



Detection of *Plasmodium* Infection by the *illumigene* Malaria Assay Compared to Reference Microscopy and Real-Time PCR

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ABSTRACT Malaria is one of the leading causes of infectious disease in travelers returning from the tropics. The diagnosis of malaria is typically performed by examining Giemsa-stained thick and thin peripheral blood smears, which is time consuming, labor intensive, and requires high levels of proficiency. Alternatively, loop-mediated isothermal amplification (LAMP) is a new molecular method, which is rapid, sensitive, and requires less capital equipment and technological training. We conducted a retrospective study comparing two formats of a commercial LAMP assay (Meridian *illumigene* malaria [M] and malaria Plus [MP]) versus reference microscopy on archived blood specimens ($n = 140$) obtained from unique returning travelers suspected of having malaria. Discrepant results were resolved by either repeat testing or a laboratory developed ultrasensitive real-time PCR method. On initial testing, the Meridian *illumigene* M and MP kits had sensitivities of 97.3% (95% confidence interval [CI], 90.7 to 99.7%) and 100.0% (95.1 to 100.0%) and specificities of 93.8% (84.8 to 98.3%) and 91.5% (81.3 to 97.2%), respectively, versus reference microscopy. We project a significant cost reduction in low prevalence settings where malaria is not endemic with LAMP-based malaria screening given the excellent negative predictive value achieved with LAMP.

KEYWORDS diagnostics, LAMP, malaria

Malaria is a common disease globally. The World Health Organization estimates the *Plasmodium* parasite infected 212 million people in 2015 (1). Malaria is also one of the most common infectious diseases found in travelers returning from the tropics. Between 2004 and 2014, CanTravNet surveillance, a network of travel clinics in large urban centers, reported that malaria was the most common specific cause of fever in returning travelers (2). Malaria is often preventable during travel using readily available chemoprophylaxis or personal protective measures such as insecticide-treated bed nets and clothing (3). *Plasmodium falciparum* is the most virulent species for malaria and must be promptly diagnosed to prevent infectious complications or death due to a delay in diagnosis, particularly in returning travelers (4). Malaria-related hospitalizations in the United States are more frequent than once thought according to a recent study which identified 22,029 hospitalizations in a 15-year period, with an average length of stay of 4.36 days at a cost of US\$25,789 (5).

Malaria diagnosis in most clinical microbiology laboratories continues to rely on microscopic examination of Giemsa-stained thick and thin peripheral blood smears, often in combination with rapid diagnostic tests (RDTs), which detect the circulating

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parasite antigen. However, this method is time consuming, labor intensive, and challenging in areas where malaria is not endemic, as it requires continuous proficiency training where positivity rates are low. Microscopy may also miss malaria infections with low peripheral blood parasitemia levels (such as asymptomatic cases and those involving pregnancy or cerebral malaria), as it cannot reliably detect parasite densities <100 parasites/ μl depending on the technologist's experience, which is often limited in areas where malaria is not endemic (6, 7). In fact, a four-year retrospective study in our region demonstrated that microscopy is only able to detect 90.4% (150/166) of high-risk *P. falciparum* cases compared with that of quantitative PCR (qPCR) (8). Numerous studies have demonstrated that molecular testing using PCR has increased the analytical sensitivity compared with that of microscopy (9–12). The limit of detection (LOD) of PCR is lower than that of microscopy, and can detect at or below 1 parasite/ μl depending on the specific molecular approach used (e.g., nested PCR or real-time PCR) (11). Both microscopy and molecular testing are time consuming and challenging to perform in smaller diagnostic laboratories or as near-patient tests. RDTs rely on antigen detection from a drop of blood and are easy to perform. However, RDTs lack analytical sensitivity with limits of detection higher than 100 parasites/ μl , resulting in low sensitivities reported especially for non-*falciparum* *Plasmodium* species (12). Molecular-based isothermal tests for field diagnosis of malaria have recently been well studied in resource-limited settings with acceptable clinical results (12–24).

Loop-mediated isothermal amplification (LAMP) is a one-step amplification technique requiring specialized primers, which utilize self-recurring strand displacement to amplify DNA at a single temperature parameter (25). Sensitivity and specificity are dependent on primer development, species, sample type, target gene choice (e.g., mitochondrial DNA or ribosomal DNA), extraction methods (RNA, DNA, or both), and blood source (filter paper blood dots or whole blood). Reported analytical sensitivities vary from study to study but typically are at 1 parasite per μl or less (22, 26–33). Limits of detection of 0.3 to 2 parasites/ μl for *P. falciparum* and 0.1 for *P. vivax* μl have been reported, although the manufacturer's product insert reports even lower limits of detection (see Materials and Methods) (34). The commercially available *illumigene* malaria LAMP assay (Meridian Bioscience) was tested in Senegal, coupled with molecular confirmation in a reference laboratory in the United States, and yielded promising results in terms of assay performance, ease of use, reagent stability, and simplified extraction technology (7). However, no data have yet been published on the performance of the *illumigene* malaria test in a large clinical laboratory in a setting where malaria is not endemic. Moreover, no information exists on how LAMP technology would be most efficiently used within current diagnostic malaria algorithms in settings where malaria is not endemic or on the potential impact on turnaround time, cost, and feasibility in a 24-h 7-day laboratory service. The *illumigene* malaria LAMP assay has the potential to allow for more sensitive detection of *Plasmodium* parasites, with a rapid turnaround time, with decreased capital investment, and without requiring molecular specialist expertise.

This study retrospectively assessed collected *Plasmodium* samples ($n = 140$) from returning travelers between 2003 and 2014 using the *illumigene* malaria LAMP method and compared the results with those from reference microscopy. Results that were initially discrepant were resolved by an in-house validated real-time PCR (9). A revised, cost-effective alternative diagnostic algorithm is proposed based on our finding that incorporates the routine use of LAMP to provide more-sensitive screening of possible malaria cases than current rapid methods provide.

RESULTS

Initial detection of malaria using routine microscopy versus LAMP. A total of 140 patient blood samples were studied using both LAMP methods (Table 1). The performance of routine microscopy versus the M/MP LAMP assays is shown in Table 2. Thick and thin microscopy was performed on all 140 blood samples, and 76 (54%) of the samples were positive for *Plasmodium* parasites in asexual stages. *P. falciparum* was

TABLE 1 Thick and thin film microscopy results for patient samples enrolled in the study

Result	No. of samples							Total
	<i>P. falciparum</i>	<i>P. falciparum</i> / <i>P. malariae</i> mixed	<i>P. vivax</i>	<i>P. ovale</i>	<i>P. vivax</i> / <i>P. ovale</i> mixed	<i>P. malariae</i>	Negative	
Positive	38	2	25	8	2	1		76
Negative							64	64
Total	38	2	25	8	2	1	64	140

the most common species and occurred as a mono-infection in 38 (27%) of the samples. Mono-infections with *P. vivax*, *P. ovale*, and *P. malariae* also occurred in 25 (18%), 8 (6%), and in 1 (1%), respectively. Four samples had dual mixed infections: 2 with *P. falciparum* and *P. malariae* and 2 other samples with *P. vivax* and *P. ovale*. Compared with that of microscopy, the M kit had a sensitivity of 97.3% (95% confidence interval [CI], 90.7 to 99.7%) and a specificity of 93.8% (84.8 to 98.3%). The MP kit compared with reference microscopy had a sensitivity of 100.0% (95.1 to 100.0%) and a specificity of 91.5% (81.3 to 97.2%). There was no significant difference between the initial performance of the M and MP assays, suggesting that DNA sample extraction was equivalent for both kit methods.

Resolution of discrepant results with illumigene M kit. There were 6 discrepancies between the M kit and microscopy (Table 3). Of these, 2 occurrences were detected with microscopy alone and 4 cases were detected with the M kit only. Both of the false-negative M kit results were positive on repeat testing. Of the 4 samples that were positive by the M kit and negative by microscopy, 3 were positive by qPCR, suggesting they were true-positive results. The remaining specimen was negative with repeat testing.

Resolution of discrepant results with illumigene MP kits. There were 4 discrepancies between the results of the MP kit and microscopy, all due to false positives (Table 4). Of the 5 that were falsely positive, qPCR confirmed two were actually positive. Two of the falsely positive tests were negative on repeat testing, in line with the microscopy result, and one remained falsely positive due to insufficient sample volume to repeat the test.

Resolution of invalid results. Of note, there were 8/140 (5.7%) occurrences where the MP kit results were considered “invalid” by the instrument and 1/140 (<0.01%) invalid results on the M kit. When repeated from freshly thawed new aliquots as per the product insert, 7 were resolved with successful runs, while 1 sample remained invalid in each kit. An invalid result is where the reaction is completed and the final result is

TABLE 2 Performance of the illumigene malaria M and MP kits

Result ^a	illumigene malaria kit	
	M	MP
True positive (<i>n</i>)	73	73
False positive (<i>n</i>)	4	5
False negative (<i>n</i>)	2	0
True negative (<i>n</i>)	60	54
Invalid result ^b (<i>n</i>)	1	8
Total ^c (<i>n</i>)	139	132
Sensitivity (% [95% CI])	97.3 (90.7–99.7)	100.0 (95.1–100.0)
Specificity (% [95% CI])	93.8 (84.8–98.3)	91.5 (81.3–97.2)
Negative predictive value ^d (%)	99.8	100
Positive predictive value ^d (%)	45.2	38.2
Invalid rate (%)	<0.01	5.7

^aResults were compared with those from reference microscopy.

^billumigene result was recorded as “invalid” due to a failed run. “Empty well” and instrument errors were rerun from a fresh extract as per the product insert and included in the performance calculations.

^cInvalid results were not included in the sensitivity and specificity calculations.

^dBoth negative and positive predictive values are calculated based on a prevalence of malaria of 0.05 (5%) in the returning traveler population.

TABLE 3 Discrepant resolution by repeating *illumigene* M test or in-house qPCR to detect *Plasmodium* spp.

Study ID ^a	Result ^b				
	Microscopy	<i>illumigene</i> malaria test		In-house qPCR	Final
		Original	Repeat		
N02	<i>P. vivax/P. ovale</i> ^c	NEG	POS	POS (<i>P. ovale</i>)	TP
N17	<i>P. vivax</i>	NEG	POS	POS (<i>P. vivax</i>)	TP
N93	NEG	POS	Not done ^d	POS (<i>P. falciparum</i>)	TP
N55	NEG	POS	NEG	NEG	TN
S32	NEG	POS	Not done ^d	POS (<i>P. falciparum</i>)	TP
S62	NEG	POS	Not done ^d	POS (<i>P. falciparum</i>)	TP
N119	<i>P. ovale</i>	Invalid	Invalid	POS (<i>P. ovale</i>)	Invalid

^aID, identifier.

^bNEG, negative; POS, positive; TP, true positive; TN, true negative.

^c*P. vivax/P. ovale* implies the microscopist was unable to distinguish the two species and gave a slash call result.

^d*illumigene* test was not repeated if the real-time qPCR agreed with original M kit result.

"invalid." Both types of invalid results were resolved after repeat testing from a freshly thawed specimen with particular attention to sample mixing. The invalid rate was reduced to 1 in 140 (<0.01%) samples with both kits. Invalid results were not included in the initial sensitivity and specificity calculations.

Cost analysis. A detailed cost analysis (Table 5) was performed to evaluate the impact of a revised malaria testing algorithm (Fig. 1). The current algorithm relies on both thin and thick film microscopy coupled with RDT (BinaxNOW malaria test) to evaluate EDTA blood submitted for malaria testing with repeat microscopy on negatives. The RDT serves as an adjunct to confirm microscopic results. This algorithm is typical in the setting where malaria is not endemic. Costs were attributed to medical laboratory assistant and technologist times for preparing and reading smears and performing RDTs and to consumables and kits. The costs were based on current labor charges and benefits in our setting for 2017. The kit and consumable costs were based on approximate prices. The major finding is that by reducing the need for repeat microscopy on negative initial results (3 times every 6 to 8 h), labor costs are reduced

TABLE 4 Discrepant resolution by repeating *illumigene* MP test and in-house qPCR to detect *Plasmodium* spp.

Study ID ^a	Result ^b				
	Microscopy	<i>illumigene</i> malaria Plus test		In-house qPCR	Final
		Original	Repeat		
N118	NEG	POS	Not done ^e	NEG	FP
N55	NEG	POS	NEG	NEG	TN
N34	NEG	POS	NEG	NEG	TN
S32	NEG	POS	Not done ^c	POS (<i>P. falciparum</i>)	TP
S62	NEG	POS	Not done ^c	POS (<i>P. falciparum</i>)	TP
N17	<i>P. vivax</i>	Invalid	POS	Not done ^d	TP
N23	<i>P. vivax</i>	Invalid	POS	Not done ^d	TP
N57	NEG	Invalid	NEG	Not done ^d	TN
N59	NEG	Invalid	NEG	Not done ^d	TN
N75	NEG	Invalid	NEG	Not done ^d	TN
N80	NEG	Invalid	Invalid	NEG	Invalid
N93	NEG	Invalid	POS	POS (<i>P. falciparum</i>)	TP
S82	<i>P. ovale</i>	Invalid	POS	Not done ^d	TP

^aID, identifier.

^bNEG, negative; POS, positive; TP, true positive; FP, false positive; TN, true negative.

^c*illumigene* test not repeated if real-time PCR agreed with original MP kit result.

^dqPCR was not done if *illumigene* MP repeat result was concordant with microscopy.

^eInsufficient sample volume to repeat test.

TABLE 5 Cost analysis performed on current malaria testing algorithm at our institution compared to a proposed workflow where LAMP is conducted as the initial screen^a

Malaria testing algorithm	No. of specimens per year	MLA FTE	MLT FTE	Cost (US\$)			
				Yearly			Per specimen
				Materials	Labor	Total	
Current							
Malaria thick and thick film microscopy and RDT to screen, repeat thick and thin film microscopy on negatives	1,776	0.03	0.98	12,497.06	82,531.76	95,029.06	53.51
Proposed^b							
LAMP to screen and report negatives as final, work-up for positives only with RDT and thick and thin film microscopy	1,440	0.02	0.24	35,947.06	22,118.82	58,065.88	40.32
Cost/FTE differential	336	0.01	0.74	23,450.00	60,412.94	36,963.18	13.19
	↓	↓	↓↓	↑↑	↓↓	↓↓	↓

^aAll values are in US dollars as of April 2017. Yearly costs are based on 2017 estimates for labor and materials. MLA, medical laboratory assistant; MLT, medical laboratory technologist; FTE, full-time equivalent; RDT, rapid diagnostic test (BinaxNOW malaria test); ↓, decreased costs; ↑, increased costs. Assumptions made include 4 thick and thick films made per patient and 3 microscopy repeat tests performed per patient when negative. Testing from patients with an initial positive microscopy was also repeated until parasitemia cleared. A positivity rate of less than 5% is assumed.

^bSee Fig. 1 for proposed workflow.

dramatically. Therefore, overall, in spite of an increase in material costs with the LAMP algorithm, a per-patient cost savings of US\$13.19 is obtained.

DISCUSSION

We have conducted the first diagnostic study in North America with *illumigene* Malaria—a commercially available LAMP-based malaria test—on returning travelers. LAMP-based methods for the detection of malaria have been available on a research-use-only basis for over a decade (28). Results from studies performed both in settings

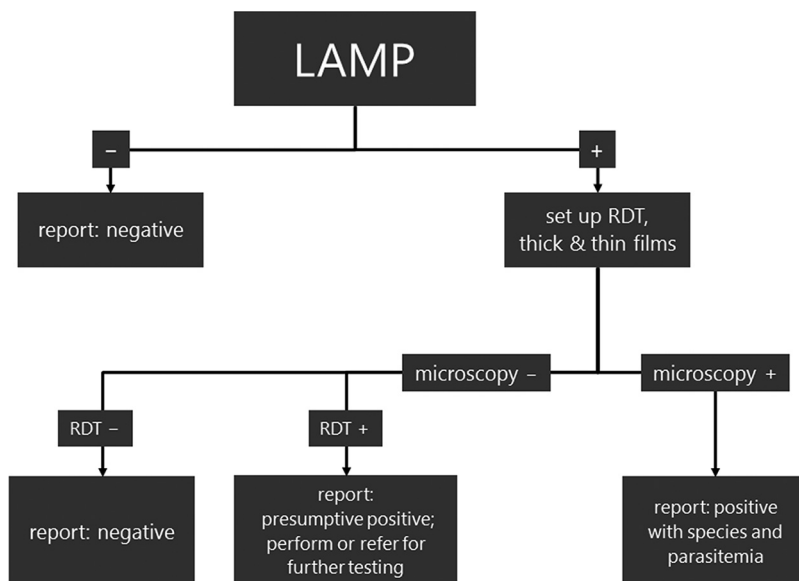


FIG 1 A proposed algorithm for malaria testing using loop-mediated amplification (LAMP) technology as a screen is presented. In the new algorithm, no repeat testing is required to rule out malaria due to the excellent sensitivity. A microbiologist consultation is recommended when LAMP results are positive and results for other tests are negative. Positives require confirmation for species and parasitemia using traditional methods. In a setting where malaria is not endemic, positives are rare and thus a cost-savings is projected due to the reduced labor associated with making thick and thin peripheral blood films. Validation of this proposed algorithm would require a prospective trial design.

where malaria is endemic and where it is not endemic have suggested that LAMP is more sensitive than rapid diagnostic tests (RDTs) such as the BinaxNOW malaria test, especially for the non-*falciparum* species (13, 17, 30, 35). LAMP appears to perform on an equal footing with other molecular techniques such as nested PCR (35). LAMP may be especially useful for the screening of low-level parasitemia due to its analytical sensitivity (17). LAMP has the distinct advantage of relying on a strand-displacing enzyme that performs at a single temperature (usually 62°C to 65°C), tolerates less-stringent nucleic acid extraction methods, and can be detected with simple turbidity measurements (25). At least two LAMP kits are now commercially available for malaria diagnosis, the Loopamp malaria "Pan/Pf" detection kit (Eiken Chemical Co. Japan) and the *illumigene* malaria assay tested here, both targeting mitochondrial DNA of *Plasmodium* spp. for genus-level identification. Of note, the Loopamp malaria Pan/Pf detection kit can distinguish *P. falciparum* (using 18S rRNA) from other species, whereas the *illumigene* malaria test cannot, and has demonstrated excellent sensitivity in published studies (13, 30, 34). Identifying *P. falciparum* is clinically useful because of this organism's high pathogenic potential. Both kits rely on lyophilized reagents that enable these assays to be performed without the refrigeration of reagents, a major advantage in areas where malaria is endemic. The *illumigene* assay comes in two versions: the M kit, which relies on simple lysis filtration for extraction, and the MP kit, which has an extra gravity-flow column. Neither format requires centrifugation or thermal treatment, which again is a major advantage in situations where sophisticated equipment is a barrier. The only published study to date of the *illumigene* malaria assay is by Lucchi and colleagues (34), who demonstrated a sensitivity of 97.2% for both extraction methods and a specificity of 87.7% with the simplified extraction (M kit) and a specificity of 93.8% for the MP kit in comparison to a PCR gold standard. The work was performed in a clinical laboratory in Senegal (a resource-limited setting in Africa where malaria is endemic). Their study also showed no difference in performance between banked retrospective specimens and fresh specimens when using the *illumigene* malaria assay.

Our study was performed in a clinical laboratory in North America using fresh-frozen specimens from returning travelers. All species except *P. knowlesi* are represented in our sample set. Our results demonstrate that excellent sensitivity was achieved by both simplified (M, 97.3%) and gravity-flow (MP, 100%) extraction kits in comparison to that of reference microscopy. A high invalid rate of 5.7% was observed with the MP kit. The reasons for this are unclear, but we speculate this may have to do with the resin beds and filters that may be disturbed during transportation, affecting performance and/or the amount of heme present in the thawed sample used here. We noted that many of our frozen specimens had considerable erythrocyte lysis. A prospective study using fresh specimens may shed light on this issue. Repeat testing with the same assay lot and instrument resolved 7 of 8 MP kit invalid results and gave the correct identification in relation to that from qPCR, which suggests the technologist's level of training may also play a factor.

Both M (99.8%) and MP (100%) kits achieved excellent negative predictive values, assuming a 5% prevalence in returning febrile travelers. False positives were noted with both M and MP kits in comparison to that with microscopy, even after discrepant analysis with qPCR. While contamination has been suggested to be a significant risk with LAMP technology, this was not observed in our study with strict adherence to decontamination and a one-way flow format, where closed-tube reaction products are discarded in a location different from where the extraction takes place (15). The assay is simple to perform and can be completed in 60 min. By comparison, the malaria RDTs used in our laboratory (BinaxNOW malaria test; Alere, Waltham, MA) can be completed in 25 min. The excellent sensitivity and negative predictive value in a setting of low prevalence and the ease of performance suggest that this test may be best used as a screen for malaria. Positives would require further confirmation with microscopy given the lower positive predictive value and inability to determine the identity to the species level or to quantify infections. Our previous study demonstrated that LAMP method-

ology is superior to that of RDTs, especially in terms of sensitivity—RDT sensitivity was on the order of 70% for non-*falciparum* species and 90% for *P. falciparum* in a similar archived sample set (35). RDTs rely on antigens which vary between species and thus affect test performance. Deletions in key genes encoding diagnostic antigens are a growing concern (12).

Limitations of this study include the retrospective nature of the design. Discrepant resolution was performed only on a subset of specimens using qPCR, which may introduce bias. Frozen samples may not perform in the same way as fresh samples, although our in-house experiments suggest no loss in signal after four freeze-thaw cycles with both kits, even at a parasitemia of <0.1%. Other limitations include not having evaluated the impact of “empty well” errors, instrument errors, and invalid results in real time. Delays due to repeat testing could be significant, as physicians expect a rapid turnaround (<2 h) for this critical test. Furthermore, we did not have *P. knowlesi* specimens to test in our bank. Determinations of whether an infection results from pathogens at a specific sexual stage (gametocytes) cannot be made with the assay evaluated here. Therefore, microscopy is still required. Infections with gametocytes only do not require treatment and often reflect previous antimalarial therapy.

For LAMP, the major outstanding question is whether a negative result with LAMP implies no further testing is required. Lucchi and colleagues obtained a limit of detection of 2 parasites/ μ l with the *illumigene* malaria assay, which is far superior to those of microscopy and RDT and is in keeping with other reports on LAMP technology (22, 27–32, 34, 41). In our setting, a highly sensitive screen, excellent LOD, and ease of use would mean no repeat testing is required and no peripheral blood film examination is required on over 95% of our specimens, based on current positivity rates. Our current algorithm for malaria testing relies on 4 thick and thin slides being made together with a rapid diagnostic (BinaxNOW malaria) test for testing. The RDT result is released as a preliminary result until thin and thick films are read for final confirmation of species and parasitemia. Initial negatives are repeated every 6 to 8 h on average 3 times to ensure no parasites are seen, as per CDC recommendations (36). While this is prudent given the limitations of microscopy, this adds a significant labor cost.

Given the significant gain in analytical sensitivity over RDTs and microscopy reported in the literature, we proposed an alternative algorithm for malaria screening in a setting where malaria is not endemic (Fig. 1). In a four-year review conducted in our jurisdiction, a sensitivity of 90.4% (150/166) for the detection of *P. falciparum* was achieved using our current algorithm (microscopy and RDT) compared with that from qPCR (8). This poses a significant mortality risk given the risk of fulminant disease with this species. The LAMP approach we propose, with its ease of sample preparation, rapid result (60 min), and improved negative predictive value compared with that from traditional RDTs, would be used as a screen and reported as a final result if it is negative and a preliminary genus-level result if it is positive. Presumptive positives (<5% of samples in most settings where malaria is not endemic) would then be confirmed with thin and thick film microscopy for determination of species and parasitemia. A rapid diagnostic test can also be used on positives if microscopy expertise is not present to distinguish *P. falciparum* from other species. If the revised algorithm were to be implemented, this would amount to a significant cost savings of US\$13.19 per malaria test in the setting where malaria is not endemic. A prospective trial in this regard would be of great value in cementing the utility of this approach, resolving the issue of repeat testing and obtaining regulatory approval for use of this assay in North America.

MATERIALS AND METHODS

Patient population and sample collection. Calgary Laboratory Services (CLS) collected samples and performed all of the described malaria testing. CLS is a large centralized microbiology laboratory service covering 1.3 million people. All of the previously archived stored study samples ($n = 140$ blood samples) were collected between 2003 and 2014 from individual returning travelers presenting in our health care region with acute febrile symptoms soon after being in a region where malaria is endemic. Specimens were stored at -80°C . The demographic details and countries visited by our catchment population have been described previously (37). In brief, the majority of patients were in the 20 to 44 years age group and were visiting friends and relatives, with the majority of *P. falciparum* cases imported from sub-Saharan

Africa and *P. vivax* cases from the Indian subcontinent. Table 1 summarizes the malaria species present in this sample set. *P. falciparum* and *P. vivax* are the predominant species, followed by *P. ovale* and *P. malariae*. The relative contribution of each species is typical of most North American laboratories. No *P. knowlesi* was present in the sample set, although the assay is designed to detect this fifth species. Blood aliquot samples were stored in cryovials at -80°C until being enrolled into this study.

Microscopy and illumigene malaria assay. Microscopy was performed using standard Giemsa-stained thick and thin peripheral films at the time of initial diagnosis (38). Aliquots of freshly thawed venous EDTA whole blood were tested with the illumigene malaria (M) and illumigene malaria PLUS (MP) (Meridian Bioscience, Cincinnati, OH) assays, which are CE marked and Health Canada-approved for diagnostic use. Both LAMP assays use different proprietary centrifugation-free techniques for DNA extraction that are included as part of their kit: either a simple filtration method (M assay) or a gravity-driven gel filtration column (MP assay). Amplification is carried out using the illumipro-10 incubator/reader that detects the production of magnesium pyrophosphate, which in turn increases the turbidity of the samples. The M/MP LAMP assays detect the presence or absence of *Plasmodium* spp., but cannot differentiate to the species level. The manufacturer claims increased analytical sensitivity with the MP assay (0.063 *P. vivax* parasites/ μl and 0.25 *P. falciparum* parasites/ μl) versus the M assay (0.125 *P. vivax* parasites/ μl and 2 *P. falciparum* parasites/ μl). The product insert reports a sensitivity of 100% (M and MP kits) and specificities of 89.3% (M kit) and 82.7% (MP kit) versus microscopy. The assay has only been verified on purified genomic DNA of *P. knowlesi*. M and MP LAMP assays were run in parallel on single blood samples strictly according to the manufacturer's instructions. According to the manufacturer's product insert, if the illumipro-10 has an "empty well" error on the instrument panel prior to amplification, an "invalid" result reported after the amplification, or "instrument error" result, then the specimen should be reextracted from the beginning and rerun.

Resolution of discrepancies. Discrepant tests occurred when the initial results of microscopy and either the M/MP LAMP assay results were different. If an invalid, empty well, or instrument error occurred, repeat M/MP LAMP assays were performed using another frozen aliquot of the patient's blood sample. A fully validated in-house real-time PCR (qPCR) method was subsequently used to verify discrepant results that were not resolved by repeat LAMP testing (9, 39). The qPCR assay has a sensitivity and specificity of 100% in mono-infections (27). Primers and probes target the 18S rRNA gene and comprise a genus-specific target coupled with a species-specific target which enables identification to the species level (27). The assay has been further optimized for the detection of *P. ovale* and *P. malariae* as described previously (40).

Cost analysis. A cost comparison was performed for our current testing algorithm, which includes microscopy and rapid diagnostic tests (BinaxNOW malaria test), with a proposed algorithm with LAMP as the screening test (Fig. 1). The assumption made is that LAMP with its negative predictive value approaching 100% would permit a final negative result to be reported. Only positives would require further work-up by microscopy for identification to the species level and quantitative parasitemia. In our current algorithm, negative initial microscopy results are repeated every 6 to 8 h up to a maximum of 3 times as per CDC recommendations (36).

Statistical analysis. Data were entered into Microsoft Excel (Microsoft Professional Office 2010; Microsoft, Seattle, WA), and diagnostic test performance was determined by standard statistical methods executed using SISA online statistical software (<http://www.quantitativeskills.com/sisa/>).

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