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Note

Insufficient sensitivity of laser desorption-time of flight mass spectrometry-based detection of hemozoin for malaria screening

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ABSTRACT

Laser desorption-time of flight (LD-TOF) mass spectrometry-based detection of hemozoin was assessed for its performance characteristics as a rapid screening test for malaria. In spite of good specificity of > 95%, poor sensitivity of 80.2% for microscopically positive samples makes the easy-to-apply and rapid approach unsuitable for the routine diagnostic setting.

1. Short communication

Suspicion of malaria is an emergency situation. Therefore, diagnostic testing has to be performed without delay. Although staining of thick and thin blood films according with Giemsa (1904) is still the reference standard after > 100 years, experience with malaria microscopy is difficult to maintain in low prevalence settings where positive samples are scarcely available. Therefore, rapid and easy-to-apply diagnostic alternatives are desirable.

Laser desorption-time of flight (LD-TOF) mass spectrometry for the detection of hemozoin, which is sequestered by the malaria parasite during hemoglobin digestion (Sullivan, 2002), is such an alternative approach. The technique was first described in 2002 (Demirev et al., 2002) and a low detection limit of < 10 parasites per micro-liter blood was postulated. In a mouse model of malaria infection, LD-TOF-based detection of hemozoin even preceded the microscopic detection of parasites in blood of freshly infected animals by 2–4 days (Scholl et al., 2004). In comparison with PCR of samples from pregnant women with submicroscopic parasitemia, hemozoin screening using LD-TOF showed sensitivity of 52% and specificity of 92% (Nyunt et al., 2005). To assess the usefulness of the approach for the routine diagnostic setting, we adopted the technique to a widely-used benchtop mass spectrometer and conducted a prospective study with clinical samples.

The measurement protocol was established and optimized with *P. falciparum* strain Welsh, non-synchronically cultured in RPMI 1640 growth medium (1.58% RPMI 1640 (AppliChem, Darmstadt, Germany, A1538,9010), 12 mM $NaHCO_3$ (Sigma, Kawasaki, Japan, S5761), 6 mM D-glucose (Merck, Darmstadt, Germany 1.08342.100), 0,5% Albumax (Gibco / Thermo Fisher Scientific, Waltham, MA, USA, 11021-045), 0.2 mM hypoxanthine (Sigma, Kawasaki, Japan, H9636), 0.4 mM gentamicin (Ratiopharm, Ulm, Germany), adjusted to pH 7.2, sterile filtrated) applying the standard protocol (Trager and Jensen, 1976). Analytical sensitivity was confirmed with spiked blood samples (Demirev et al., 2002) (Fig. 1).

Clinical evaluation of LD-TOF was performed over a period of 14 months with 158 EDTA blood samples, sent for malaria testing by the University Medical Center Hamburg-Eppendorf, the Bernhard-Nocht Institute for Tropical Medicine Hamburg and various physicians and laboratories from all over Germany. Samples had been collected from 129 febrile (≥ 38.5 °C) patients (70% males and 30% female, aged 6 to 72 years) after stays in (sub-) tropical areas of endemicity in accordance with the recommendations by the German Society for Tropical Medicine and International Health (https://www.dtg.org/images/Leitlinien_DTG/Leitlinie_Malaria_2016.pdf, last accessed 15th March

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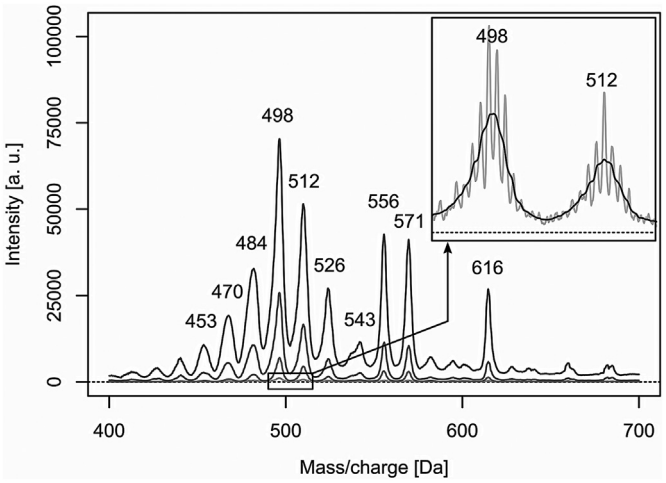

Fig. 1. LD mass spectra of *P. falciparum* from unsynchronized culture diluted in human blood to parasitemia values of 30, 3, 0.3 and 0.03% (dark to light grey). Peaks representing the heme molecular ion (*m/z* 616) and prominent fragment ions produced via consecutive cleavages of the molecule's two propionic acid side chains are labeled with their respective *m/z*-values. Insert: detailed view of raw (grey) and smoothed (black) spectra from the 0.03% parasitemia sample. a.u. = arbitrary units.

2019). Travel history was available for 39 patients returning from Ghana (16), Cameroon (4), Nigeria (3), Tanzania (3), Gambia (2), Pakistan (2), Togo (2), Guinea (1), Ivory Coast (1), Kongo (1), Mozambique (1), Niger (1), Senegal (1), and Uganda (1). Besides results from malaria testing, no additional information on the potential causes of disease was available for this study.

All samples were analyzed by microscopy of Giemsa-stained thick and thin blood films at the Bernhard Nocht Institute for Tropical Medicine Hamburg, the German National Reference Center for Tropical Pathogens. 145 of 158 samples (including 66 of 72 microscopically negative samples) were additionally tested by in-house SYBR-green real-time PCR for malaria on a Corbett Rotor-Gene 6000 (Qiagen, Hilden, Germany) after automated nucleic acid extraction using the EZ1 DNA Blood 200 µL kit (Qiagen) (Mangold et al., 2005; Frickmann et al., 2015; Hagen et al., 2015). For 13 out of 158 samples, no residual material for PCR was available. Volumes of 200 µL EDTA blood were subjected to nucleic acid extraction with 2 out of 50 µL of eluate used in the PCR reactions. For LD-TOF assessment, five microliter from each sample were deposited in one micro-liter aliquots on MSP microscout polished steel targets (Bruker Daltonics, Billerica, MA, USA) and air-dried at room temperature. Mass spectra were acquired on a microflexLT benchtop mass spectrometer (Bruker Daltonics) in linear positive mode with 20 kV extraction potential, 65% laser power and 20 ns delay. From each spot, traces from 3000 consecutive laser shots were aggregated into a sum spectrum, which was analyzed for the presence of characteristic heme signals (Fig. 1).

Based on microscopy, 86 samples tested positive, comprising 71 cases of *P. falciparum*, 6 cases of *P. vivax*, 2 cases of *P. malariae*, 1 case of *P. ovale*, 1 case which could not be identified by microscopy, two mixed infections with *P. falciparum* and *P. malariae* as well as 3 cases for which microscopic discrimination between *P. vivax* and *P. ovale* failed. In the latter three cases, PCR allowed confirmation as *P. vivax* in two out of three cases, while sufficient sample material for PCR was not available in the third instance. The sample with the non-identified *Plasmodium* species was positive for *P. falciparum* by PCR. No additional cases of *Plasmodium* infection were detected by PCR among microscopically negative samples. Detected parasitemia ranged from few parasites in the whole thick blood film to parasitemia up-to 10%.

In our hands, sensitivity of LD-TOF hemozoin detection was 80.2% (69/86) and specificity was 95.8% (69/72) as compared to microscopy.

Table 1
Comparison of LD-TOF analysis and parasitemia as assessed by microscopy.

Microscopy	LD-TOF mass spectrometry		Parasitemia	LD-TOF positive
	Positive	Negative		
Negative	3[a]	69	NA	3/72 (4.2%)
Positive	69	17	< 0.01%	5/14 (35.7%)
			0.01–0.1%	16/18 (88.9%)
			0.1–1%	35/39 (89.7%)
			> 1%	13/15 (86.7%)

NA = not applicable.
[a] Clinical history suggested cases of successfully cured malaria.

Focusing on parasitemia, sensitivity was 35.7% (5/14) in samples with parasitemia < 0.01% and remained stable between 85% and 90% in case of higher parasitemia (Table 1). Regarding the three apparently false positive results, PCR was negative in two out of three instances while sufficient sample material for PCR was not available in the third instance. Clinical history, however, indicated that the blood samples were from patients with successfully treated malaria, comprising two samples with a mixed infection of *P. falciparum* and *P. malariae* as well as a sample from a patient with *P. falciparum*. Assuming that residual hemozoin was still circulating in the blood stream of these patients, specificity was increased to 100%.

Although our simple protocol was capable of detecting parasitemia below 0.01% in *Plasmodium* cultures and individual patient samples, the achieved analytical sensitivity could not be translated into acceptable accuracy in a diagnostic setting. While detection rates in low parasitemia samples could probably be addressed by sampling larger volumes of blood, false negative results observed in eight samples with high *P. falciparum* parasitemia is bothersome. As we did not observe significant differences regarding duration of illness (Mann-Whitney $U = 75.5$, $p = .63$) or the fraction of patients on antimalarial treatment ($\chi 2 = 0.073$, $p = .79$) as compared to cases with true positive tests, our results strengthen concerns about inherent shortcomings of hemozoin-based malaria detection. Delahunt et al. observed that hemozoin content of early ring stages was insufficient for parasite detection by dark-field microscopy which led to false negative results in five of ten field samples with high parasitemia (Delahunt et al., 2014). Reliable detection of tightly synchronized infections would therefore require multiple samples from different time-points over the parasite's life-cycle or a much higher number of investigated sample spots to allow for the detection of scarce hemozoin-rich gametocytes. The observed lack of overall sensitivity currently precludes the use of LD-TOF as a single diagnostic test to rule out malaria. Although the procedure showed excellent specificity in our patient population, potential confounders like molecular remnants of previous infections or hemozoin from other blood-inhabiting hematophagous parasites like *Shistosoma* spp. (Egan, 2008; Lvova et al., 2016; Xiao and Sun, 2017) would have to be addressed in endemic settings.

Even if sensitivity issues could be resolved, LD-TOF would face tough competition by well-established immunochromatographic rapid diagnostic tests (World Health Organization, 2018) and a plethora of molecular point-of-care test systems which are already commercially available or under investigation. In a recent assessment, we have described good performance characteristics and – in particular – excellent negative predictive value of a LAMP-based screening approach for *Plasmodium* spp. without sophisticated nucleic acid extraction (Frickmann et al., 2018). Unlike hemozoin based detection, automated molecular assays can also be designed to discriminate *P. falciparum* from *P. vivax/ovale* (https://clinicaltrials.gov/ct2/show/NCT02968355, last accessed 29th February 2018), offering additional diagnostic value.

Aside from individual patient testing, LD-TOF could be an alternative to nucleic acid amplification for high throughput analyses in

epidemiological studies or entomological surveys (Laroche et al., 2017) given its good specificity and very low costs per sample. As demonstrated by Rogan and Gladen (1978), diagnostic accuracy adjusted methods are useful to calculate prevalence and incidence in point-prevalence and surveillance assessments even in case of limited diagnostic sensitivity and specificity of the applied test.

In summary, LD-TOF based hemozoin detection provided high specificity and short turnaround times for the screening for malaria. However, due to relevant concerns regarding sensitivity, the technique cannot be recommended for the diagnosis or exclusion of malaria in individual patients.

Competing interests

The authors have declared that no competing interests exist.

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