitivity of microscopy but with a reduced number of false positives and false negatives with respect to currently available lateral-flow devices. In this paper, we present an innovative diagnostic test that provides fast yet sensitive and low-cost detection of malaria. It is based on the magnetophoretic separation and electrical counting of the i-RBCs. The system is called TMek, from "Tid Mekii", the name of "malaria" in the local language of Mbalmayo, the small village of Cameroon where the first preclinical validation of the test has been carried out.

2. – Materials and methods

TMek test is based on paramagnetic properties of malaria i-RBCs and Hemozoin Crystals (HCs) which allow their selective magnetophoretic separation from healthy ones, driven by an external magnetic field gradient [5]. The origin of RBC paramagnetism is connected to the degradation of hemoglobin into free heme during the *Plasmodium* parasite intra-erythrocytic development. This molecule, highly toxic to the parasite, is converted in an insoluble form, known as hemozoin or malaria pigment, paramagnetic nanocrystals found both within the i-RBCs and free in the blood, after RBCs lysis [6-16]. Noteworthy, the number of the hemozoincrystals in the RBCs depends on the stage of the parasite development: a lower amount of crystals in the ring stage with respect to the schizont stage is found [17].

The infected blood corpuscles, after separation, are concentrated at the surface of gold interdigitated electrodes thanks to magnetic concentrators placed in closed proximity whereas healthy ones sediment under the action of gravity (fig. 1). Then, a change in resistivity, proportional to the amount of attracted particles (i-RBCs and HCs) is detected as a current variation by an electronic circuit. Following a lab-on-chip approach, the test is based on a silicon microchip (fig. 2(a)) composed by an array of micrometric nickel pillars allowing the magnetophoretic separation and planar interdigitated gold electrodes for the counting of the captured corpuscles.

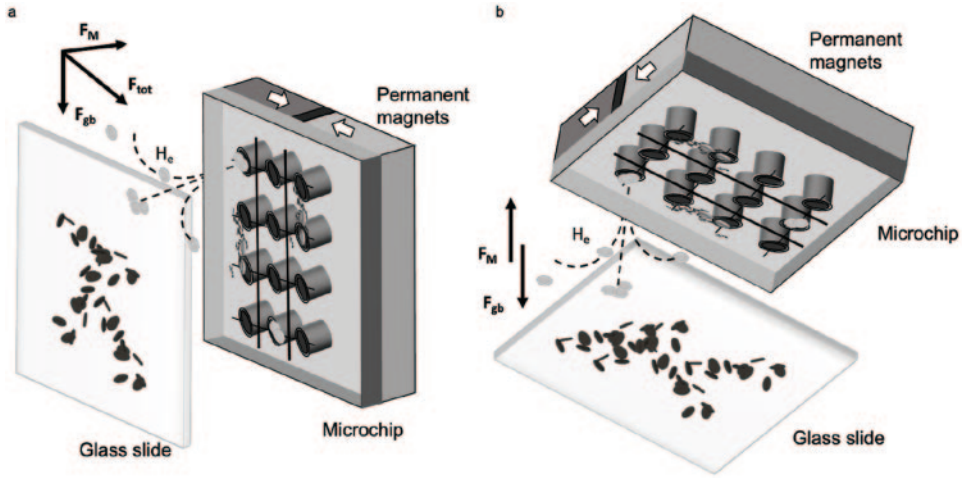

Fig. 1. – TMek schematic concept in vertical (a) and horizontal (b) configuration: i-RBCs (light grey) and HCs are attracted towards the chip, while healthy h-RBCs (dark) sediment.

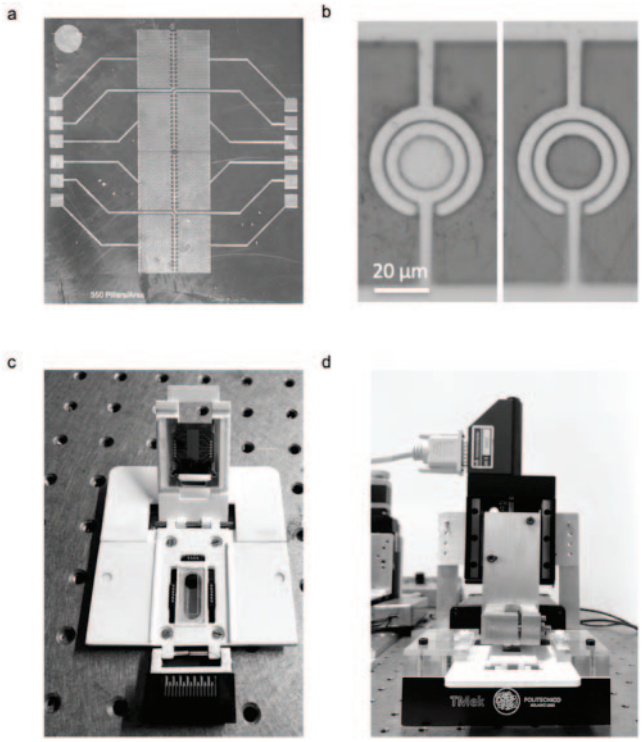

Fig. 2. – (a) Picture of the microchip with measurement (left) and reference electrode (right) areas [18]. (b) Top view of the interdigitated gold electrodes on top of magnetic concentrators (measurement) and without the Ni posts underneath (reference) [18, 19]. (c) Photo of the cartridge where the chip and the blood sample are loaded. When the cartridge is closed, and a perfect sealing is performed, it is inserted within the reader (d), such that the experiment can start.

An identical number of reference electrodes are placed nearby, in a region without magnetic concentrators underneath (fig. 2(b)). The blood sample to be analyzed is dispensed on a glass slide where a PDMS confinement gasket is prefabricated. The microchip covers the gasket with the electrodes in contact with the blood sample, and permanent magnets move in the proximity of the microchip enabling the magnetophoretic capture. In order to perform the experiments in the most reproducible and automated way possible, a mechanical setup (fig. 2(d)) has been designed: the chip and the glass slide with the gasket are placed in a cartridge (fig. 2(c)), while a motorized linear stepper motor allows the external magnets to approach the back surface of the chip in a controlled way. The measurement protocol is based on the magnets motion "downward" and "upward" in order to attract or release blood corpuscles. An electronic board, for the readout of the electrical signals, a portable power supply and a PC complete the setup configuration.

The capability of TMek to perform the selective detection of i-RBCs and HCs has been investigated by multiphysics simulations and tested by means of capture experiments on bovine RBCs treated with $NaNO_2$ to induce the full transformation of hemoglobin in methaemoglobin and mimicking the behavior of malaria-infected ones, and on suspensions of synthetic HCs. The magnetic force exerted on a particle immersed in a fluid can be expressed as

$$F_m = \frac{1}{2}\mu_0 V_p \Delta\chi \nabla H^2, \tag{1}$$

where μ_0 is the vacuum permeability, V_p is the particle volume, $\Delta\chi = \chi_{particle} - \chi_{fluid}$ is the difference between the magnetic susceptibility of the particle and the surrounding medium, and ∇H^2 represents the gradient of the magnetic field squared. Looking at F_m we understand that in order to attract a particle magnetophoretically what is important is not the χ value of the particle itself but rather its difference with respect to the surrounding medium. Table I reports the relative magnetic susceptibilities $\Delta\chi$ of i-RBCs at the various stages of development of the malaria parasite (ring, trophozoite, or schizont) that depend on their peculiar HC content, when the medium in which the blood particles are suspended is Phosphate-Buffered Saline (PBS) which has an absolute magnetic susceptibility of $\chi_{PBS} = -9.05 \cdot 10^{-6}$.

In the table, Met-Hb t-RBC represents red blood cells treated with $NaNO_2$, in which hemoglobin is fully oxidized into methemoglobin, giving rise to a relative magnetic susceptibility, two times that of the schizont stage. Due to the strong similarity between

TABLE I. – *Net magnetic susceptibilities of hemozoin and RBCs with respect to PBS.*

Corpuscle	$\Delta\chi(\cdot 10^{-6})$	Minimum ∇H^2 value (A^2/m^3)
healthy h-RBC [20, 21]	0.01	$1.56 \cdot 10^{17}$
ring i-RBC [21]	0.82	$1.9 \cdot 10^{15}$
trophozoite i-RBC [21]	0.91	$1.72 \cdot 10^{15}$
schizont i-RBC [14, 17, 20, 21]	1.82	$8.6 \cdot 10^{14}$
met-Hb t-RBC [20]	3.9	$4 \cdot 10^{14}$
hemozoin crystals HCs [5]	320	$2.26 \cdot 10^{13}$

i-RBCs and t-RBCs, the latter have been used in this paper to mimic i-RBCs behavior in experiments carried out to optimize the diagnostic test [20].

3. – Results and discussion

The test has been carried out in different configurations: a vertical configuration, with the chip surface perpendicular to the floor (fig. 1(a)) and a horizontal configuration with the chip surface parallel to the floor and facing downwards (fig. 1(b)). Since the field gradient provided by the external magnets is in the order of $\nabla H^2 = 7 \cdot 10^{14} \mathrm{A}^2 \cdot \mathrm{m}^{-3}$, thanks to the higher magnetic susceptibility of HC ($3.2 \cdot 10^{-4}$) with respect to i-RBCs ($1.8 \cdot 10^{-6}$) when the chip is operated in the horizontal configuration and the gravity force opposes the magnetic one, only HCs are detected. In the vertical configuration, instead, there is no threshold: the magnetic and gravity force are perpendicular and both HC and i-RBCs can be captured with concentrations down to, respectively, $5 \cdot 10^3$ HCs/μl and 10 iRBCs/μl in 10 minutes [19].

The sample preparation protocol suitable for validation only requires 8 μl of whole blood from patients taken with a venous or peripheral sampling and diluted in 72 μl of a heparin-PBS solution in order to avoid coagulation and RBCs aggregation and achieve a hematocrit around 4% which favors magnetophoretic capture (fig. 3(b)). Then, 70 μl of diluted blood are dispensed on the glass slide with a PDMS rectangular gasket precharged on the cartridge. Once the cell sealing is created, the cartridge is inserted in the reader and, using a stepper motor, the permanent magnets are moved in proximity to the chip. The measurement protocol is the one qualitatively represented in fig. 3(a) [18]. The first capture of corpuscles typically lasts 300 seconds, after which the cyclic movement of the magnets, taken far away and then repositioned close to the microchip, starts. The cycle is repeated twice in order to increase the reliability of the measurement.

As confirmation, under the TMek idea, in the first capture phase the current decreases as a result of the capture of the i-RBC between the electrodes, while the rise in the current signal at 300 s fully reflects the release of i-RBCs previously captured. Preliminary tests on patients with malaria were successfully performed at the Sacco Hospital in Milano and the possibility to perform a follow up of the treatment has emerged. In fig. 3(c), (d), (e) we report in particular the case of a patient affected by *Plasmodium vivax* during the illness evolution. TMek tests have been carried out on samples taken at the patient admission, 24 and 48 hours after the beginning of the treatment with chloroquine. Microscopy analysis indicates an initial parasitemia of 1%, which decreases to about 0.1% after 24 h and is negligible 48 h after beginning of the treatment. TMek signals show an initial amplitude $A1$ of 1 μA, gradually decreasing to zero at 48 h [18].

A study on cultured RBCs at different infection stages of *Plasmodium* (ring, trophozoite, gametocyte) has been done at Istituto Superiore di Sanitá in Roma (fig. 3(f), (g), (h)). The results suggest the possibility of a discrimination of the infection stage by analyzing the waveform of the impedimetric signal in response to the application/removal of the external magnetic field [18]. This could provide clinicians with a better evaluation of the infection, it could be crucial in monitoring the effects of treatment and it could be crucial in blocking the infection transmission associated to gametocytes.

In April 2019, a preclinical validation has been carried out at Hôpital Saint Luc of Mbalmayo, in Cameroon on 75 suspected malaria patients [18]. Using RDT Bioline in parallel limits and performances of the TMek device have been evaluated. Within the limits of this preliminary study, the lack of false negative results for TMek indicates a

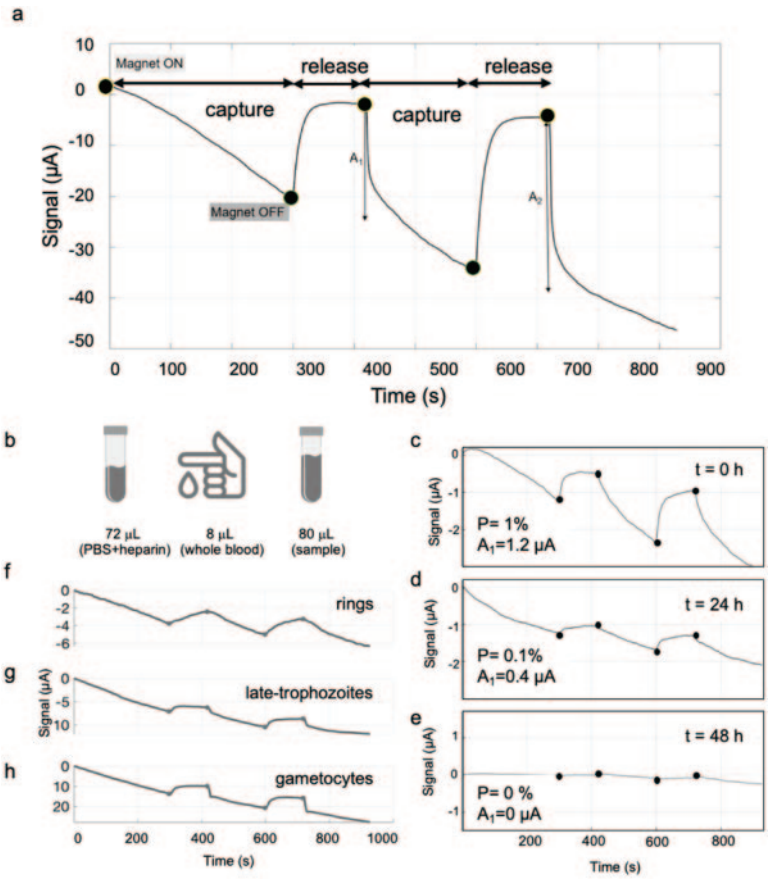

Fig. 3. – (a) Typical shapes of diseased patient current signal *vs.* time according to the measurement protocol: approach of the external magnets (Magnet ON) and corpuscles are captured, disengagement of magnets (Magnet OFF) and corpuscles are released. (b) Sample preparation protocol: 8 μl of whole blood diluted in a 72 μl heparin-phosphate buffered saline (PBS) solution. ((c), (d), (e)) TMek signal *vs.* time on a whole blood sample from a patient affected by *P. vivax*, at $t = 0$ h, 24 h and 48 h upon treatment with choloroquine. Starting from the top, $P = 1\%$, 0.1% and 0%, and the corresponding amplitudes $A1 = 1$ μA, 0.4 μA and 0 μA. ((f), (g), (h)) Waveforms measured on blood samples infected by cultured *P. falciparum* at different stages [18].

high sensitivity, 100% (93.3–100.0), both for venous and capillary samples. This means that the device did not miss any diseased patient, and this is crucial in order to avoid that a patient affected by malaria is not specifically treated with antimalarials. 9 false positive are found in venous samples, leading to a specificity of 69% (49.2–84.7) while no false positive results have been detected in case of samples from a finger prick, at least within the limited number of these samples (just 10 due to time limitations in the first prevalidation campaign). Even though the confidence intervals are very large, this preliminary study indicates the potential of TMek as quantitative, stage-selective, rapid diagnostic test for malaria.

4. – Conclusions

In this paper a new rapid diagnostic test for malaria diagnosis, based on the selective magnetophoretic capture and electrical impedance detection of malaria-infected red blood cells in less than 10 minutes with a limit of detection of 10 parasites/μl, which is the same of the gold standard for malaria diagnosis, has been presented. A preliminary study carried out at Sacco Hospital on three blood samples, drawn 0, 24 and 48 hours after the treatment from the same malaria-infected patient, demonstrated the possibility to use the quantitative nature of the measurement to also perform a follow up of the status of the disease before and after the treatment. Moreover, the current signal morphology allows to distinguish the different particles in the samples thus the stage of the disease which depends on the HC content. This feature has been demonstrated through experiments performed at Istituto Superiore di Sanitá in Rome on cultured red blood cells infected by different stages of *Plasmodium*. Finally, a preclinical validation campaign has been conducted on 75 patients at Hôpital Saint Luc of Mbalmayo in Cameroon, an African region where malaria is endemic. A sensitivity of 100% (confidence interval: 93.3–100.0) and specificity of 69% (confidence interval: 49.2–84.7) attested the potential of the TMek system to become a successful tool for malaria diagnosis.

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