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Detection of histidine-rich protein 2- and/or 3-deleted *Plasmodium falciparum* using the automated hematology analyzer XN-31: A proof-of-concept study

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ABSTRACT

Rapid diagnostic tests (RDTs) based on immunochromatographic detection of *Plasmodium falciparum* histidine-rich protein 2 (HRP2) have been frequently used for malaria diagnosis. The HRP2-based RDTs are highly sensitive and easy to use; however, their sensitivity may be low in detecting *P. falciparum* strains carrying deletion of the *pfhrp2* and *pfhrp3* genes encoding HRP2 and HRP3, respectively. The automated hematology analyzer XN-31, developed by Sysmex (Kobe, Japan) to aid in malaria diagnosis, has higher sensitivity than RDTs owing to a unique automated nucleic acid staining technology that has shown great potential in clinical settings. In this study, we compared the performance of the XN-31 analyzer and two RDTs to detect *pfhrp2*- and/or *pfhrp3*-deleted parasites cultured in vitro. The analyses showed that the analyzer was not only as sensitive to *pfhrp2*- and/or *pfhrp3*-deleted strains as it was to the wild-type strain but also had higher sensitivity than the RDTs. These results suggested that the XN-31 analyzer is useful for rapid and reliable detection of *pfhrp2*- and/or *pfhrp3*-deleted parasites in clinical settings.

Malaria, tuberculosis, and acquired immunodeficiency syndrome are among the deadliest infectious diseases worldwide. The World Health Organization (WHO) reported approximately 241 million cases of malaria in 2020; of them, 627,000 resulted in death [1]. Although microscopic diagnosis has been considered the gold standard for malaria diagnosis, simple methods providing rapid and sensitive detection of malaria parasites are necessary to ensure timely treatment and prevent further infection spread. Immunochromatography-based rapid diagnostic tests (RDTs) detect malaria parasite antigens in blood; RDTs that detect histidine-rich protein 2 (HRP2), abundantly produced only by *Plasmodium falciparum*, have high specificity and sensitivity [1]. Anti-HRP2 antibodies on test strips often cross-react with HRP3, an HRP2 paralog with a highly similar amino acid sequence contributing to *P. falciparum* detection [2]. However, the sensitivity of HRP2-based RDTs varies depending on genetic variations caused by deletion of HRP2- and HRP3-encoding genes *pfhrp2* and *pfhrp3*, respectively [1]. Deletions of *pfhrp2* and/or *pfhrp3* genes of the parasites have been reported in 37 countries, and WHO Malaria Policy Advisory Group emphasizes the necessity to address this challenge in endemic areas [1]. A meta-analysis on 23 studies for the prevalence of the *pfhrp2*- and *pfhrp3*-deleted parasites indicated that the global pooled prevalence of the parasites was 4%, with the prevalence reported by WHO region ranging from 0 to 25% in South and Central America, from 0 to 62% in Africa, and from 0% to 4% in Asia [3]. HRP2-based RDTs can often give false negative results at low parasitemia (<1,000 parasites/μL) [2,4]. Although alternative RDTs that recognize other parasite antigens, such as parasite's lactate dehydrogenase and aldolase, have been developed, HRP2-based RDTs are most frequently utilized.

Single-color flow cytometers that measure nucleic acid staining are used to detect malaria-infected red blood cells (iRBCs) [5]. An automated hematology analyzer XN-31 recently developed by Sysmex (Kobe, Japan) uses a unique automated malaria detection technology and has

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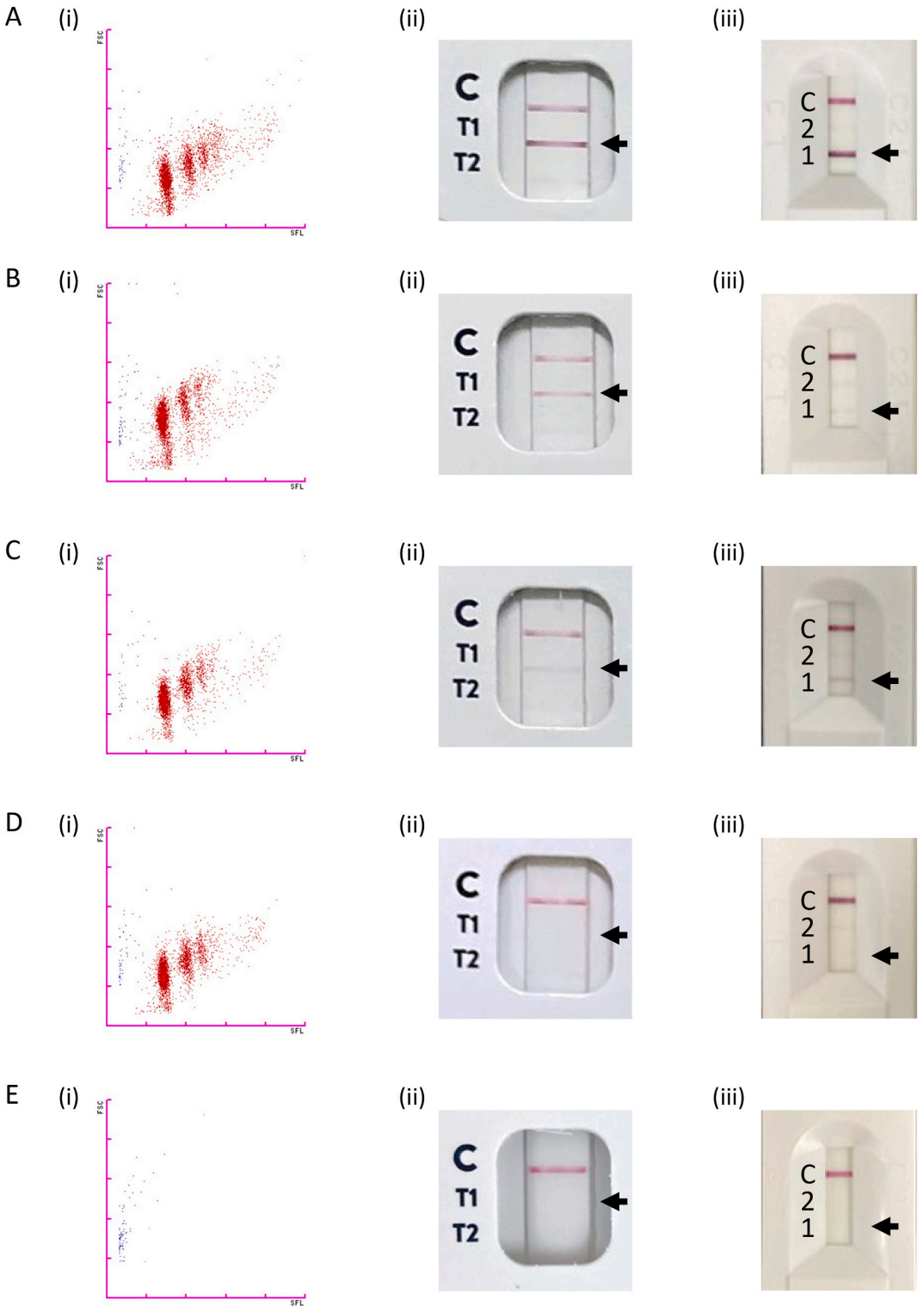

Fig. 1. Comparison of the results of XN-31 and two rapid diagnostic tests. (A) 3D7. (B) Dd2. (C) HB3. (D) 3BD5. (E) Red blood cell (RBC). (i) XN-31. On the scattergrams, the vertical and horizontal axes indicate the intensities of forward scattered light (FSC), corresponding to the size of the malaria-infected red blood cells (iRBCs), and side fluorescent light (SFL), indicating the nucleic acid content, respectively. The red and blue dots represent iRBCs and debris, respectively. The three red populations represent RBCs infected with 1 (left), 2 (middle), and 3 (right) ring-form parasites per single RBC. (ii) BinaxNOW™ Malaria. The "C", "T1", and "T2" denote the control, *P. falciparum*, and Pan (or mixed), respectively. (iii) Malaria Pf/Pan Antigen Test Kit. The "C", "2", and "1" denote the control, Pan (or mixed), and *P. falciparum*, respectively. The arrows show the position of "T1" and "1" indicating *P. falciparum* infection.

shown great potential in research [6–8] and clinical settings [9–11]. XN-31 has been approved as a malaria diagnostic device in Kenya, Ghana, European Union, Japan, and several other countries. The technology is based on nucleic acid staining with a fluorescent dye [6–8,12]. The analyzer automatically calculates parasitemia shown as "MI-RBC%" in approximately 1 min [6,12]; it can distinguish between *P. falciparum* and *P. vivax* [8]. In this study, we evaluated the potential of XN-31 to detect *P. falciparum*, cultured in vitro, that lacks *pfhrp2* and/or *pfhrp3* genes.

For in vitro malaria culture, RBCs were obtained from healthy Japanese volunteers. This study was approved by the institutional review committee of the Research Institute for Microbial Diseases, Osaka University (approval number: 22–3); written informed consent was obtained from all participants. *P. falciparum* strains 3D7 (*pfhrp2*+/*pfhrp3*+), Dd2 (*pfhrp2*−/*pfhrp3*+), HB3 (*pfhrp2*+/*pfhrp3*−), and 3BD5 (*pfhrp2*−/*pfhrp3*−) were cultured in vitro in RBCs at 3% hematocrit level in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 0.5 g/L L-glutamine, 5.96 g/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 g/L $NaHCO_3$, 50 mg/L hypoxanthine, 10 mg/L gentamicin, and 10% heat-inactivated human serum in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 37 °C [13]. For detection analysis, iRBCs with ring-form were collected using the sorbitol synchronization technique [14]. RBCs were harvested by centrifugation at 840 ×*g* for 5 min, resuspended in a 5-fold volume of 5% D-sorbitol (Nacalai Tesque, Kyoto, Japan), and washed twice with RPMI 1640 to remove D-sorbitol. Ring-form iRBCs with the final parasitemia of approximately 1.0, 0.3, 0.1, 0.03, and 0.01% were prepared.

XN-31 was used according to the manufacturer's instructions. Each sample was analyzed five times at the whole blood (WB) mode. The operation was automatically conducted as follows: aspiration of samples, dilution with diluent (CELLPACK DCL), treatment with lysis solution (Lysercell M), and nucleic acid staining with dye solution (Fluorocell M). Then, the number of iRBCs (indicated as "MI-RBC#") was determined by flow cytometry with 405 nm semiconductor laser beam, parasitemia (indicated as "MI-RBC%") was calculated as the ratio of MI-RBC#, and total RBCs were counted using the sheath flow DC detection method. The analyzer automatically raised a "Positive" judgment for MI-RBC# of ≥30/μL. The results were compared with those of two RDTs (BinaxNOW™ Malaria [Abbott, Abbott Park, IL, USA] and Malaria Pf/Pan Antigen Test Kit [Artron Laboratories, Burnaby, Canada]), which were used according to the manufacturer's instructions. To conduct these tests, 15 μL (BinaxNOW™ Malaria) or 5 μL (Malaria Pf/Pan) of each sample was loaded on a pad at a specified position, and the bands were detected visually after 15 or 20 min, respectively. The RDTs have test lines to detect *P. falciparum*-specific HRP2 and a pan-malarial antigen common to four malarial species, *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, causing human infections.

The XN-31 analyzer detected 3D7, Dd2, HB3, and 3BD5 in the same level of parasitemia at approximately 1% parasitemia (Fig. 1 panel (i)) and raised the "Positive" judgment (Table 1). This similarity was consistent with the MI-RBC% data: 1.19 ± 0.022%, 1.24 ± 0.024%, 1.12 ± 0.020%, and 1.29 ± 0.038% for 3D7, Dd2, HB3, and 3BD5, respectively (Table 1). However, analysis of the same samples with BinaxNOW™ Malaria RDT showed that the "T1" band indicative of *P. falciparum* was strong for 3D7, weaker for Dd2 and HB3, and not detected for 3BD5 (Fig. 1 panel (ii)). Similar results were obtained with Malaria Pf/Pan RDT ("1" band indicative of *P. falciparum* in Fig. 1 panel (iii)). These findings suggested that the analyzer could effectively detect *pfhrp2*- and/or *pfhrp3*-deleted parasites, whereas RDTs showed poor performance with strains lacking one of the genes and could not detect those lacking both.

The detection limits of the methods were determined using serially diluted samples. The results in Table 1 indicate that XN-31 detected parasites even at 0.01% parasitemia and raised the "Positive" judgment for each strain irrespective of the presence of *pfhrp2* and/or *pfhrp3* genes. However, the RDTs had low sensitivity: both tests detected 3D7 at 0.03% parasitemia and Dd2 and HB3 at 0.3% parasitemia but could not detect 3BD5 at any parasitemia level. Both RDTs recognized four plasmodial species as the pan-malarial antigen at 1% parasitemia. These results showed that the XN-31 analyzer could detect *pfhrp2*- and/or *pfhrp3*-deleted strains with the same high sensitivity as the wild-type strain.

The analyzer detects parasites at lower parasitemia levels in an easier and more rapid manner and with higher sensitivity than the RDTs. The limits of detection (LoDs) of XN-31 are ≥18, ≥11, and ≥25/μL for MI-RBC# in WB, Low Malaria (LM), and Pre-Dilute (PD) modes,

Table 1
Comparison of data between XN-31 and two RDTs.

Strain*	Therological parasitemia (%)	XN-31			RDT1**		RDT2***	
		MI-RBC% (%)		Flagging *P. falciparum*	Pf	Pan	Pf	Pan
		mean	SD					
3D7	1.00	1.19	0.022	Positive	+	+	+	+
	0.30	0.39	0.0048	Positive	+	−	+	−
	0.10	0.13	0.0049	Positive	+	−	+	−
	0.03	0.042	0.0021	Positive	+	−	+	−
	0.01	0.013	0.0014	Positive	−	−	−	−
Dd2	1.00	1.24	0.024	Positive	+	+	+	+
	0.30	0.40	0.018	Positive	+	−	+	−
	0.10	0.14	0.0030	Positive	−	−	−	−
	0.03	0.042	0.010	Positive	−	−	−	−
	0.01	0.017	0.0021	Positive	−	−	−	−
HB3	1.00	1.12	0.020	Positive	+	+	+	+
	0.30	0.36	0.011	Positive	+	−	+	−
	0.10	0.13	0.0064	Positive	−	−	−	−
	0.03	0.042	0.0052	Positive	−	−	−	−
	0.01	0.013	0.0028	Positive	−	−	−	−
3BD5	1.00	1.29	0.038	Positive	−	+	−	+
	0.30	0.40	0.018	Positive	−	−	−	−
	0.10	0.13	0.0073	Positive	−	−	−	−
	0.03	0.044	0.0042	Positive	−	−	−	−
	0.01	0.015	0.0033	Positive	−	−	−	−
RBC	0	----	----	Negative	−	−	−	−

Abbriviations: RDT, rapid diagnostic test; SD, standard deviation; RBC, red blood cell.
* 3D7 (*pfhrp2*+/*pfhrp3*+), Dd2 (*pfhrp2*−/*pfhrp3*+), HB3 (*pfhrp2*+/*pfhrp3*−), and 3BD5 (*pfhrp2*−/*pfhrp3*−).
** RDT1, BinaxNOW™ Malaria.
*** RDT2, Malaria Pf/Pan Antigen Test Kit.

respectively. Furthermore, the limits of quantification (LoQs) are ≥30, ≥20, and ≥ 40/μL of MI-RBC# in WB, LM, and PD modes, respectively. The LoD for Pf of BinaxNOW™ Malaria is approximately 1,001–1,500 parasites/μL as indicated in the manufacturer's manual. In this study, we confirmed the high sensitivity of XN-31 with this setting; however, this efficiency should be validated under clinical settings.

Microscopic examination of stained blood smears is the "gold standard" for malaria diagnosis, showing excellent sensitivity, which is as low as 5–10 parasites/μL of blood (approximately 0.0001% parasitemia) [15]. However, diagnostic errors occur as oversight increases in the cases of low-density parasitemia (10–100 parasites/μL of blood), and quantification errors also occur in the cases of high-density parasitemia (>5,000 parasites/μL and especially >20,000 parasites/μL of blood) [16]. These challenges are exacerbated in non-endemic regions where microscopic detection of malaria is rarely performed. The XN-31 analyzer, which is easy to operate and has a low detection limit, may potentially overcome these challenges.

In addition to the drawbacks of insufficient detection due to the deletion of *pfhrp2* and/or *pfhrp3* and low parasitemia, the prozone effect (or high doses-hook phenomenon) due to high parasitemia should be considered when faced with HRP2-RDT negative results in patients with suspected malaria. This effect has been previously demonstrated in laboratory studies of both clinical isolates and laboratory cultures [17]. Another study has reported prozone-like effects at parasitemia levels of 5.5–35% [18]. Another study elucidated that high concentrations of patient antibodies against HRP2 and HRP3 antigens may also contribute to the inhibition of RDT recognition of antigens [19]. On the other hand, our previous study on rodent malaria infection indicated that MI-RBC% from XN-30 (a research version of XN-31) highly correlated with the parasitemia from microscopy at the range of 2.5–60% [7], suggesting that XN-31 has potential to overcome these drawbacks.

HRP2-based RDTs may demonstrate persistent false positivity due to residual antigenemia, which can last for weeks after effective treatment and disappearance of clinical symptoms [4]. False positive rates for an HRP2-based assay can be as high as 98.2% and 94.6% on days 14 and 21 of follow-up, respectively, after recovery from the febrile illness [20], and reach 26.4% on day 28, whereas effective parasite clearance is indicated on day 35 [21]. Thus, HRP2-based RDTs have limited use in assessing treatment results or detecting reinfection within 1 month. Contrastingly, the XN-30 analyzer shows the same efficiency as microscopy in detecting rodent malaria in infected mice treated with artemisinin [7], suggesting that XN-31 is useful for assessing the therapeutic effect of antimalarial drugs.

The XN-31 analyzer detected *pfhrp2*- and/or *pfhrp3*-deleted parasites with the same sensitivity as it detected the intact parasites and showed higher sensitivity than the RDTs, indicating its potential to overcome the drawbacks of HRP2-based RDTs. Our results were obtained using in vitro cultures and should be validated using samples from areas endemic to *pfhrp2*- and/or *pfhrp3*-deleted parasites to authenticate the use of XN-31 analyzer for rapid and reliable diagnosis of malaria in clinical settings.

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Declaration of Competing Interest

F.H. is an employee of Sysmex Corporation.

Data availability

Data will be made available on request.

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