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Evaluation of Ebola Virus Inactivation Procedures for *Plasmodium falciparum* Malaria Diagnostics

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***Plasmodium falciparum* malaria is highly endemic in the three most affected countries in the current epidemic of Ebola virus disease (EVD) in West Africa. As EVD and malaria are clinically indistinguishable, both remain part of the differential diagnosis of ill travelers from returning from areas of EVD transmission. We compared the performances of a rapid diagnostic test (BinaxNOW) and real-time PCR with *P. falciparum*-positive specimens before and after heat and Triton X-100 inactivation, and we documented no loss of sensitivity.**

The current outbreak of Ebola virus disease (EVD) in West Africa is the largest on record (1), and guidance has emerged in hospital and outpatient settings around screening for EVD in ill travelers who have returned from areas of EVD transmission (2, 3). *Plasmodium falciparum* malaria is highly endemic in the three most affected countries, Sierra Leone, Liberia, and Guinea, which contribute >3 million cases annually to the global burden of malaria (4). Malaria can cause severe morbidity and mortality if diagnosis and/or treatment are delayed (5); yet, hospital laboratories are often ill-prepared to handle specimens from an individual suspected to have a filoviral infection (6). Laboratory safety must be balanced against the timely exclusion of malaria in ill travelers who have returned from EVD-affected regions in order to avoid adverse outcomes in patients. The Centers for Disease Control and Prevention (CDC) has recommended the addition of Triton X-100 and heat inactivation at 56°C prior to testing specimens from patients suspected to have filoviral infection, in addition to performing enhanced safety procedures, such as using personal protective equipment (PPE) and a certified class II biosafety cabinet (BSC) (7, 8). The inactivation of blood prior to the preparation of malaria thin smears is unnecessary, as filoviruses are inherently susceptible to methanol (7, 9), the solvent in which malaria thin smears are fixed prior to staining. However, rapid diagnostic tests (RDT) for malaria are widely and routinely used in hematology and microbiology laboratories and have no corresponding fixation step. Similarly, the extraction of nucleic acid for molecular diagnostic assays is presumed to inactivate filoviruses, although data on this are lacking.

The available data on the effects of filoviral inactivation procedures on the performance characteristics of malaria diagnostic assays are limited and variable (10, 11). We sought to evaluate the performance characteristics of both RDT and quantitative real-time PCR for detecting *P. falciparum* malaria following Triton X-100 and heat inactivation compared to those using the standard operating procedure. We documented no loss of sensitivity for either assay when performing filoviral inactivation procedures.

Thirty-one *P. falciparum*-containing whole blood EDTA specimens, with parasitemia levels ranging from <0.1% to 6.6% and confirmed by microscopy and RDT (BinaxNOW Malaria, Alere, ME), were included, along with 10 negative-control EDTA specimens. Nineteen specimens were obtained from our malaria biobank containing anonymized specimens stored at −80°C, and 22 were fresh EDTA blood samples stored at 4°C within 1 week of routine malaria diagnostics performed by our clinical parasitology department.

We performed a filovirus inactivation procedure according to the CDC's guidance for managing patients with suspected viral hemorrhagic fever in U.S. hospitals (7). EDTA whole blood was incubated in a 57°C heat block for 1 h, followed by the addition of 10 μl of 10% Triton X-100 (catalog no. X100; Sigma-Aldrich) per 1 ml of blood (final concentration, 0.1%), which was further incubated at room temperature for 1 h.

A rapid diagnostic test (RDT) was conducted with 15 μl of whole blood before and after the inactivation procedure, according to the manufacturer's instructions (BinaxNOW Malaria, Alere, ME). The T1 band of the BinaxNOW RDT detects the presence of histidine-rich protein 2 (HRP2), which is specific for *P. falciparum*, and the T2 band detects pan-*Plasmodium* aldolase, which can be found in all four species of human malaria. The expert medical laboratory technologists who read the RDT were blinded to inactivation and positivity statuses.

DNA was extracted with 200 μl of whole blood before and after the inactivation procedure using the DNA minikit blood or body fluid spin protocol (Qiagen, Germantown, MD). DNA was eluted with 60 μl of AE buffer (10 mM Tris-Cl, 0.5 mM EDTA [pH 9.0]) and stored at −20°C prior to use.

Plasmodium-specific real-time PCR targeting the 18S rRNA gene (quantitative PCR [qPCR]) was performed, as previously described (12, 13), using the ABI 7900HT real-time PCR system and under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and then 60°C for 1 min. We used 12.5 μl of TaqMan Universal PCR master mix (Life

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TABLE 1 BinaxNOW and qPCR results of 31 *P. falciparum* and 10 negative samples with and without the filovirus inactivation procedure

			Control (preinactivation)						With inactivation							
			BinaxNOW (RDT) detecting band:		qPCR				BinaxNOW (RDT) detecting band:		qPCR					
Sample	Source	Parasitemia level (%)	T1	T2	Mean log DNA	SD	%CV	Mean DNA copy no.	T1	T2	Mean log DNA	SD	%CV	Mean DNA copy no.	Log difference (inactivation − control)	% copy no. of inactivated/ control
1	Fresh	<0.1	+	−	4.4565	0.0914	2.05	28,609	+	−	4.4037	0.0952	2.16	25,335	−0.0528	89
2	Fresh	<0.1	+	−	4.1750	0.0549	1.32	14,962	+	+	4.1486	0.0806	1.94	14,080	−0.0264	94
3	Fresh	<0.1	+	−	3.5065	0.0457	1.30	3,210	+	−	3.5416	0.0305	0.86	3,480	0.0352	108
4	Fresh	<0.1	+	+	3.0578	0.1056	3.45	1,142	+	−	3.0138	0.0762	2.53	1,032	−0.0440	90
5	Fresh	<0.1	+	−	3.6472	0.0806	2.21	4,438	+	−	3.5460	0.0187	0.53	3,516	−0.1012	79
6	Fresh	<0.1	+	−	4.8189	0.0262	0.54	65,905	−	−	4.8102	0.0151	0.31	64,593	−0.0087	98
7	Fresh	<0.1	+	−	4.7665	0.0454	0.95	58,413	+	−	4.7316	0.0303	0.64	53,898	−0.0349	92
8	Frozen	<0.1	+	+	5.4301	0.0791	1.46	269,220	+	+	5.8682	0.0570	0.97	738,325	0.4381	274
9	Frozen	<0.1	+	+	4.4534	0.0548	1.23	28,407	+	−	4.4991	0.0158	0.35	31,554	0.0456	111
10	Frozen	<0.1	+	−	4.4808	0.0987	2.20	30,255	+	−	3.9970	0.0316	0.79	9,932	−0.4838	33
11	Frozen	<0.1	+	+	4.8824	0.0158	0.32	76,284	+	+	4.8459	0.0570	1.18	70,133	−0.0365	92
12	Frozen	<0.1	+	−	4.4258	0.0524	1.18	26,659	+	−	4.2511	0.0605	1.42	17,829	−0.1747	67
13	Frozen	<0.1	+	+	4.6267	0.0151	0.33	42,340	+	−	4.6617	0.0000	0.00	45,887	0.0349	108
14	Frozen	<0.1	+	+	5.2994	0.0659	1.24	199,229	+	−	5.3518	0.0303	0.57	224,784	0.0524	113
15	Frozen	<0.1	+	−	4.7228	0.0546	1.16	52,824	+	−	4.6704	0.0400	0.86	46,819	−0.0524	89
16	Frozen	0.1	+	+	4.7820	0.0152	0.32	60,530	+	−	4.8172	0.0403	0.84	65,639	0.0352	108
17	Frozen	0.2	+	+	5.3114	0.0570	1.07	204,854	+	+	5.7861	0.0418	0.72	611,078	0.4747	298
18	Frozen	0.2	+	+	5.2567	0.0570	1.08	180,583	+	+	5.2932	0.0880	1.66	196,421	0.0365	109
19	Frozen	0.3	+	+	5.3954	0.0262	0.49	248,565	+	−	5.2994	0.0400	0.76	199,229	−0.0961	80
20	Fresh	0.4	+	+	5.4153	0.2365	4.37	260,217	+	+	5.5121	0.0264	0.48	325,162	0.0968	125
21	Fresh	0.4	+	+	5.3977	0.0762	1.41	249,886	+	+	5.2130	0.0403	0.77	163,310	−0.1847	65
22	Frozen	0.4	+	+	5.3480	0.0418	0.78	222,821	+	+	5.5031	0.0570	1.04	318,516	0.1552	143
23	Frozen	0.4	+	+	4.9098	0.0880	1.79	81,249	+	+	5.0194	0.0962	1.92	104,557	0.1095	129
24	Frozen	0.5	+	+	5.7405	0.0725	1.26	550,119	+	+	5.6400	0.0791	1.40	436,566	−0.1004	79
25	Fresh	0.6	+	+	5.8904	0.0609	1.03	776,887	+	+	5.8464	0.0152	0.26	702,060	−0.0440	90
26	Frozen	0.6	+	+	5.8147	0.0262	0.45	652,721	+	+	5.7623	0.0454	0.79	578,518	−0.0524	89
27	Fresh	0.9	+	+	6.1279	0.0609	0.99	1,342,359	+	+	6.1543	0.0403	0.66	1,426,458	0.0264	106
28	Frozen	1.1	+	+	5.9982	0.0262	0.44	995,785	+	+	6.0331	0.0151	0.25	1,079,211	0.0349	108
29	Frozen	1.7	+	+	6.2242	0.0158	0.25	1,675,855	+	+	6.3885	0.0316	0.49	2,446,465	0.1643	146
30	Fresh	6.1	+	+	7.1599	0.0151	0.21	14,452,421	+	+	7.0726	0.0454	0.64	11,819,228	−0.0874	82
31	Frozen	6.6	+	+	7.0639	0.0756	1.07	11,583,877	+	+	6.9678	0.0454	0.65	9,284,688	−0.0961	80
32	Fresh	Neg[a]	−	−	Neg				−	−	Neg					
33	Fresh	Neg	−	−	Neg				−	−	Neg					
34	Fresh	Neg	−	−	Neg				−	−	Neg					
35	Fresh	Neg	−	−	Neg				−	−	Neg					
36	Fresh	Neg	−	−	Neg				−	−	Neg					
37	Fresh	Neg	−	−	Neg				−	−	Neg					
38	Fresh	Neg	−	−	Neg				−	−	Neg					
39	Fresh	Neg	−	−	Neg				−	−	Neg					
40	Fresh	Neg	−	−	Neg				−	−	Neg					
41	Fresh	Neg	−	−	Neg				−	−	Neg					

[a] Neg, negative.

Technologies), 5 μl of DNA, and primers and probes, with concentrations as previously reported (12), for a final volume of 25 μl per reaction mixture. All qPCR amplification curves were analyzed using a manual threshold cycle (C_T) of 0.02 and an automatic baseline. A result was called positive if the C_T value was <40 in the presence of a logarithmic amplification curve. Each sample was run in triplicate, and a standard curve with a *P. falciparum* sample of known copy number 10-fold serially diluted from 11.7 to 11,700,000 copies/reaction was included in each qPCR run. The 18S rRNA copy number for each sample was determined by taking the average qPCR C_T value and using the equation generated by the standard curve to calculate the log DNA copy number. The actual copy number detected per sample was calculated by taking the inverse log. The PCRs of the inactivated and corresponding noninactivated samples were performed concurrently to avoid run to run variation.

Descriptive statistics (including the mean and range) were calculated for parasite copy number. Pre- and postinactivation copy number were compared by a Wilcoxon matched-pairs signed-rank test. All statistical computations were performed using GraphPad Prism 5 (GraphPad, La Jolla, CA), and the level of significance was set at a P value of <0.05.

All *P. falciparum*-positive samples were detectable by *Plasmodium* real-time PCR both before and after the filovirus inactivation procedure (Table 1). The T1 band of the BinaxNOW RDT, which is specific to *P. falciparum* infection, was evident in all positive-control samples and 30 out of 31 positive samples after inactivation (Table 1). The T2 band of BinaxNOW RDT, which is specific to pan-*Plasmodium* aldolase, varied by the level of parasitemia, with the T2 band being absent in 9 of 16 (56%) specimens with a *P. falciparum* parasitemia level of ≤0.1% prior to inactivation and 13 of 16 (81%) specimens with a parasitemia level of ≤0.1% following inactivation (Table 1). Thus, the sensitivity of the T1 band for *P. falciparum* was 100% prior to inactivation and 97% following inactivation. However, the sensitivity of the T2 band for *P. falciparum* at very low parasitemia levels (≤0.1%) dropped from 44% to 19% following the inactivation procedure. The sensitivity of qPCR remained at 100% with and without inactivation. The specificities of the T1 and T2 bands of BinaxNOW RDT and qPCR were all 100%, regardless of inactivation status.

The parasite copy number varied by parasitemia on microscopy and ranged from 1,142 to 14,452,421 copies (log 3.0578 to log 7.1599) per reaction prior to inactivation, to 1,032 to 11,819,228 copies (log 3.0138 to 7.0726) per reaction following the inactivation procedures (Table 1). The lowest 18S rRNA copy number of 1,142 copies per reaction is equivalent to 69 parasites per μl of blood, or 0.0014% parasitemia. Low intra-assay variability was observed, with an average % coefficient of variation (CV) of 1.07. The real-time PCR C_T values and log DNA copy number of the standard curves used to calculate the copy number for our samples were highly correlated, with an R^2 of 0.996. Using an arbitrary value of 0.01 for parasitemia levels of <0.1%, DNA copy number and parasitemia were also correlated for both the control and inactivated samples, with R^2 values of 0.958 and 0.961, respectively. The average percent copy number detected in the inactivated compared to noninactivated specimens was 109% (range, 33 to 298%), suggesting a small increase in sensitivity after inactivation, but this was not significant (P = 0.977). To evaluate whether the storage state of samples (i.e., fresh versus frozen) had any effect on the detectability of *P. falciparum* by qPCR, the copy number from fresh or frozen samples was grouped in the analysis, but this was also not significantly different (fresh samples, P = 0.15; frozen samples, P = 0.56).

The current EVD epidemic in West Africa is occurring in a region where malaria is highly endemic (4). To enhance laboratory safety and ensure the prompt exclusion of malaria in ill travelers returning from areas of ongoing EVD transmission, specimens from patients suspected to have EVD should be inactivated prior to use in routine diagnostic assays, including RDT and PCR for malaria. We evaluated the effect of a recommended inactivation procedure with whole-blood clinical samples using a combination of heat and Triton X-100 on the performance of malaria RDT and real-time PCR, and we determined that the detection of *P. falciparum*-specific histidine-rich protein 2 antigen and 18S rRNA gene copy number were unaffected by inactivation. In one sample with a parasitemia level of <0.1%, a very faint T1 band was lost following inactivation, although this was not observed with the 14 other samples with similarly low parasitemia levels. Although the sensitivity for detecting pan-*Plasmodium* aldolase (T2 band) was reduced by the inactivation procedure, this loss of sensitivity would not result in a clinical impact due to the superior sensitivity of HRP-2 detection for *P. falciparum*, regardless of inactivation status. Our results may not extend to different RDTs that detect other plasmodial antigens.

Prior studies of Triton X-100 or heat inactivation have been shown to decrease the sensitivity for detecting *P. falciparum* by histidine-rich protein 2 RDT in reconstituted cultured isolates in whole blood; however, there was no reported commensurate effect on clinical samples when treated with Triton X-100 (10). In another study of plasma analytes, heat inactivation decreased the measured concentrations of analytes to 70 to 80% of the noninactivated controls, whereas Triton X-100 had a minimal effect, with concentrations of 91 to 107% of the controls (11). These data support our finding that *P. falciparum* nucleic acid copy number varied similarly with inactivation procedures.

In summary, we evaluated the effect of CDC-recommended inactivation procedure with a combination of heat and Triton X-100 on the detection of *P. falciparum* by BinaxNOW RDT and real-time PCR (qPCR), and we noted no loss of performance for either test following inactivation. This inactivation procedure enhances safety for laboratory staff handling specimens suspected to contain Ebola virus without compromising the validity of diagnostic tests for malaria. Along with the use of enhanced PPE and a class II BSC, filoviral inactivation procedures should be considered for diagnostic specimens from patients suspected to have EVD, all of which are likely achievable in most hospital core laboratories (8). As malaria must be promptly excluded in any ill traveler who returns from an EVD-affected area in order to avoid adverse outcomes, including death, balancing laboratory safety with efficient and sensitive diagnostic testing is of paramount importance.

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