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NINA-LAMP compared to microscopy, RDT, and nested PCR for the detection of imported malaria

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Abstract

Microscopy and field adaptable rapid diagnostic tests (RDTs) are not sensitive and specific in certain conditions such as poor training of microscopists, lack of electricity, or the inability to detect non-*falciparum* malaria. More sensitive point of care testing (POCT) would reduce delays in diagnosis and initiation of therapy. In the current study, we have evaluated the efficacy of non-instrumented nucleic acid amplification (NINA) coupled with LAMP for detection of traveler's malaria (n = 140) in comparison with microscopy, nested PCR, and the only FDA-approved rapid diagnostic test. NINA-LAMP was 100% sensitive and 98.6% specific when compared to nested PCR. For non-*falciparum* detection, NINA-LAMP sensitivity was 100% sensitive compared to nested PCR whereas RDT sensitivity was 71%. LAMP is highly sensitive and specific for symptomatic malaria diagnosis regardless of species in a POCT setting.

Keywords

malaria; point of care test; LAMP

Introduction

Due to the effective implementation of Malaria Control Programs (MCPs), global malaria cases, and the associated mortality have decreased by 26% and 46% respectively since 2000 (42). As evidence of progress, 39 countries have now refocused their programs toward elimination (12). However, artemisinin resistant *Plasmodium falciparum* has already

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emerged in South-east Asia and has become a major concern for global health (3, 23). Gold standard microscopy can only achieve a maximum of 80–90% accuracy when used in the field and only when conducted by expert malaria microscopists (34). Failure to detect submicroscopic infections results in untreated patients can be a driving force for the spread of resistant and slow clearing parasites throughout South-east Asia and promulgates the risk of the global spread of drug resistant malaria. Similarly, RDTs currently used in field settings also have limitations such as low sensitivity, false positivity due to cross reactivity, and variable stability at the field site (2, 25). RDTs have especially poor performance in the detection of non-*falciparum* malaria (7, 39). Although PCR based methods are accurate for low parasitemia *Plasmodium* infection detection, logistical issues often make this technique incompatible as a POCT (24). The number of malaria cases imported into non-endemic countries such as Canada, the US and Europe continues to increase, likely as a result of globalization and changing immigration and travel patterns (8, 19, 29). In 2009, 35% of Canadian travelers bound to destinations other than the United States travelled to regions with a risk of malaria, a 131% increase from 2000 (11). In addition to the increased incidence of malaria cases seen in Canada, the number of cases severe malaria due to *P. falciparum* is also increasing each year (19, 21). Travelers to malaria endemic areas may not always appreciate their risk of malaria and do not take malaria prophylaxis or use personal protective measures against mosquito bites (5). Travel for the purpose of “visiting friends and relatives” (VFR) represents a particularly high-risk group of travelers for the acquisition of malaria and is becoming an increasingly important group of travelers (4, 10, 18). Even in well-resourced settings adverse outcomes including death due to malaria may still occur. Misdiagnosis or delay in diagnosis leading to delay in initiating appropriate anti-malarial therapy are factors that contribute to adverse outcomes (26). Diagnosis of malaria in non-endemic settings such as Canada may also be challenging because physicians and laboratory personnel outside of major tertiary care centers may have limited access to gold standard testing and rely on RDTs.

Loop Mediated Isothermal Amplification (LAMP) as an approach for isothermal nucleic acid amplification was first developed in 2000 (28). The LAMP assay for *P. falciparum* was introduced in 2006 (33) and subsequently a genus and species specific LAMP assay was developed by Han and co-authors targeting 18S rDNA sequence (13). The visual detection system of LAMP amplified nucleic acids was improved by pre-addition of calcein and Hydroxynaphtholblue (HNB) to the LAMP reaction mixture (24, 41). A mitochondrial DNA LAMP assay for *Plasmodium* species was also developed to increase the sensitivity (32). Along with in-house methods, a commercial kit for LAMP assay was developed by Eiken Chemical Company, Japan and evaluated (14, 31). Real-time fluorescence LAMP with a portable, rechargeable fluorescence reader was also used for malaria detection in the field (30). Importantly, the LAMP method is robust and tolerant to amplification in the presence of blood impurities and therefore does not require pure RNA/DNA like PCR. Hemoglobin, antibodies, and other blood content that strongly inhibit common commercial PCR DNA polymerases (e.g. *Taq*, *Pfu*) do not have as strong an effect on *Bst* DNA polymerase adapted for LAMP reactions. *Bst* polymerase amplifies DNA with great accuracy at constant temperature via inherent strand displacement activity, which eliminates the requirement for thermal cycling (1).

Despite these developments in nucleic acid based detection of malaria parasites, LAMP assays, including sample preparation and amplification detection, still require a heat block or water bath, a centrifuge, and optionally an expensive fluorescent reader, all of which often require an electricity supply. Program for Appropriate Technology in Health (PATH) has developed non-instrumented nucleic acid amplification (NINA) platform heater (Figure 1) to facilitate isothermal amplification of nucleic acids at the point of care. This heater does not require any electricity or other instrumentation as heat generation derives from Mg-Fe exothermic chemical reaction and is tuned to the target isothermal assay temperature via coupling to an engineered phase change material (PCM) (16, 17, 36, 37, 40). Previously our group evaluated NINA-LAMP performance in Ethiopia for symptomatic malaria diagnosis in comparison with microscopy and nested PCR (35). In the current study, we sought to answer two questions: (1) How does the performance of the latest version of the NINA heater compare to a standard PCR thermo-cycler for detecting malaria parasite infection in returning travelers using microscopy and nested PCR as gold standard, and (2) How does LAMP compare to the BinaxNOW malaria RDT head-to-head in order to determine the feasibility of the LAMP as a POCT in the non-endemic setting for returning travelers.

Materials and Methods

Sample collection

The returning traveler samples used in the present study were previously collected, curated, and stored at Calgary Laboratory Service, Alberta, Canada (CLS). Individuals who travel to malaria endemic regions may become infected by the *Plasmodium* parasite and develop the characteristic febrile symptoms of infection only following their returning to the Calgary region. Based on clinical symptoms and CLS standard procedure, travelers returning to Calgary from malaria endemic areas are routinely evaluated for malaria parasite infection via microscopy and the BinaxNOW malaria RDT. All samples were collected from 2003 to 2014. Whole blood samples were collected and aliquoted in EDTA tubes and stored at −80°C until use. The sample size was pre-calculated assuming 90% sensitivity and specificity according to the method described by Malhotra *et al.*, 2010 using a two-sided z-test at a significance level of 0.05 by considering precision at 10% (20). As a result, it was determined that at least 69 positive and 69 negative samples were needed to achieve significance in the analysis. Therefore, a total 69 microscopy positive and 71 microscopy negative samples were included.

Microscopy and RDT

Microscopy slides were prepared immediately after blood collection using standard methods. The Giemsa stained slides were then used to assess the proportion of red blood cells that were infected. Parasitaemia were determined by the proportion (%) of the red blood cells (RBC) that were infected and number of RBCs per micro-liter of blood. BinaxNOW malaria RDT tests were carried out following two different schemes. For samples collected from 2010–2014, RDT testing was performed immediately after collection. For previously collected samples (before 2010), RDT testing was carried out on frozen whole blood stored at −80°C according to the manufacturer’s instructions.

DNA extraction

Two different protocols were followed to isolate DNA for amplification. For nested PCR amplification, pathogen DNA was isolated using the QiaAmp blood DNA extraction kit (Qiagen, Germany) to elute an equal volume of DNA from whole blood. For LAMP amplification, the boil and spin method was used (32). Briefly, 20μL of the whole blood specimen was mixed with 20μL of extraction buffer (40 mM Tris (pH 6.5)-0.4% sodium dodecyl sulfate (SDS)) followed by incubation at 95°C for 5 minutes. The boiled specimen was then centrifuged at 10,000g for 10 minutes. Fifteen μL of the supernatant was diluted 10 times with distilled water and directly used for LAMP amplification. Figure 2 details the work flow used in the evaluation of all four methods performed in this study and described below.

Nested PCR

Nested PCR was carried out on QiaAmp extracted DNA according to the method described by Snounnu (38) with a minor modification. One micro-liter of DNA was used as template for the first amplification step. That amplification product was then diluted 10× and 2 μL was used for the next round of amplification. *Taq* DNA polymerase and other reagents from New England Biolabs (NEB) were used for amplification and products were visualized via SYBR Safe staining after amplicon separation on a 2% agarose gel.

LAMP assay

The LAMP assay was carried out using the Loopamp Pan/Pf Malaria detection kit (stable at room temperature) produced by Eiken Chemical Company (Taito-ku, Tokyo, Japan). Thirty μL of extracted and diluted DNA was added to each reaction tube. Duplicate reaction tubes were prepared for each sample for Pan/Pf detection according to the manufacturer's instructions. One tube was heated in the PCR thermo-cycler (Eppendorf Mastercycler, Eppendorf, Germany) set at 65°C and the other placed inside the NINA platform heater preheated to 65°C. LAMP reactions were carried out for 40 minutes. After 40 minutes, turbidity was measured by naked eye by three independent readers. Consensus from any two readers was considered as the final result. One *P. falciparum* (initial parasitaemia 125,000/μL) and one *P. vivax* (initial parasitaemia 25,000/μL) positive whole blood specimen (collected from two different patients) were diluted with uninfected blood to 10000, 1000, 100, 10, 5, and 1 parasite(s)/μL. Subsequently, the LAMP assay was carried out by NINA and PCR machine.

Results

In the case of serially diluted specimens, the LAMP assay in NINA and thermo-cycler were equally successful in detecting *P. falciparum* up to a lower limit of five parasites /μL for both Pan and Pf primer sets. On the other hand, for *P. vivax* minimum level of detection was one parasite/μL for both LAMP platforms. LAMP data, whether obtained from isothermal DNA amplification in the NINA platform heater or in the thermo-cycler, were consistent with the exception of one false negative with the LAMP platform. However, both NINA-LAMP and thermo-cycler-LAMP resulted in 2 false positive *P. falciparum* cases (negative by microscopy and nested PCR) each along with one false negative (positive by microscopy and

nested PCR) by NINA-LAMP. Pan LAMP assay results were consistent on all platforms throughout the study (Table 1). Importantly, nested PCR detected two additional positive cases from microscopy negatives and also three mixed infections. The results of the discordant analysis resolved are depicted in Figure 2. A mismatch where one or more assays differed from the original microscopic diagnosis was observed with NINA-LAMP 3 times, LAMP performed on the thermo-cycler (3), nested PCR (3), and RDT (12). Ability to detect *Plasmodium* genus (Pan) was considered in the overall accuracy analysis of LAMP performance. LAMP detected all microscopy positive samples resulting in an overall sensitivity of 100% (95% CI, 93.4–100; 69/69) and an overall specificity of 98.6% (95% CI, 91–99) compared to nested PCR considered as gold standard (Table 2). In contrast, the BinaxNOW malaria RDT had an overall sensitivity of 85.9% (95% CI, 74.5–92.4; 59/69) and specificity 98.6% (95% CI, 91.1–99.9) compared to nested PCR.

Discussion

The data presented in the current study demonstrate the excellent accuracy of the LAMP assay. The results of LAMP were equally accurate whether conducted in the electricity-free NINA platform heater or the PCR thermo-cycler. Irrespective of the standard (microscopy or nested PCR), the accuracy of the NINA-LAMP assay was satisfactory for all species. Performance of BinaxNOW RDT against non-*falciparum* cases was less successful with sensitivity of 74.2% (23/31) and 71% (22/31) as compared to microscopy and nested PCR respectively. Of the four *P. falciparum* cases missed by the BinaxNOW RDT, only one sample was previously frozen at −80°C before performing RDT while the other three samples were fresh blood. LAMP with NINA and thermo-cycler successfully identified all these cases. As the BinaxNOW test was carried out on fresh samples in the case of all non-*falciparum* RDT testing, low sensitivity is not attributable to the long storage time of the samples. The inability of the BinaxNOW RDT to detect non-*falciparum* (aldolase based detection) cases has already been demonstrated. Particularly, groups from Japan and France have reported poor performance in detecting *P. ovale* (6, 39). This lack of sensitivity can perhaps be attributed to lower production of aldolase by *P.ovale* (6, 7). In the present study, BinaxNOW RDT could detect only 80% of P. *ovale* cases. Overall (pan-specific) sensitivity of the BinaxNOW malaria RDT (85.5% and 85.9%) approximate the most recent evaluation in a US hospital (84.2%) and a previous study conducted in Toronto (83.7%) (9, 15). However, high sensitivity and specificity (97.2% and 93.6%) was reported from Ghana (27). We have observed excellent specificity of both the BinaxNOW RDT test and the Loopamp Malaria Pan/Pf assay. False positive cases used in the calculation came from the failure of standard methods (microscopy and nested PCR) to detect specific cases. False positive results for both the BinaxNOW and Loopamp have been reported previously (22, 35). Throughout the present study, LAMP positivity was determined by consensus visualization of turbidity in the reaction tube by naked eye for three independent readers. Only one instance of discrepant visual readout (only 2 of 3 readers agreed) was observed out of all 140 tests performed either with the NINA or thermo-cycler. Calcein fluorescence could not be utilized because samples were collected in EDTA coated collection tubes which quench the Calcein fluorescence as per manufacturer guidelines.

We observed results similar to that observed previously in a similar traveler's clinic at London (31). Similarly sensitivity of LAMP were demonstrated in field settings in Uganda (14) and Gondar, Ethiopia (35) by using the same Loopamp Malaria Pan/Pf kit from Eiken Chemical Company, Japan. Additionally, the high sensitivity and specificity of non-*falciparum* diagnosis observed in the current study is similar with previous controlled laboratory studies conducted in Calgary and London (31, 35). A major benefit of LAMP over RDT is the identification of non-*falciparum* cases which is critical for regions approaching elimination in Southeast Asia and Latin America (30). We have demonstrated equal efficacy of LAMP-based pathogen infection detection utilizing the NINA electricity-free platform heaters and a programmable PCR thermo-cycler for consistent maintenance of temperature throughout the assay. The single-use Mg-Fe fuel pack and saline buffer and the semi-reusable phase change material (PCM) required for NINA operation are very inexpensive costing approximately $0.11/test (10 samples). Currently, the PCM for this particular application can be used for at least 15 cycles. To our knowledge, the NINA platform heater is the least expensive DNA amplification system for point-of-care use at US $13 for the device start up cost. In comparison, a real time turbidity meter currently costs approximately US$28,000, a fluorescent scanner costs approximately US$6300, and a thermo-cycler can cost US$3000–8000. Other less expensive battery powered heaters do exist (Diagenetix, Hawaii, USA), yet even a very simple heat block starts at US$200 (30). The Eiken Loopamp Malaria Pan/Pf kit is produced in a 48-sample pack. The potential for cross contamination of samples exists. We have seen compromised specificity in the field (84.3% and 81.2%) due to contamination (35) also observed by others in Uganda (84.9%), although a specific reason has not been given in the latter study (14). As a result, we suggest an alternate individual use "blister" packaging of the kit, similar to RDT kits, to minimize the risk of contamination.

We are still dependent on electrical centrifugation and heat block for the proscribed boil and spin DNA extraction protocol. New extraction approaches suitable for coupling with the NINA-LAMP are necessary. Several improvements to the current NINA platform are likely required. The NINA used in this study was 12-well however greater throughput may be required for mass screening in elimination campaigns. Finally, an integrated sample-to-results closed system would be optimal to reduce the risk of contamination. We have not studied the effect of external temperature and humidity on the efficacy of NINA-LAMP in the present study.

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Highlights

- LAMP can be applied as a point of care test for malaria in returning travelers
- LAMP is more sensitive than standard RDTs especially for non-falciparum malaria
- LAMP is cost effective and requires minimal capital equipment investment

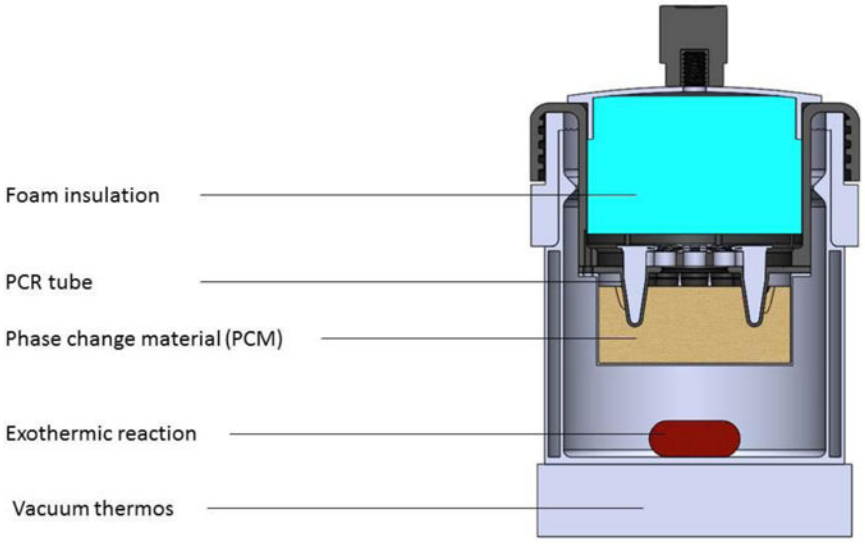

Figure 1.
Depiction of the Non-instrumented nucleic acid amplification (NINA) device used in the present study.

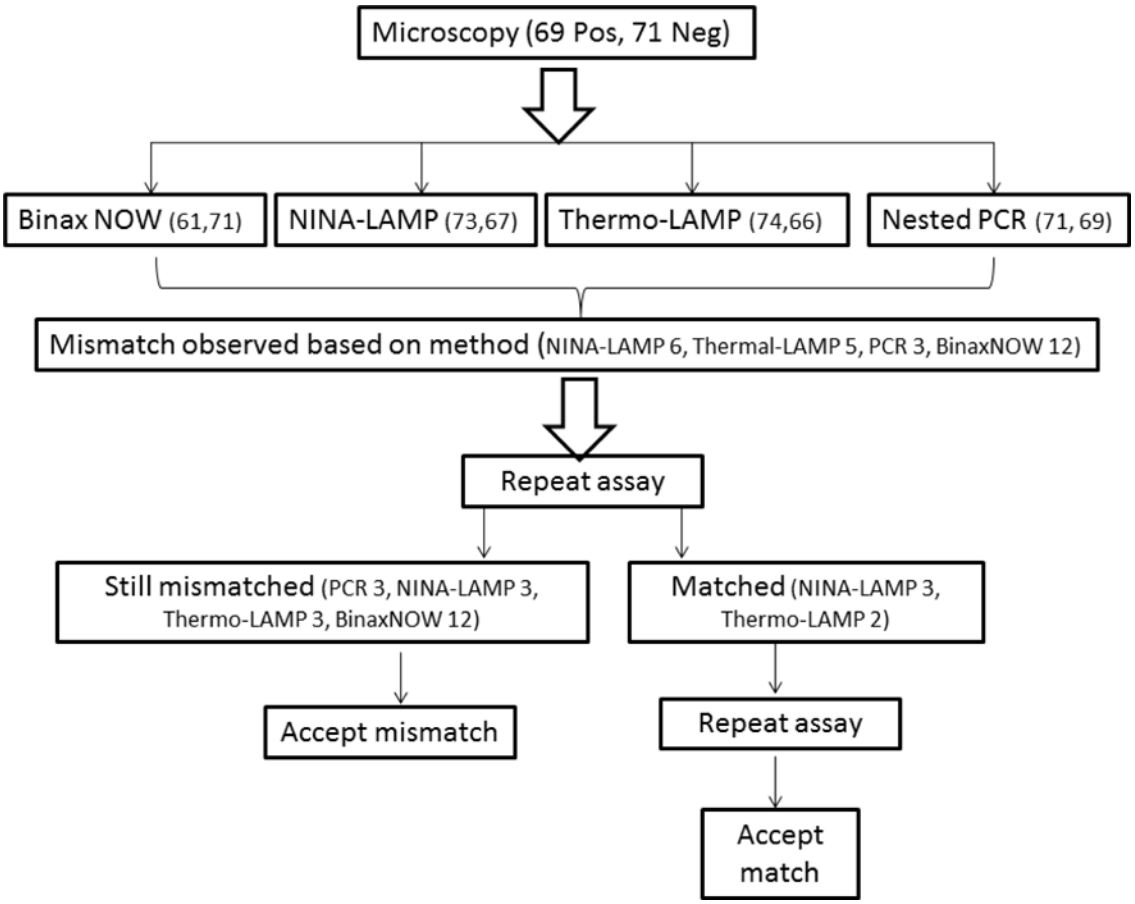

Figure 2.

Workflow used in this study when resolving discordance (mismatch results) between the NINA-LAMP, thermal-LAMP (performed in a thermocycler), BinaxNOW (RDT), and nested PCR compared to initial microscopy for the detection of malaria parasites. Number of positive (pos) and negative (neg) results are indicated in parentheses.

Table 1

Microscopy, nested PCR, NINA-LAMP and BinaxNOW malaria RDT results for 140 patients suspected of having malaria.

Result	Microscopy	Nested PCR	NINA-LAMP	LAMP-thermocycler	RDT (BinaxNOW)
Total Positive	69	71	72	72	61
P. falciparum	38	40 (42 inc. mixed)	41	41	38
Total non-*falciparum*	31	28 (31 inc. mixed)	31	31	23
P. vivax	25	24 (26 inc. mixed)	N/A	N/A	N/A
P. ovale	5	4 (5 inc. mixed)			
P. malariae	1	0 (1 inc. mixed)			
Mixed	0	3: *P. falciparum* + *P. vivax* (1) *P. falciparum* + *P. malariae* (1) *P. vivax* + *P. ovale* (1)	NA	NA	0
Negative	71	69	68	68	79

Table 2

Sensitivity and Specificity of LAMP and BinaxNOW in comparison with microscopy and nested PCR.

Test		Nested PCR	
		Sensitivity (%)(95% CI)	**Specificity (%)(95% CI)**
LAMP	Overall	100(93.6–100)	98.6(91.1–99.9)
	P. falciparum	97.6(85.9–99.9)	100(95.3–100)
	non-*falciparum*	100(86.3–100)	99.1(94.3–100)
BinaxNOW	Overall	85.9(75.2–92.7)	98.6(91.1–99.9)
	P. falciparum	90.5(76.5–96.9)	100(95.3–100)
	non-*falciparum*	71.0(51.8–85.1)	99.1(94.3–100)