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METHODOLOGY

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Enhancing safety of rapid and accurate malaria diagnosis in patients at risk for viral haemorrhagic fever

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Abstract

Background Malaria and viral haemorrhagic fever (VHF) have overlapping symptomatology, creating clinical and diagnostic challenges within, and in travellers returning from, co-endemic areas. Rapid and accurate malaria diagnosis is critical, yet complicated by safety measures required for VHF.

Methods Diagnostic accuracies of the rapid Alethia Malaria loop-mediated isothermal amplification (LAMP) assay and EasyNAT Malaria cross-primer assay (CPA) were assessed for the detection of malaria in whole blood mixed with guanidine isothiocyanate (GITC)-based virucidal DNA/RNA Shield buffer.

Results Both the LAMP assay and the CPA performed excellent and detected DNA of *Plasmodium* spp. in all 47 mixtures. All mixtures without *Plasmodium* spp. DNA (n = 5) tested negative in both assays. Technical detection limit of both assays for *P. falciparum* was between 0.005 and 0.0005 parasites per microlitre of mixture.

Conclusions Rapid and accurate malaria diagnosis remains possible on whole blood mixed with this virucidal buffer. The buffer is available in blood collection tubes, which allows for enhanced safety of sample handling from bedside to bench. These tubes can be used in patients with high suspicion of malaria, but in whom VHF cannot be completely excluded.

Keywords Malaria, Viral haemorrhagic fever, Rapid testing, LAMP, CPA

Background

Malaria is a febrile illness caused by protozoans from the genus *Plasmodium* causing about 600,000 deaths annually [1]. In some regions, mostly in sub-Saharan Africa, endemic malaria coexists with viral haemorrhagic fever (VHF) viruses, such as Ebola, Lassa and Marburg [2, 3]. Patients from those regions, and those returning from travel there, can present with symptoms that overlap between malaria and VHF, thus posing a diagnostic challenge [4]. The likelihood of malaria in these patients is substantially higher than that of VHF, indicating the need for prompt malaria diagnosis to prevent morbidity and mortality [4]. However, VHF suspicion requires stringent safety measures, including sample handling in at least a biosafety level (BSL) 3 facility, which complicates and delays malaria diagnosis.

For rapid malaria diagnosis, rapid nucleic-acid amplification tests (NAATs), such as loop-mediated isothermal amplification (LAMP) assays [5, 6] and

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cross-primer assays (CPA) [7], are available. These assays have low detection limits around or even below one parasite per microlitre of whole blood for all *Plasmodium* spp., rendering excellent clinical performance [5–7]. Lysis of whole blood is an essential first step for NAATs, allowing the use of guanidine isothiocyanate (GITC)-based lysis buffers with the added benefit of inactivating potential infectious agents [8, 9]. This approach could allow safe malaria diagnostics in BSL-2 facilities, instead of higher-containment BSL-3 facilities in situations when VHF cannot be ruled out.

DNA/RNA Shield (Zymo Research, Irvine, CA, USA) is a GITC-based buffer designed for stabilization of nucleic acids, as well as pathogen inactivation. Durable stabilization of *Plasmodium* DNA was shown in an earlier study, in which it remained detectable after prolonged storage at various conditions [10]. The virucidal effect of the buffer was confirmed by the company for ebolavirus and other viruses (unpublished datasheets available upon request from the company). A published report shows virucidal effect for robust murine parvovirus within various sample types, including whole blood, after 30 min of incubation at room temperature [11]. The buffer is available in tubes for bedside blood collection, which allows safe transport to a BSL-2 facility for rapid malaria diagnostics. For this study, diagnostic accuracies of a LAMP assay and a CPA were assessed after mixture of whole blood with buffer.

Methods

A total of 47 residual EDTA whole blood samples of individual patients with microscopically confirmed malaria, and five samples of individual patients without malaria, were included in the study. All samples had been drawn for malaria diagnostics from patients with suspected malaria after travelling between 2017 and 2023. The samples had been cryopreserved at −80℃ since the initial diagnostic phase.

Detection and determination of asexual stages of malaria had been achieved during the initial diagnostic phase with a combination of fluorescent microscopy on a quantitative buffy coat assay and light microscopy on smears prepared from the whole blood samples. Parasite densities for infections with *Plasmodium falciparum* had been assessed twice during the initial diagnostic phase with light microscopy on smears of each patient, using standardized approaches based on guidelines from the British Society of Hematology and the World Health Organization [12, 13]. For the current study, *P. falciparum* densities were confirmed on the same smears by an experienced technician, who was blinded for the initial results. Parasite densities for infections with non-*falciparum* spp. were assessed twice for the study by the same technician. In case of discrepancies between assessments, smears were reassessed a final time and the result of this reassessment was used for this study. For interconversion between percentages of infected red blood cells and number of parasites per microlitre, red and white blood cell counts were assumed to be 5.0×10^6 and 8.6×10^3 per microlitre of whole blood, respectively, based on median counts in patients with malaria.

The tubes for bedside blood collection contain 6 millilitre of DNA/RNA Shield buffer. In the clinical setting, a maximum of 3 millilitre of whole blood is drawn into these tubes via venipuncture achieving an ideal 2:1 mixture of buffer and whole blood. Smaller volumes of stored whole blood were available of each sample. Thus, for this study, two volumes of buffer were mixed with one volume of whole blood of each sample to mimic concentrations that would ideally be present in a full collection tube after a clinical venipuncture. The mixtures were then kept at room temperature before further handling in a laminar flow cabinet in a BSL-2 facility. After 30 min, the Alethia Malaria LAMP assay (Meridian Bioscience, OH, USA) and the EasyNAT Malaria CPA (UStar Biotechnologies, Hangzhou, China) were performed with the mixtures according to the manufacturer's instructions.

For the LAMP assay, 50 µl of mixture is used within an assay-specific tube-extraction method based on sodium hydroxide, followed by a tube-purification step. The extracted DNA is then transferred to a cartridge, containing two chambers that contain primers for detection of either *Plasmodium* or human mitochondrial DNA, respectively. The cartridge is then placed in an assay-specific reader for detection of turbulence. For the CPA, 50 µl of mixture was placed in the top chamber of a cartridge and mixed with assay-specific extraction solution containing guanidine salts and beads that bind DNA. The cartridge is then placed in the assay-specific analyser, in which DNA extraction and purification take place. Ultimately, in the lowest chamber of the cartridge *Plasmodium* and human β-actin DNA amplification renders fluorescence. Both the LAMP assay and the CPA are easy to perform and results can be reported within one hour. The assay-specific analysers report qualitative results for presence of *Plasmodium* spp. (i.e., 'negative' or 'positive', or 'invalid' due to technical issues or when the human control is not detected), yet do not differentiate between species.

To assess technical detection limits of the LAMP assay and CPA in mixtures, a whole blood sample of a patient with *P. falciparum* malaria and known parasite density of 5% infected red blood cells was diluted with uninfected blood, also taking into account additional dilution due to buffers used during the LAMP assay or CPA procedures.

Each dilution was then mixed with DNA/RNA Shield buffer at a 2:1 ratio of buffer and whole blood, resulting in final dilutions containing 5, 0.5, 0.05, 0.005 and 0.0005 parasites per microlitre of mixture, respectively. These mixtures were incubated at room temperature for 30 min and then tested with the LAMP assay and the CPA. Detection limits for non-*falciparum* spp. were not assessed, because earlier studies have shown that these do not vary significantly between different *Plasmodium* spp. [5–7, 10].

Results

Results are shown in Table 1. All *Plasmodium*-positive mixtures (n = 47) tested positive in both the LAMP assay and the CPA. All five mixtures without *Plasmodium* DNA tested negative in both assays. The dilution series revealed technical detection limits between 0.005 and 0.0005 parasites per microliter of mixture for both the LAMP assay and the CPA (Table 2).

Discussion

Performance of both the LAMP assay and the CPA in detecting DNA of all *Plasmodium* spp. in the mixtures was excellent. Although the tested cohort for this study was relatively small, it consisted of clinically representative samples containing all four major *Plasmodium* spp. with low to high parasite densities. Technical detection limits of the LAMP assay and CPA for *P. falciparum* were far below one parasite per microliter of mixture, which is even further below clinical parasite densities for all *Plasmodium* spp. in this set of samples. These detection limits are in line with low detection limits in whole blood found in earlier validation studies of 0.075 and 0.04 parasites per microlitre for the LAMP assay and CPA, respectively [6, 7]. In addition, in an earlier study, whole blood stored under various conditions and durations in DNA/RNA Shield buffer still resulted in *P. falciparum* and *Plasmodium vivax* DNA detection at a limit as low as 25 parasites per millilitre [10]. Here, it should be emphasized that the performance of the LAMP assay and the CPA in relation to higher dilutions of whole blood with buffer was not assessed extensively. This could be relevant when suboptimal venipuncture results in a smaller volume of blood in the collection tubes. In those cases, negative results should be interpreted with caution.

Both the Alethia Malaria LAMP assay and EasyNAT CPA have been validated with prospectively collected whole blood samples without buffer [14]. Sensitivity of both assays was 100%, while specificity was lower at 98.3% and 97.4% for the LAMP assay and CPA, respectively, due to several false-positive results [14]. Other validation studies also reported occasional false-positive results of the LAMP assay [5, 6]. This was not expected to be different for the current study and, therefore, only a small number of five EDTA whole blood samples of patients without malaria were included. Even though these samples all tested negative, it should be stressed that false-positive results can occur posing a small, yet acceptable, risk in clinical practice. Moreover, it should be emphasized that results of this study may not be directly transferable to other rapid nucleic-acid amplification tests (NAATs) as validation and protocol development will depend on the context of the setting. For example, in malaria-endemic settings, the positive predictive value for malaria of rapid NAATs may be lower due to the presence of *Plasmodium* spp. DNA in asymptomatic individuals. In such settings, results should be interpreted with caution, especially as malaria and VHF may occur simultaneously in febrile patients [3].

Collecting blood directly into virucidal buffer is a highly practical approach as it enhances safety of sample handling from bedside to bench, while maintaining speed and accuracy of malaria diagnostics. Therefore, in the non-endemic setting, the use of this buffer in blood collection tubes could be introduced in a protocol for returning travellers with high suspicion of malaria, yet in whom VHF cannot be completely excluded based on aspects of travel history and exposures, clinical presentation, or physical examination during their stay in the Emergency Room. Rapid assessment of presence of malaria in these patients is critical. Given the reported virucidal activity for ebolavirus of other GITC-based lysis buffer [8] and the reported virucidal activity of DNA/RNA Shield buffer for robust non-enveloped murine parvovirus [11], safety of sample transport and handling is enhanced and allows the biosafety risk to be reduced to BSL-2. However, other studies have shown that virucidal activity of certain GITC-based buffers (e.g., buffer AVL, RLT or TCL) may be incomplete and may require additional steps such as longer incubation, heating or ethanol treatment [9, 15, 16]. For these buffers, additional inactivation protocols and sample handing in BSL-3 facilities may still be rational in case of a reasonable suspicion of VHF.

Conclusion

Both the Alethia Malaria LAMP assay and the EasyNAT Malaria CPA performed excellent in mixtures of whole blood with DNA/RNA shield buffer. This buffer in blood collection tubes can be used for rapid and accurate malaria diagnosis in patients in whom VHF cannot be completely excluded. This relatively simple approach renders enhanced safety of sample handling from bedside to bench.

Table 1 Results of rapid nucleic acid detection in mixtures of whole blood of patients with DNA/RNA Shield buffer

Sample	*Plasmodium* spp.[a]	Parasite density[b]		Alethia Malaria LAMP assay	EasyNAT Malaria CPA
		Parasites/µL	%		
1	*P. falciparum*	172	0.003	+	+
2	*P. falciparum*	172	0.003	+	+
3	*P. falciparum*	430	0.01	+	+
4	*P. falciparum*	430	0.01	+	+
5	*P. falciparum*	774	0.02	+	+
6	*P. falciparum*	1462	0.03	+	+
7	*P. falciparum*	2236	0.05	+	+
8	*P. falciparum*	6450	0.1	+	+
9	*P. falciparum*	7826	0.2	+	+
10	*P. falciparum*	10,234	0.2	+	+
11	*P. falciparum*	15,480	0.3	+	+
12	*P. falciparum*	17,630	0.4	+	+
13	*P. falciparum*	20,038	0.4	+	+
14	*P. falciparum*	22,274	0.5	+	+
15	*P. falciparum*	35,000	0.7	+	+
16	*P. falciparum*	40,000	0.8	+	+
17	*P. falciparum*	65,000	1.3	+	+
18	*P. falciparum*	125,000	2.5	+	+
19	*P. falciparum*	125,000	2.5	+	+
20	*P. falciparum*	170,000	3.4	+	+
21	*P. falciparum*	270,000	5.4	+	+
22	*P. falciparum*	325,000	6.5	+	+
23	*P. falciparum*	470,000	9.4	+	+
24	*P. falciparum*	485,000	9.7	+	+
25	*P. falciparum*	780,000	15.6	+	+
26	*P. falciparum*	1,095,000	21.9	+	+
27	*P. vivax*	86	0.002	+	+
28	*P. vivax*	258	0.005	+	+
29	*P. vivax*	860	0.02	+	+
30	*P. vivax*	1462	0.03	+	+
31	*P. vivax*	9030	0.2	+	+
32	*P. vivax*	17,028	0.3	+	+
33	*P. vivax*	37,066	0.7	+	+
34	*P. vivax*	Unknown[c]	Unknown[c]	+	+
35	*P. ovale*	129	0.003	+	+
36	*P. ovale*	516	0.01	+	+
37	*P. ovale*	7396	0.2	+	+
38	*P. ovale*	7654	0.2	+	+
39	*P. ovale*	15,652	0.3	+	+
40	*P. ovale*	15,738	0.3	+	+
41	*P. ovale*	16,340	0.3	+	+
42	*P. ovale*	Unknown[c]	Unknown[c]	+	+
43	*P. malariae*	65	0.001	+	+
44	*P. malariae*	86	0.002	+	+
45	*P. malariae*	1892	0.04	+	+
46	*P. malariae*	6020	0.1	+	+
47	*P. malariae*	7310	0.1	+	+
48	No malaria	NA	NA	–	–

Table 1 (continued)

Sample	*Plasmodium* spp.[a]	Parasite density[b]		Alethia Malaria LAMP assay	EasyNAT Malaria CPA
		Parasites/µL	%		
49	No malaria	NA	NA	–	–
50	No malaria	NA	NA	–	–
51	No malaria	NA	NA	–	–
52	No malaria	NA	NA	–	–

LAMP loop-mediated isothermal amplification, *CPA* cross-primer assay, *NA* not applicable

[a] Detected and determined with fluorescent microscopy on a quantitative buffy coat assay, and light microscopy on thick smear and thin smears

[b] Determined on Fields-stained thick and Giemsa-stained thin smears

[c] Unknown: smears for microscopy no longer available; samples were confirmed to contain *P. vivax* or *P. ovale* DNA using real-time PCR [17]

Table 2 Technical detection limits of the Alethia Malaria LAMP assay and the EasyNAT Malaria CPA in mixtures of patient whole blood with *Plasmodium falciparum* and DNA/RNA Shield buffer

Parasite density[a] (Parasites/µL)	Results	
	Alethia Malaria LAMP assay	EasyNAT Malaria CPA
5	+	+
0.5	+	+
0.05	+	+
0.005	+	+
0.0005	–	–
0.00005	–	–

LAMP loop-mediated isothermal amplification, *CPA* cross-primer assay

[a] In whole blood of a patient with *P. falciparum* malaria diluted with uninfected blood and mixed with DNA/RNA Shield buffer

Abbreviations

VHF Viral hemorrhagic fever
NAAT Nucleic-acid amplification test
LAMP Loop-mediated isothermal amplification
CPA Cross-primer assay

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Author contributions
RZ and MW conceptualized and designed the study, interpreted the data, and drafted the manuscript. KT, EWB and JF performed the testing, acquired the data and performed initial analyses. JF, SM, SJ, JS and GB contributed to the design of the study and interpretation of the data. All authors have substantially contributed to revisions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The Medical Ethical Review Committee of Amsterdam University Medical Center waived the need for informed consent (2024.0389) for the use of anonymized samples.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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