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Combining Parasite Lactate Dehydrogenase-Based and Histidine-Rich Protein 2-Based Rapid Tests To Improve Specificity for Diagnosis of Malaria Due to *Plasmodium knowlesi* and Other *Plasmodium* Species in Sabah, Malaysia

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Plasmodium knowlesi **causes severe and fatal malaria in Malaysia. Microscopic misdiagnosis is common and may delay appropriate treatment. *P. knowlesi* can cross-react with "species-specific" parasite lactate dehydrogenase (pLDH) monoclonal antibodies used in rapid diagnostic tests (RDTs) to detect *P. falciparum* and *P. vivax*. At one tertiary-care hospital and two district hospitals in Sabah, we prospectively evaluated two combination RDTs for malaria diagnosis by using both a pan-*Plasmodium*-pLDH (pan-pLDH)/*P. falciparum*-specific-pLDH (*Pf*-pLDH) RDT (OptiMAL-IT) and a non-*P. falciparum* VOM-pLDH/*Pf*-HRP2 RDT (CareStart). Differential cross-reactivity among these combinations was hypothesized to differentiate *P. knowlesi* from other *Plasmodium* monoinfections. Among 323 patients with PCR-confirmed *P. knowlesi* ($n = 193$), *P. falciparum* ($n = 93$), and *P. vivax* ($n = 37$) monoinfections, the VOM-pLDH individual component had the highest sensitivity for nonsevere (35%; 95% confidence interval [CI], 27 to 43%) and severe (92%; CI, 81 to 100%) *P. knowlesi* malaria. CareStart demonstrated a *P. knowlesi* sensitivity of 42% (CI, 34 to 49%) and specificity of 74% (CI, 65 to 82%), a *P. vivax* sensitivity of 83% (CI, 66 to 93%) and specificity of 71% (CI, 65 to 76%), and a *P. falciparum* sensitivity of 97% (CI, 90 to 99%) and specificity of 99% (CI, 97 to 100%). OptiMAL-IT demonstrated a *P. knowlesi* sensitivity of 32% (CI, 25 to 39%) and specificity of 21% (CI, 15 to 29%), a *P. vivax* sensitivity of 60% (CI, 42 to 75%) and specificity of 97% (CI, 94 to 99%), and a *P. falciparum* sensitivity of 82% (CI, 72 to 89%) and specificity of 39% (CI, 33 to 46%). The combination of CareStart plus OptiMAL-IT for *P. knowlesi* using predefined criteria gave a sensitivity of 25% (CI, 19 to 32%) and specificity of 97% (CI, 92 to 99%). Combining two RDT combinations was highly specific for *P. knowlesi* malaria diagnosis; however, sensitivity was poor. The specificity of pLDH RDTs was decreased for *P. vivax* and *P. falciparum* because of *P. knowlesi* cross-reactivity and cautions against their use alone in areas where *P. knowlesi* malaria is endemic. Sensitive *P. knowlesi*-specific RDTs and/or alternative molecular diagnostic tools are needed in areas where *P. knowlesi* malaria is endemic.**

Human malaria due to the simian parasite *Plasmodium knowlesi* has now been reported in all countries throughout Southeast Asia except Laos and East Timor (1–9). In Malaysian Borneo, *P. knowlesi* is the most common cause of human malaria (10, 11) and commonly causes severe disease, with fatal cases also reported (12–16). Early diagnosis of *P. knowlesi* infection is important; with its 24-h life cycle, it can rapidly cause high parasitemia and is three times as likely as *P. falciparum* to cause severe malaria (15). Microscopy remains the gold standard for malaria diagnosis across Asia. However, microscopic diagnosis of *P. knowlesi* remains problematic, since the species is nearly indistinguishable from *P. malariae* (17) and is frequently confused with *P. falciparum* and *P. vivax* (18). Misdiagnosis of *P. knowlesi* has important clinical implications, including inappropriate treatment in regions without unified treatment strategies, failure to administer antihypnozoite treatment for *P. vivax* malaria (18), and failure to recognize patients at risk of severe disease (16). Public health surveillance of *P. knowlesi* is also compromised by inaccurate microscopic reporting.

Malaria rapid diagnostic tests (RDTs) using immunochromatographic capture techniques provide an alternative method of diagnosis and have been increasingly deployed in areas where malaria is endemic. The tests can be conducted by staff with minimal training, and results are rapidly available. In the most recent WHO round 4 malaria RDT product testing results, composite test positivity had considerably increased, with the best-performing tests for *P. falciparum* and *P. vivax* approaching 100% even at a parasite count of 200/μl (19). These RDTs utilize monoclonal antibodies targeting specific antigens such as *P. falciparum* histidine-rich protein 2 (*Pf*-HRP2) or *Plasmodium* genus- or species-specific parasite lactate dehydrogenase (pLDH). Although no *P. knowlesi*-specific monoclonal antibody has been developed, *P. knowlesi* has

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TABLE 1 Predefined clinical specificity reference for RDT component combinations

	Combined specificity[d] of:			
	CareStart		OptiMAL-IT	
Parasite	VOM-pLDH	*Pf*-HRP2	Pan-pLDH	*Pf*-pLDH
P. knowlesi[a,b]	±	−	±	+
P. falciparum[c]	−	+	±	±
P. vivax	+	−	+	−
P. malariae	+	−	+	−
P. knowlesi-P. vivax	+	−	+	+
P. knowlesi-P. falciparum	+	+	+	+
P. falciparum-P. vivax	+	+	+	+

[a] *P. knowlesi* monoinfection or mixed *P. knowlesi-P. vivax* infection cannot be differentiated.
[b] VOM-pLDH and pan-pLDH are not required to differentiate *P. knowlesi* from *P. falciparum* or *P. vivax* monoinfection.
[c] *Pf*-HRP2 is highly specific for *P. falciparum*; therefore, a positive *Pf*-HRP2 result indicates *P. falciparum* infection regardless of the OptiMAL-IT result.
[d] Symbols: +, specific; −, not specific; ±, equivocal.

been shown to cross-react with *P. falciparum*- and *P. vivax*-"specific" pLDH (20).

Several case reports of returned travelers with PCR-confirmed *P. knowlesi* monoinfection have demonstrated this cross-reactivity, with *Plasmodium* genus pLDH-, *P. falciparum* pLDH-, and *P. vivax* pLDH-based RDTs all yielding positive results but with poor sensitivity at low parasite counts (21–24). In a previous prospective evaluation of RDTs for *P. knowlesi* malaria, a pan-pLDH-based RDT demonstrated a moderate overall sensitivity for *P. knowlesi* of 74% (95/129; 95% confidence interval [CI], 65 to 80%), which improved for pretreatment samples (88%; 30/34; CI, 73 to 95%) and for severe disease (95%; 36/38; CI, 83 to 99%) (25). Neither this test nor a more poorly performing aldolase-based RDT also evaluated in that study approached a sensitivity of 100% for parasitemias of >100/μl, which was previously defined as necessary for use as a clinically sufficient malaria diagnostic tool (26, 27). Moreover, neither RDT was able to distinguish *P. knowlesi* from *P. vivax*.

As *P. knowlesi* cross-reacts with a proportion of monoclonal antibodies targeting both *P. falciparum* and *P. vivax* pLDHs but does not cross-react with *Pf*-HRP2, we and others (20) have hypothesized that a combination of RDTs containing these test components may be used for differentiating *P. knowlesi*, *P. falciparum*, and *P. vivax* malaria infections (Table 1). Current commercial RDTs do not routinely specify the exact epitopes present on the monoclonal antibodies used to target *Plasmodium* species pLDHs and whether they are shared (20), meaning that it is not possible to ascertain the potential *P. knowlesi*-binding capacity of previously untested pLDH-based RDTs. We therefore prospectively evaluated the sensitivity and specificity of a combination of two RDTs for the diagnosis of *P. knowlesi* malaria.

MATERIALS AND METHODS

Study sites and referral system. This study was conducted at three hospitals in Sabah, Malaysian Borneo: Kudat District Hospital (KDH), Kota Marudu District Hospital (KMH); and Queen Elizabeth Hospital (QEH), the corresponding adult tertiary-care referral center in the state capital Kota Kinabalu. QEH services a catchment area along the northwestern coast of Sabah with a population of 1.14 million people in six districts. Malaria patients from this area are either admitted to their primary district hospital or referred to QEH with an accompanying pretreatment blood film and whole-blood sample if they meet state transfer guidelines. These include a thick blood film reported as 4+ (indicating >10 parasites/high-power microscopy field) or any evidence of severe malaria, with treatment prior to transfer. Malaria patients from the local Kota Kinabalu area are admitted directly to QEH.

Subjects. All of the patients presenting to QEH from September 2011 to July 2013 or to KDH and KMH from October 2012 until July 2013 with a microscopic diagnosis of malaria were eligible for inclusion in this study. This included 14 adults with severe ($n = 3$) or nonsevere ($n = 11$) malaria previously reported (15, 18). District sites were included to allow pretreatment evaluation of children >1 year of age and adults in a primary-care setting where, in contrast to a tertiary-care referral hospital, malaria cases are more often nonsevere and have lower parasite counts (12, 28). All of the participants were nonpregnant with no major comorbidities and had not previously been enrolled in this study. On the basis of PCR testing, patients who were negative ($n = 19$), did not have a result ($n = 6$), or had mixed infections with *Plasmodium* species ($n = 3$) were retrospectively excluded. Malaria severity was defined by modified 2010 WHO criteria (15, 29). Written informed consent was provided by patients or their relatives. Approvals were obtained from the Ethics Committees of the Malaysian Ministry of Health and the Menzies School of Health Research.

RDT selection. On the basis of the WHO round 1 and 2 malaria RDT product test results available at the time of study design (30) (Table 2), RDTs were chosen according to the combined potential specificity of the individual test components for *P. knowlesi* malaria (Table 1). The test components with the highest reported sensitivities for *P. falciparum*-specific and non-*P. falciparum* pLDHs were selected. They were OptiMAL-IT (DiaMed AG), which detects *P. falciparum*-specific and pan-*Plasmodium* sp. pLDHs, and CareStart Malaria (Pf/VOM) Combo (Access Bio Inc.), which detects the *Pf*-HRP2 antigen and non-*P. falciparum* pan-pLDH.

Despite potential positive cross-reactivity with *Pf*-LDH, it may be possible to differentiate a *P. knowlesi* monoinfection from a *P. falciparum* monoinfection by using its lack of reactivity with the *Pf*-HRP2 component. It may be possible to differentiate a *P. knowlesi* monoinfection from a *P. vivax* monoinfection by using the lack of reactivity of the latter with *Pf*-LDH. While this RDT combination will not distinguish a *P. knowlesi* monoinfection from a mixed *P. knowlesi-P. vivax* infection, the latter appears rare in Malaysian Borneo, accounting for 5/387 (1.3%) (15) and 1/188 (0.5%) (14) PCR-confirmed malaria cases in two recent studies.

Study procedures. Demographic and clinical information was recorded on standardized case record forms. Pretreatment venous blood

TABLE 2 Test positivity in WHO RDT product testing rounds 1 and 2[a]

	% Test positivity with:			
	200 parasites/μl		2,000 or 5,000 parasites/μl	
Test	*P. falciparum*	*P. vivax*	*P. falciparum*	*P. vivax*
Pf-HRP2/VOM-pLDH (CareStart)	89	80	100	100
Pf-pLDH/pan-pLDH (OptiMAL-IT)	37	95	96	100

[a] Data are from reference 30.

was collected in a labeled EDTA or citrate-theophylline-adenosine-dipyridamole tube. RDTs were transported directly to the study sites by registered courier from the manufacturer in the United States (CareStart) and through a local distributer (OptiMAL-IT). RDTs were subsequently stored out of direct sunlight in monitored, air-conditioned research laboratories at 22°C. Batch numbers and expiration dates were documented. RDTs were taken to the patient enrollment area, where the temperature and humidity did not exceed 32°C and 74%, respectively. All of the RDTs were packaged and sealed individually with desiccant and used immediately after opening, in accordance with the manufacturers' instructions. Results were recorded by one of two research laboratory technicians blinded to the microscopy diagnosis, with regular cross-checking by the study clinician to ensure consistent reporting. Interobserver agreement was tested at the district sites, with a research staff member reading the test first, followed 5 min later by a second person blinded to the initial reading. In the event of discordant results, a third person also blinded read the test, with the final result being the most common reading. Results were recorded as follows: negative, no clearly visible line; 1, faint line; 2, line darker than 1 but lighter than the control; 3, same as the control line; 4, line darker than the control. Thick and thin blood films were prepared on enrollment and examined by microscopists at referring district hospitals or at QEH, with slides later cross-checked by an experienced research microscopist. Parasite density was quantified by the research microscopist using pretreatment slides and reported as the number of parasites per 200 leukocytes or per 1,000 erythrocytes and converted to the number of parasites per microliter by using the patient's leukocyte count or hematocrit, respectively. Where pretreatment slides were unavailable (13/327 [4%]), referring hospital microscopy was used and the grades 1+ to 4+ were converted into numbers of parasites per microliter by using the relevant median parasite density. Parasite DNA was extracted, and PCR was performed as previously described for *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* (31) or *P. knowlesi* (32) detection.

Statistical analysis. Data were analyzed by using STATA, version 12 (StataCorp LP, College Station, TX). Intergroup differences were compared by using the Kruskal-Wallis test for nonnormally distributed continuous variables and a χ^2 test for categorical variables. The variables measured included the numbers of true positives, true negatives, false positives, and false negatives. PCR results were used as the gold standard, with *Plasmodium* species monoinfections compared against all of the other PCR-positive *Plasmodium* species. Test sensitivity was defined as true positives/(true positives + false negatives), and test specificity was defined as true negatives/(true negatives + false positives). Ninety-five-percent confidence intervals (CIs) were estimated by Wilson's method. Logistic regression was used to assess the relationship between sensitivity and parasite count and also the usefulness of RDT band intensity as a predictor of severity. Interobserver agreement between the first and second readings was measured by using the kappa coefficient. Spearman's correlation coefficient was used to assess the association between RDT band intensity and the parasite count.

RESULTS

Baseline demographic and clinical features. From October 2012 until July 2013, RDTs were conducted for 193, 93, 37, and 3 patients with PCR-confirmed *P. knowlesi*, *P. falciparum*, *P. vivax*, and *P. malariae* monoinfections, respectively. This included 321 patients (*P. knowlesi*, $n = 189$; *P. falciparum*, $n = 92$; *P. vivax*, $n = 37$; *P. malariae*, $n = 3$) tested with the pan-pLDH/*Pf*-pLDH (OptiMAL-IT) test. The VOM-pLDH/*Pf*-HRP2 (CareStart) test was performed for 301 patients (*P. knowlesi*, $n = 178$; *P. falciparum*, $n = 85$; *P. vivax*, $n = 35$; *P. malariae*, $n = 3$). Their baseline demographics are shown in Table 3. Patients with *P. knowlesi* malaria were older than those with *P. falciparum* or *P. vivax* malaria (median ages, 40, 30, and 27 years, respectively; $P < 0.001$) and more likely to be male (81, 73, and 68%, respectively; $P = 0.031$). The majority of the malaria patients were enrolled at the QEH tertiary-care study site for all of the *Plasmodium* species. *P. knowlesi* patients enrolled at district hospitals were younger (median age, 33 versus 44 years; $P = 0.001$), were less likely to have severe malaria (5 versus 22%; $P = 0.001$), and had lower median parasite counts (1,436 versus 3,802/μl; $P = 0.001$) than those enrolled at the tertiary-care hospital. For nonsevere malaria, *P. knowlesi* malaria patients had a lower median parasite count (1,701/μl) than *P. falciparum* malaria patients ($P = 0.033$) but not *P. vivax* malaria patients ($P = 0.918$).

Microscopy. Among 193 patients with PCR-confirmed *P. knowlesi* infection, only 33 (17%) were accurately reported as *P. knowlesi* or "*P. malariae*/?*P. knowlesi*" by hospital microscopists, while 109 (56%) were reported as *P. malariae* (morphologically indistinguishable from *P. knowlesi* and the default reporting nomenclature from previous state guidelines). Considered as a single group, this gave a sensitivity of 74% (CI, 67 to 80%) and a specificity of 89% (CI, 82 to 94%) for *P. knowlesi*. Our dedicated research microscopist correctly identified 158 of 193 *P. knowlesi* blood films (sensitivity, 83% [CI, 76 to 88%]; specificity, 93% [CI, 87 to 96%]).

RDT sensitivity. The VOM-pLDH test component had the highest sensitivity (77/178; 43%; CI, 36 to 51%) for *P. knowlesi* malaria overall but still performed poorly (Table 4). For *P. vivax*, despite a median parasite count of 3,366/μl, the VOM-pLDH test performance (29/35; 83% sensitivity; CI, 70 to 96%) was more in line with the reported WHO composite test positivity of 80% at 200 parasites/μl than the reported 100% with 2,000 to 5,000 parasites/μl (30). For *P. falciparum*, three false-positive VOM-pLDH results were recorded, with parasite counts ranging from 9,079 to 21,972/μl. For the PCR-confirmed *P. malariae* cases, VOM-pLDH demonstrated test positivity (2/3; 67%; CI, 9 to 99%); however, the small numbers did not allow meaningful evaluation. The pan-pLDH test component was insufficiently sensitive for all of the species, with sensitivities of 30% (CI, 24 to 37%), 68% (CI, 52 to 83%), 78% (CI, 70 to 87%), and 67% (CI, 0 to 100%) for *P. knowlesi*, *P. vivax*, *P. falciparum*, and *P. malariae*, respectively. The *Pf*-pLDH test also demonstrated poor sensitivity for *P. knowlesi* (55/190; 29%; CI, 22 to 35%) and suboptimal stand-alone performance for *P. falciparum* (74/92; 80%; CI, 72 to 89%), despite a median *P. falciparum* parasitemia of 5,832/μl. The overall sensitivity of the OptiMAL-IT RDT (i.e., a positive *Pf*-pLDH and/or pan-pLDH test result) for the detection of *P. knowlesi* (32% [CI, 25 to 39%]) was only a marginal improvement on the individual test components. The CareStart overall sensitivity for *P. knowlesi* of 42% (CI, 34 to 49%) was slightly less than the VOM-pLDH standalone sensitivity of 43% because of four false-positive *Pf*-HRP2 results. The sensitivities of the VOM-pLDH, pan-pLDH, and Pf-pLDH tests for *P. knowlesi* malaria were all lower at the district sites than at the tertiary-care site (35 versus 49% [$P = 0.061$], 13 versus 42% [$P < 0.0001$], and 13 versus 40% [$P < 0.001$], respectively).

Sensitivity and parasitemia. Increasing *P. knowlesi* parasitemia correlated with test sensitivity for VOM-pLDH ($P < 0.001$), pan-pLDH ($P = 0.001$), and *Pf*-pLDH ($P = 0.004$) (Fig. 1A and B). Thirty-one percent of the *P. knowlesi* patients had a parasite count of <1,000/μl, and in this group only 12% had a positive VOM-pLDH test. Among the 27% of *P. knowlesi* patients with a parasite count of >10,000/μl, 81% (39/48) had a positive VOM-pLDH test result, which increased to 100% (13/13) for those with

TABLE 3 Baseline demographics, clinical severity, and parasitemia of enrolled subjects

Species (no. of patients) or parameter	No. (%) of patients		Median age, yr (IQR[a]) [range]			No. (%) of males		
	Tertiary-care Hospital	District hospital	Total	Tertiary-care hospital	District hospital	Total	Tertiary-care hospital	District hospital
P. knowlesi (193)	114 (59)	79 (41)	40 (26–51) [3–94]	44 (30–52) [16–81]	33 (20–48) [3–72]	157 (81)	97 (85)	60 (75)
P. falciparum (93)	91 (98)	2 (2)	30 (21–44) [13–75]	30 (21–44) [13–75]	43 (15–71) [15–71]	68 (73)	68 (75)	0
P. vivax (37)	23 (62)	14 (38)	27 (11–39) [1–62]	31 (27–42) [15–62]	11 (4–12) [1–39]	25 (68)	18 (78)	7 (50)
P. malariae (3)	1 (33)	2 (66)	21 (13–25) [13–25]	25	17 (13–21) [13–21]	1 (33)		
P value for:								
P. knowlesi vs *P. falciparum*			0.006	<0.001	0.52	0.133	0.064	0.019
P. knowlesi vs *P. vivax*			<0.001	0.023	<0.001	0.069	0.418	0.058

[a] IQR, interquartile range.

a parasite count of >100,000/μl. Despite the lowest recorded parasite count for a positive VOM-pLDH test result being 50/μl, the median positive result was 7,089/μl and the highest negative result was 40,807/μl. For both pan-pLDH and *Pf*-pLDH, the lowest *P. knowlesi* parasitemia detected was 26/μl and the highest was 641,464/μl. The highest negative result for *P. knowlesi* with both pan-pLDH and *Pf*-pLDH was 425,784/μl. The relationship between test positivity and parasitemia was also apparent for *P. falciparum* with the pan-pLDH ($P = 0.048$) and *Pf*-pLDH ($P = 0.045$) tests. In contrast, *P. vivax* had a smaller range of parasite counts (76 to 60,288/μl) and no statistically significant relationship between parasitemia and the sensitivity of any of the different test components.

Sensitivity and clinical severity. The VOM-pLDH test had a higher sensitivity among severe *P. knowlesi* malaria patients (24/26; 92%; CI, 81 to 100%) than the pan-pLDH (20/28; 71%; CI, 54 to 89%; $P = 0.049$) and *Pf*-pLDH (17/28; 61%; CI, 41 to 80%; $P = 0.006$) test components. For *P. vivax*, both the VOM-pLDH and pan-pLDH tests of all three severe-malaria patients were positive. All of the severe *P. falciparum* malaria patients tested with *Pf*-HRP2 were positive (14/14; 100%); however, only 14/16 (86%; CI, 69 to 100%) and 13/16 (81%; CI, 60 to 100%) had positive *Pf*-LDH and pan-pLDH test results, respectively.

Line intensity. The intensity of the test line was strongly associated with an increasing parasite count for *P. knowlesi* malaria with all of the test components, with Spearman correlation coefficients of 0.616 ($P < 0.001$), 0.408 ($P < 0.001$), and 0.376 ($P < 0.001$) for VOM-pLDH, pan-pLDH, and Pf-pLDH, respectively (Fig. 2). For positive VOM-pLDH tests, the highest line intensity reading of 4+ did not independently predict severe *P. knowlesi* malaria ($P = 0.595$).

Interobserver agreement. Both pan-pLDH/*Pf*-LDH ($n = 95$) and VOM-pLDH/*Pf*-HRP2 ($n = 89$) demonstrated excellent interobserver agreement, with kappa values of >0.9 for all of the

TABLE 4 Sensitivity and specificity of RDTs and their components for each *Plasmodium* species

Test and parameter	Proportion, % (CI)				*P* value
	P. knowlesi	*P. vivax*	*P. falciparum*	*P. malariae*	
CareStart					
VOM-pLDH, sensitivity	77/178, 43 (36–51)	29/35, 83 (70–96)	3/85, 4 (0–8)[d]	2/3, 67 (0–100)	<0.0001[a]
Pf-HRP2, sensitivity	4/183, 2 (0–4)[d]	1/35, 3 (0–9)[d]	86/87, 99 (97–100)	0/3, 0	
OptiMAL-IT					
Pan-pLDH, sensitivity	57/189, 30 (24–37)	25/37, 68 (52–83)	72/92, 78 (70–87)	2/3, 67 (0–100)	<0.0001[b]
Pf-pLDH, sensitivity	55/190, 29 (22–35)	5/37, 14 (2–25)[d]	74/92, 80 (72–89)	0/3, 0	<0.0001[c]
CareStart (VOM-pLDH + *Pf*-HRP2)					
Sensitivity	74/178, 42 (34–49)	29/35, 83 (66–93)	82/85, 97 (90–99)	2/3, 67 (9–99)	
Specificity	91/123, 74 (65–82)	189/266, 71 (65–76)	214/216, 99 (97–100)	194/298, 35 (30–41)	
OptiMAL-IT (pan-pLDH + *Pf*-pLDH)					
Sensitivity	61/191, 32 (25–39)	22/37, 60 (42–75)	75/92, 82 (72–89)	2/3, 67 (9–99)	
Specificity	28/132, 21 (15–29)	276/285, 97 (94–99)	141/231, 39 (33–46)	290/319, 91 (87–94)	

[a] *P. knowlesi* compared against *P. vivax* only.
[b] *P. knowlesi* compared against other *Plasmodium* species.
[c] *P. knowlesi* compared against *P. falciparum* only.
[d] False positives.

TABLE 3 (Continued)

No. (%) of patients with severe malaria			Median no. of parasites/μl (IQR[a]) [range]				
Total	Tertiary-care hospital	District hospital	All patients	Nonsevere malaria	Severe malaria	Tertiary-care hospital	District hospital
29 (15)	25 (22)	4 (5)	2301 (672–641464) [30–641464]	1701 (493–7573) [30–90683]	56784 (5300–144968) [979–641464]	3802 (1152–18442) [88–641464]	1436 (320–7515) [30–143184]
16 (17)	16 (100)	0	5832 (1464–19000) [26–606108]	4386 (1464–17208) [26–247897]	17912 (6554–106070) [33–606108]	6254 (1464–21972) [26–606108]	570 (33–1107) [33–1107]
3 (8)	3 (100)	0	3366 (371–13593) [76–60288]	3004 (371–12544) [76–60288]	4453 (4025–19520) [4025–19520]	3381 (1332–6581) [210–19520]	3004 (600–12544) [76–60288]
0			675 (77–1212) [77–1212]	675 (77–1212) [77–1212]		1212 (1212–1212) [1212–1212]	376 (77–675) [77–675]
0.623	0.329	0.009	0.033	0.009	0.308	0.388	
0.271	0.337	0.395	0.918	0.483	0.165	0.342	0.417

tests. No tests were repeated at the district hospital sites; however, this was not documented at the tertiary-care site.

Combined test specificity and sensitivity. Individual test component specificity for *P. knowlesi* is not clinically useful, as the individual test components cannot differentiate *P. knowlesi* from other *Plasmodium* species. However, a combination of the tests could theoretically distinguish a *P. knowlesi* monoinfection from a *P. vivax*, *P. falciparum*, or *P. malariae* monoinfection (Table 1). The use of this predefined RDT combination to diagnose PCR-confirmed *P. knowlesi*-infected patients yielded 45/181 positive results, with a sensitivity for *P. knowlesi* diagnosis lower than that of hospital microscopy (25% [CI, 19 to 32%] versus 74% [CI, 67 to 80%]; $P < 0.001$) but a higher specificity (96% [CI, 91 to 99%] versus 89% [CI, 82 to 94%]; $P = 0.030$) (Table 5). The sensitivity of the RDT combination for *P. knowlesi* diagnosis was also lower at the district hospitals (10% [CI, 5 to 19%]) than at the tertiary-care hospital (36% [CI, 26 to 46%]; $P < 0.001$).

DISCUSSION

P. knowlesi cross-reactivity was demonstrated with all of the pLDH-based test components evaluated in this study; however, the sensitivities of each test component and of the RDTs overall (32 and 42% for OptiMAL-IT and CareStart, respectively) were insufficient for the diagnosis of *P. knowlesi* malaria. Binding affinity appeared to be strongly related to parasitemia, with test sensitivity particularly poor at lower parasite counts. *P. knowlesi* patients, including both adults and children, with nonsevere malaria have lower parasitemias than those with nonsevere *P. falciparum* malaria, with *P. knowlesi* parasitemias particularly low at the district level. The lack of test sensitivity for these patients is highlighted by the finding that the median parasitemia detected by the VOM-pLDH test (7,089 parasites/μl) far exceeds the median parasitemia among nonsevere *P. knowlesi* patients presenting to a district hospital (1,040 parasites/μl) or a tertiary-care hospital (2,133 parasites/μl). Although the VOM-pLDH test was positive among all of the patients with *P. knowlesi* parasite counts of >100,000/μl and among 92% of the patients with severe *P. knowlesi* malaria, false-negative results occurred among patients with parasite counts of up to 40,807/μl. Given that the risk of severe disease with *P. knowlesi* is 11-fold higher with a parasitemia of >20,000/μl (15), false-negative VOM-pLDH test results with parasite counts above this level are clearly unsatisfactory.

The insufficient sensitivity of each of the RDTs evaluated in the present study for *P. knowlesi* diagnosis is consistent with the lack of sensitivity demonstrated in other recent series evaluating RDT performance in hospital settings in countries where *P. knowlesi* malaria is endemic. These include tests incorporating the follow-

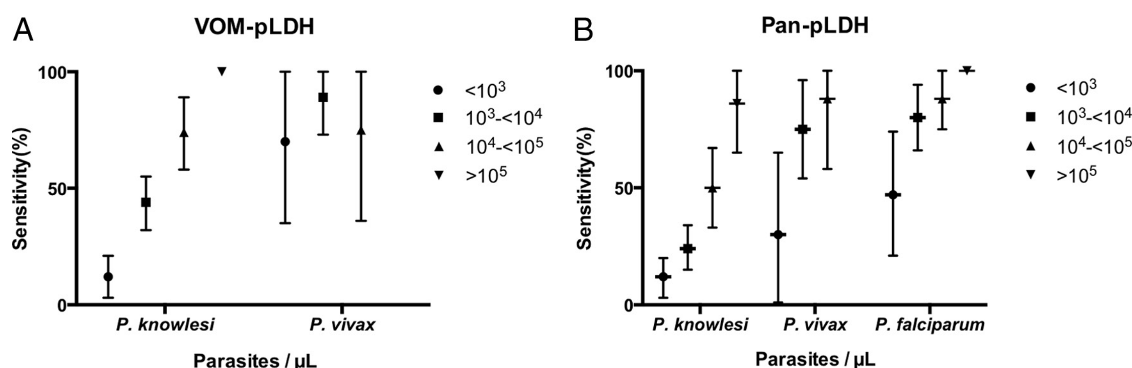

FIG 1 (A) Sensitivity of the VOM-pLDH component of the CareStart RDT by parasite count for *P knowlesi* and *P. vivax* infections. Vertical lines represent CIs. (B) Sensitivity of the pan-pLDH component of the OptiMAL-IT RDT by parasite count for *P knowlesi*, *P. vivax*, and *P. falciparum* infections. Vertical lines represent CIs.

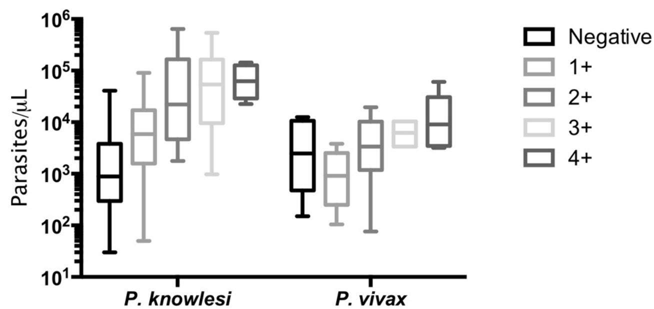

FIG 2 Parasite counts by VOM-pLDH line intensity for *P knowlesi* and *P. vivax* infections. Horizontal lines indicate medians, boxes indicate interquartile ranges, and vertical lines indicate ranges.

ing antibody components: pan-pLDH (First-Response, 74% sensitivity) (25), *Pf*-pLDH/pan-pLDH (OptiMAL-IT, 71% sensitivity with fresh isolates) (33), Pv-pLDH/pan-pLDH (Paramax-3, 40% sensitivity with fresh isolates) (33), and pan-*Plasmodium* aldolase (ParaHit, 23% sensitivity with fresh isolates [25]; BinaxNOW, 29% sensitivity with fresh isolates [33]).

The cross-reactivity of *P. knowlesi* in RDTs utilizing "species-specific" *P. falciparum* or *P. vivax* pLDH monoclonal antibodies means that their specificity for these other species is affected. This has important implications for the use of RDTs to diagnose *P. falciparum* and *P. vivax* infections in areas of Southeast Asia where *P. knowlesi* is also prevalent. We demonstrate here that the previously reported specificities of the OptiMAL-IT RDT elsewhere in Southeast Asia of 94.7% for *P. falciparum* (with or without other species) and 96.5% for non-*P. falciparum* species (34) are not applicable to areas with higher *P. knowlesi* endemicity. The OptiMAL-IT RDT, in particular, had a much lower specificity for *P. falciparum* of 39%. The CareStart RDT specificity of 71% for *P. vivax* was also lower than would be expected in areas where *P. knowlesi* is not endemic.

A limitation of the RDTs used in this study was that even when they were used in combination they could not differentiate between *P. knowlesi* and mixed *P. knowlesi*-*P. vivax* infections, which would have implications for radical *P. vivax* cure. In Malaysian Borneo, the prevalence of mixed *P. knowlesi*-*P. vivax* infections appears to be low, with only one reported PCR-confirmed case in Sarawak (14) and 5/387 (1.3%) in our recent prospective study in Sabah (15). A separate study in Sabah reported 83 *P. knowlesi*-*P. vivax* mixed infections (35) but used earlier PCR primers that can include a false-positive *P. vivax* result for true *P. knowlesi* monoinfections (32). In other areas where *P. knowlesi* malaria is endemic, such as Thailand, India, and Myanmar, mixed *P. knowlesi*-*P. vivax* infections have been reported, with prevalences ranging from <1 to 9% (3, 36, 37). Comparing specificity against controls without malaria may also have assisted in ascertaining whether false-positive results may have been interpretation or reporting errors rather than real cross-reactivity between pLDH monoclonal antibodies directed at other *Plasmodium* species. While it is recommended that RDT evaluations be conducted against the gold standard, microscopy (38), the known difficulties in microscopic diagnosis and reporting of *P. knowlesi* (18) meant that the RDT sensitivities were analyzed by using PCR results.

We hypothesized that the use of a combination of pLDH and HRP2 tests may make it possible to differentiate *P. knowlesi* from other *Plasmodium* monoinfections. While the RDT combination did give a high specificity of 96% for *P. knowlesi* against other *Plasmodium* monoinfections, the low overall sensitivities prevent these RDTs from replacing standard microscopy as a diagnostic tool for *P. knowlesi* in this region. The cost of using two commercial RDTs following positive microscopy to improve specificity is also not feasible in many settings. For *P. falciparum*, RDTs containing an HRP2 component that does not cross-react with *P. knowlesi* should therefore be used. The ideal RDT combination would also contain a *P. vivax*-specific pLDH monoclonal antibody that does not cross-react with *P. knowlesi* and would therefore enable accurate diagnosis of *P. vivax* in order to guide the need for radical primaquine therapy. Other, more sensitive, molecular diagnostic tools, such as loop-mediated isothermal PCR (LAMP) targeting the AMA-1 or beta-tubulin *P. knowlesi* gene, have demonstrated test detection limits as low as 10 to 10^2 copies, respectively, with reported sensitivities and specificities of 100% (39, 40). The sensitivity of LAMP may be ideal at a district hospital level in Malaysia, given that the majority of *P. knowlesi* patients present with very low parasite counts. LAMP is also at least as sensitive and specific as nested PCR (40), with the added advantage of being able to be conducted with less training and equipment and at isothermal temperatures. However, the benefit of improving the sensitivity of immunochromatographic RDTs for use in more remote clinics with declining rates of malaria infection due to other *Plasmodium* species and parallel deskilling of health clinic level microscopists should encourage the development of *P. knowlesi*-

TABLE 5 Performance characteristics of combined use of CareStart and OptiMAL-IT RDTs compared with those of microscopy for the diagnosis of *P. knowlesi* malaria

	No. of patients with:						Likelihood ratio	
Method and result	PCR-confirmed *P. knowlesi*	PCR-confirmed other *Plasmodium* species	% Sensitivity (CI)	% Specificity (CI)	% PPV[b] (CI)	% NPV[c] (CI)	Positive test	Negative test
RDTs[a]								
Positive	45	5	25 (19–32)	96 (91–99)	90 (78–97)	47 (40–53)	6.17	0.78
Negative	136	119						
Microscopy								
Positive	143	15	74 (67–80)	89 (82–94)	91 (85–95)	70 (63–77)	6.57	0.29
Negative	50	118						

[a] Equivocal result for VOM-pLDH, negative result for *Pf*-HRP2, equivocal result for pan-pLDH, and positive result for *Pf*-pLDH.
[b] PPV, positive predictive value.
[c] NPV, negative predictive value.

specific monoclonal antibodies. Improved diagnosis of *P. knowlesi* would assist in the collection of prevalence data to guide public health strategies for this emerging and potentially fatal infection.

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