DEVELOPING AND EVALUATING A NOVEL HIGH THROUGHPUT LAMP ASSAY FOR DETECTION OF LOW *PLASMODIUM FALCIPARUM* INFECTIONS

Kenny Kimani Kamau
Department of Medical Laboratory Science, Jomo Kenyatta University of Agriculture and Technology, Kenya

Francis Kimani
Kenya Medical Research Institute, Centre for Biotechnology Research and Development, Kenya

Dr. Amos Mbugua
Department of Medical Laboratory Science, Jomo Kenyatta University of Agriculture and Technology, Kenya

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ABSTRACT

As the prevalence of malaria continues to decrease globally, optimism is growing as countries shift from bringing down the number of new malaria cases to aiming for malaria elimination. Supporting this bold goal is the World Health Organization (WHO) which has guidelines for how this goal may be attained as well as for certification of the elimination of malaria. Thus, it endorsed an ambitious goal of achieving global malaria elimination and eradication. Achieving this milestone requires identification and treatment of all parasite carriers, both symptomatic and asymptomatic. There is therefore need to develop improved high throughput diagnostics, with capacity to detect low parasitemia infections. This would enhance identification and treatment of all asymptomatic cases in the early stages of infection especially in non-endemic areas that play a major role in malaria transmission. The aim of this project was to develop and evaluate a dry high –throughput (HT) LAMP assay for detection of low intensity *P. falciparum* infections. The assay was modified to enhance throughput, optimized by assessment by inclusion of DMSO to increase sensitivity and specificity. The assay evaluated 134 samples which were validated against Nested PCR.HT-LAMP Sensitivity of 92.1%, Specificity 100 %, Positive predictive value of 100% and Negative predictive value of 97% was achieved. The two assays showed a strong agreement between them (k= 0.943). HT LAMP showed superior diagnostic capacity just like that of Nested PCR and thus it can be used in field evaluations in the quest to eliminate malaria. Further research should also be done to make a multiplex HT-LAMP assay that can detect other strains of Plasmodium and lyophilize the reagents for ease of filed application.

Key Words: *malaria, molecular test, HT-LAMP PCR, nested PCR*

INTRODUCTION

Substantial changes have occurred in the prevalence of malaria from 2010. The cases of malaria globally have reduced from 237 million in 2010, to 216 million in 2016 indicating a decline of 8.8%. WHO African Region boasting 90% of the cases recorded against WHO South-East Asia Region (7%) and the WHO Eastern Mediterranean Region (2%) (WHO 2017). The incidence rate of malaria is estimated to have decreased by 18% globally, from 76 to 63 cases per 1000 population at risk, between 2010 and 2016. The WHO South-East Asia Region recorded the largest decline (48%) followed by the WHO Region of the Americas (22%) and the WHO African Region (20%) (WHO 2017).

However, World health organization have recorded zero malaria cases within the European countries as illustrated in figure 1 (World Health Organization, 2015), this has been attributed to concerted global efforts in application of interventions to interrupt Plasmodium parasite transmission i.e. vector control using long lasting insect treated mosquito nets, intermittent residual spraying and enhanced case management through improved detection of cases and treatment using artemisinin-based combination therapy (World Health Organization, 2015).

Increasingly, many countries have harnessed their resources with the aim of eliminating malaria, beside the fight being threatened by quite a number of factors i.e. the rise and spread of mosquito species resistant to insecticides (Sougoufara, Doucouré, Sembéne, Harry, & Sokhna, 2017). Since 2010, about 60 countries have reported incidences of resistance to at least one insecticide class used in IRS or ITNs; predominantly of pyrethroid resistance (WHO, 2017).

Moreover, field diagnosis of malaria in the past have been done using microscopy and rapid diagnostic tests (RDT), that target genus-specific aldolase and lactate dehydrogenase enzymes, the majority of them being specific for the *P. falciparum* histidine-rich protein –2 (Pf HRP-2). Recently, some *Plasmodium falciparum* parasites in parts of South America and Africa have deleted the hrp-2 gene. This has brought into question the use of the HRP-2 based RDT tests due to potential false negatives (Lucchi et al., 2013).

In addition, expert microscopy similarly has recorded 100 % sensitivity the disadvantage being it's laborious, and requires a highly trained microscopists (WHO 2017). Molecular tools have shown more sensitivity in detecting low parasite infections and correctly identifying the species of malaria parasite (Fernandes et al., 2016). these tools range from conventional PCR-based assays, real-time PCR assays and, isothermal amplification assays (Britton et al., 2016).When choosing the type of molecular assay to use, it is paramount to consider cost, robustness and ease of use. High throughput loop- mediated isothermal amplification in filed application has demonstrated such characteristics (McCreesh P., 2015).

High throughput LAMP uses a thermal stable enzyme *Bacillus stearothermophilus* that does not need repeated temperature changes that are seen in PCR. This characteristic helps adoption of this method in field evaluations and thus would be a major boost in the fight towards malaria elimination (McCreesh P. 2015).A LAMP end product detection can be done visually by checking for the establishment of magnesium pyrophosphate precipitate(Oriero et al., 2015).metal ion indicators, such as calcein, hydroxynaphthol blue and pico-green have been used to detect end point, along with melt curve analysis, a bioluminescent output in real time (BART), a lateral flow dipstick and a moveable fluorescence detection unit have also been used in real time (Oriero et al., 2015).

Moreover, in the goal to eliminate malaria the WHO projects that those scientific developments and inventions in novel methods, development of new insect control intrusions, improved diagnostics and more effective antimalarial medicines will contribute towards malaria elimination quest. It is thus calling for increased investment in the development and deployment of innovative tools – a critical strategy for reaching global malaria targets (WHO, 2017).

MATERIALS AND METHODS

Study Site

The study samples were from Busia County situated at the western region of Kenya. The population of Busia was estimated to be 816,452 (census 2012). According to a census

conducted in 2012, Busia County receives an annual rainfall of between 760mm and 2000 mm. majority of this rain occurs during the long rain season between late March and late May, while about 25% falls during the short rains between August and October. The annual temperatures range between 14oC and 26°C in this area. This county is a malaria endemic region with suitable breeding sites for the Anopheles mosquito vectors that transmit the malaria parasites.

Study Population

The current study utilized archived DBS samples from a previous drug efficacy testing study (KEMRI SSC 2276). The study population of that study comprised children between the ages of 6 months and 12yrs from Matayos, Busia County who presented with acute, symptomatic, uncomplicated P. falciparum malaria. Blood samples were collected from these children during the peak malaria transmission season from August to November 2016. From this whole blood samples DBS were prepared and archived. Children who tested positive for Plasmodium falciparum were put on Artemether-Lumefantrine and Dihydroartemisinin and followed up for 42 days testing for drug therapeutic efficacy testing. For the current study, 134 DBS were selected by simple random sampling from a total of 302 archived DBS samples collected from baseline and the first two days post treatment. The goal was to select potentially low parasitaemia infections for evaluation using HT LAMP. These would be samples which were determined to be positive for parasites at baseline by microscopy but subsequent present as negative by microscopy immediately after drug treatment. Integrity and storage of the archived samples was checked before being extracted and samples that didn't meet the criteria were excluded from the current study.

DNA Extraction from Plasmodium falciparum cultures and archived DBS field samples

Positive control DNA from Plasmodium Falciparum cultures was extracted for optimization of HT LAMP assay. First the P. Falciparum cultures were quantified by microscopy to a parasite count of 500 parasites per microliter of blood. In order to utilize an identical DNA extraction protocol for the control and archived samples, dried blood spots were then prepared using a single drop of blood and allowed to dry. DNA was then extracted from both P Falciparum cultures DBS, and archived DBS field samples from P. falciparum infected children using the Chelex method (Musapa et al., 2013) A 6 mm filter paper disc was cut using a sterile scalpel blade. This disc was incubated in 100 µl 0.5 % Saponin in PBS for 12 hours at 4° C overnight. The resulting brown solution was then discarded and substituted with 100 µl 1 × PBS followed by refrigeration at 4° C for 20 minutes. The solution was then discarded and refilled with100µl DNase free water and 50µl 20% Chelex consecutively. The tubes were heated at 98 °C and vortexed every two minutes and repeated up to 5 times on a block heater. The solution was then centrifuged for 2 min at 4000 rpm and 100 µL of the supernatant aliquoted into a 200µL Eppendorf tube and then stored at −20° C for PCR analysis (Musapa et al., 2013).

Nested PCR on archived Plasmodium falciparum DBS field samples

The Nested PCR reaction targeted the P. falciparum 18s rRNA gene. this was a 2 step amplification process. The sequences of primers used are shown in Table 1. First round outer PCR amplification used 1uL of template DNA and 2.5mM Magnesium chloride, 100 nM dNTP, 100 nM RPLU1, 100 nM RPLU5, 1 unit Taq polymerase, 1X PCR Buffer and 22.5uL PCR water, to result in a final reaction volume of 30uL. The cycling conditions for the outer PCR were set at 98°C for initial denaturation for 4 minutes followed by denaturation at 94°C for 1 min, annealing at 65°C for 2 minutes and extension at 72°C for 2 minutes and 72o C for 4 minutes. These was repeated for 30 cycles and the products stored at -4o C. (Echeverry et al., 2016). The following reaction components were used for the second round inner PCR amplification: 1uL of outer PCR amplicon was used as template DNA, 2.5mM Magnesium chloride, 100 nM dNTP, 100 nM RPLU3, 100 nM RPLU4, 1 unit Taq polymerase, 1X PCR Buffer and PCR water resulting in a final reaction of volume of 30uL. The thermal cycling conditions was 94°C for 4 minutes initial denaturation followed by denaturation at 94 ° C for 30 seconds followed by annealing at 62° C for 1 minute and extension at 72° C for 1minute. These steps were repeated for 35 cycles and then followed by a final extension at 72° C for 1 minute and 72° C for 4 minutes (Echeverry et al., 2016).

Table 1: Nested PCR oligonucleotide primers and their sequences

Primer	Sequence
fPLU1	TCAAAGATTAAGCCATGCAAGTGA
rPLU3	TTTTTATAAGGATAACTACGGAAAAGCTGT
fPLU4	TACCCGTCATAGCCATGTTAGGCCAATACC
rPLU5	CCTGTTGTTGCCTTAAACTTC

PCR product visualization by Gel Electrophoresis

Amplicons generated by nested PCR reactions were separated by gel electrophoresis on 1.5% agarose gel using 1X TAE buffer with ethidium bromide as the fluorescent stain for 30 minutes at 80 volts. A 100 base pair DNA ladder was utilized and expected band sizes for first and second round amplifications were 1400bp and 800 bp respectively. Visualization of amplicons was done using a transilluminator and the results captured using a camera.

Plasmodium falciparum High Throughput LAMP (HT LAMP) Assay

The HT LAMP assay was adapted, with modifications, from the P. falciparum malaria CZC-LAMP assay developed by (Hayashida et al 2017). The modifications included: enhancement of throughput (using 8-strip PCR tubes setup) and optimization (assessment of inclusion of DMSO) of the assay for increased sensitivity and specificity. The setup of LAMP assay followed the process as described by Hayashida et al (2017) with minor alterations. 8-strip PCR tubes were used for the reactions. Briefly, 35 nmol each of dNTPs mix and 8 U of Bst2.0 WS DNA polymerase was aliquoted on the lids of the 8-strip PCR tubes. The indicator which was 3.5 nl

SYBR green and the LAMP primers (3.2 pmol of FIP and BIP, 0.4 pmol of F3 and B3, and 1.6 pmol of LF and LB) were aliquoted in the wells of 8-strip PCR tubes. The primers used target mitochondria DNA. Primer names and sequences are indicated on Table 2 below as previously described by (Polley et al., 2010). DNA from either P. falciparum cultures of P. falciparum DBS was subsequently added to these pre-setup 8 strip tubes.

Table 2: HT LAMP Oligonucleotide primers used in and their sequences

Primer	Sequence
FIP	CAGTATATTGATATTGCGTGACGACCTTGCAATAAATAATATCTAGCGT
BIP	AACTCCAGGCGTTAACCTGTAATGATCTTTACGTTAAGGGC
F3	TATTGGCACCTCCATGTCG
B3	AACATTTTTTAGTCCCATGCTAA
LF	GTGTACAAGGCAACAATACACG
LB	GTTGAGATGGAAACAGCCGG

Testing of LAMP Assay Using a Positive Control and DMSO

DNA was extracted from a dried blood spot made from 25µL aliquot of P. falciparum culture using Chelex method and used to optimize and validate the HT LAMP assay. 10-fold Serial dilutions of this DNA were tested. In addition, various concentrations of DMSO were prepared i.e. 1%, 2%, 3% and 4%. Aliquots of 1µL of the P. falciparum culture DNA (positive control) plus 1µl of DMSO were added to the other HT LAMP reagents to make a final volume of 25µl. The PCR tube was then mixed and then incubated for 1 hour in water bath set at 63°C. Amplified DNA products were visualized using a transilluminator to detect florescence of SYBR green dye (Hayashida et al., 2017).

HT LAMP evaluation of Plasmodium falciparum DBS field samples

A pre-setup 8 strip was used to perform HT LAMP assay on DNA from the archived malaria samples as described by (Britton, Cheng, Sutherland, & McCarthy, 2015). Reactions were done in 25 µL final volumes made by adding 1µl of extracted DNA and 1µl of DMSO. The tubes were mixed thoroughly to mix the reagents and then incubated for 1 hour in water bath set at 63°C. The amplified DNA products were visualized using a transilluminator to detect florescence of SYBR green dye. Positive samples showed a bright fluorescent green colour.

Ethical approval

Ethical approval for this study was granted by scientific steering committee and Ethical Review Committee of the Kenya Medical Research Institute. The approval number was KEMRI/SERU/CBRD/185/3691.

RESEARCH RESULTS

LAMP assay development and Optimization

Optimization of HTLAMP Primer Ratio

DNA was extracted from a 500 parasites/uL aliquot culture of D6 Plasmodium falciparum using the Chelex method. The optimal primer ratio that showed optimum amplification was 8:1, ratio of the inner primers to outer primers.

The 1.5 % agarose gel plate shows amplification of plasmodium falciparum with variation of inner to outer primer ratio. Lane 1 - 1:1, Lane 2 – 2:1, Lane 3 – 4:1, Lane 4 – 6:1. Lane 5– 8:1 Lane 6 – 10:1. 8:1 ratio readily amplified DNA, setting precedence for the LAMP technique.

Temperature optimization

The optimum temperature that gave amplification was 63o C. The 1.5 % agarose gel plate shows amplification of plasmodium falciparum at various temperatures, 63o Celsius gave the best amplification hence adopted for assay.

Time optimization

The optimum time that gave amplification was found to be 45minutes. The 1.5 % agarose gel plate shows amplification of plasmodium falciparum at certain range of times. 45 minutes gave the best amplification and hence adopted for the assay.

DNA polymerase Enzyme optimization

Bacillus stearothermophilus DNA polymerase was used in the LAMP technique as it produced results under the above mentioned conditions. One unit of Bst DNA polymerase requiring an optimum temperature of 63o C to incorporate 35 nmol of dNTPs into polynucleotide fraction in 45 min.

Sensitivity and Specificity in varied DNA Concentrations with addition of DMSO

The assay was able to amplify serially diluted extracted DNA of Plasmodium falciparum species. This was confirmed by visualization under gel electrophoresis and compared to Nested PCR and Loop amp kit. A 10-fold serial dilution was done of the known standard and 4% of DMSO was added to enhance sensitivity. The limit of detection of the assay was determined to be 5 parasites per microliter. Sample 1 had 500 parasites per microliter of blood, sample 2 had 50 parasites per microliter of blood and sample 3 had 5 parasites per microliter of blood. NC is the negative control while M is the molecular ladder.

Evaluation of LAMP assay to Nested PCR

The developed LAMP assay was evaluated using 134 archived field samples and the results compared to Nested PCR.

Table 3: Contingency table comparing HT LAMP assay and NESTED PCR

HT LAMP NESTED PCR		POSITIVE	NEGATIVE	TOTAL
	POSITIVE	43	0	43
	NEGATIVE	3	88	91
TOTAL		46	88	134

Both assays detected 43 samples as positive, 3 sample were positive in Nested PCR and negative in LAMP PCR. The assays agreed that 88 samples were negative and thus the sensitivity, specificity, positive predictive value and negative predictive value was 93.5%,100 %,100% and 96.7% respectively.

Table 4: kappa agreement of LAMP assay and Nested PCR

	Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Measure of Agreement Kappa	.950	.029	11.006	.000
N of Valid Cases	134			

Cohen's κ was run to determine if there was agreement between two tests for diagnosing malaria on 134 patients. There was a strong agreement between the PCR and LAMP, $\kappa = .950$, $p < .000$.

DISCUSSION

The directive by WHO geared to malaria elimination, will only be realised if there are highly sensitive diagnostic tools that will be able to detect asymptomatic malaria infections (The malERA Consultative Group on Diagnoses 2011). In this project, we developed an assay that was able to amplify DNA at a primer ratio of 8:1 between the inner and outer primers. It worked best at temperatures of 63o Celsius for 45 minutes. This was consistent with a study done in Kenya (Mugambi et al. 2015). Additionally, the assay has a low detection threshold of 5 parasites per µl of blood. It has high throughput capacity hence many samples can potentially be handled at the same time. The developed HT LAMP PCR was able to identify 43 samples as positive, among which ten were negative in microscopy. In addition, both diagnostic methods agreed that 88 samples were negative. This meant that the three negative samples in Microscopy had a low parasite count that could have been difficult to pick under microscopy. However, Kappa comparison gave a strong agreement between the two diagnostic methods indicating that both methods can be relied on for malaria diagnosis. In general, HT LAMP PCR being a molecular technique and with a low parasite detection threshold was able to pick the parasites and hence demonstrated higher sensitivity. This is consistent with previous studies which have demonstrated HT LAMP PCR as a cheap and sensitive tool for malaria diagnosis (Tambo et al. 2018).

NESTED PCR identified 46 samples as positive among which thirteen samples were negative in microscopy. Moreover, both diagnostic methods agreed that 88 samples were negative. This demonstrates a higher sensitivity of Nested PCR to low parasite counts that were missed in microscopy. This consistently agrees with a study done in Southern Ethiopia where Nested PCR demonstrated higher sensitivity than microscopy (Mekonnen et al. 2014). Comparison between HT LAMP PCR and Nested PCR demonstrated that Nested PCR had superior diagnostic capability than LAMP PCR. This was deduced as a result of Nested PCR detecting 46 samples as positive, 3 of which read negative in HT LAMP PCR. However, Kappa comparison gave a strong agreement indicating that both molecular diagnostic methods would be relied in diagnosis of malaria parasites. These results are in consistent with the study done in Sabah, Malaysia that demonstrated Nested PCR as superior in diagnosis compared to HT LAMP (Lau et al. 2016). In addition, the turnaround time of getting results in HT LAMP was an 8th of the turnaround time for Nested PCR. HT LAMP PCR has a basic benchtop preparation process that makes it an appropriate diagnostic tool, even for low resource settings in health facilities, compared to Nested PCR which requires working on ice and a PCR cabinet to avoid contamination (Tambo et al. 2018).

Additionally, HT LAMP PCR has a number of pluses over the existing molecular diagnostic methods. The Bacillus steothermophillus polymerase enzyme that catalyzes the LAMP reaction is more robust with respect to inhibition than Taq polymerase enzyme (Notomi et al. 2000). It is therefore possible to use a simple, prompt, and low-cost procedure for sample preparation, in contrast to that required for Nested PCR (Polley et al. 2010). The ease of preparing the samples and the prompt reaction results with a simple visible endpoint makes the assay more ideal for application in field settings. For instance, detection of amplification products with ultraviolet light at the end of the incubation time, provided visual comparison of a positive test from a negative test eliminating the need for electrophoresis thus reducing cost. SYBR green, a DNA intercalating dye that turns green for positive results and color remains orange in a negative result.

A water bath was able to provide isothermal conditions and hence demonstrating the feasibility of using this assay under field conditions not only for its simplicity but also for cost effectiveness in resource poor settings where malaria is endemic (Hartmeyer et al. 2019). Majority of the identified costs in the application of LAMP technique went into buying molecular reagents such as Bacillus steothermophillus DNA polymerase, SYBR green DNA intercalating dye, dinucleotides, primers, dimethylsulphoxide, which were bought at a market price of (USD) 1000. They were estimated to run about 50 reactions. This translates into approximately (USD) 20 for analyzing one sample. This is ten times higher than microscopy which cost on average (USD) 0.2. This is however relatively cheaper than when using Nested PCR as expensive equipment will be needed.

CONCLUSION

We developed an assay with a low detection threshold of 5 parasites per microliter of blood. It has similar performance characteristics to Nested PCR, it is less expensive, easy to use, and amicable to large scale-surveillance studies in developing country settings. This assay is ideal in application of field evaluation in the quest to eliminate malaria.

RECOMMENDATIONS

The study recommends Improvement of the assay to a multiplex assay that will be able to screen other plasmodium species, Lyophilize the LAMP reagents to make the developed assay more user friendly in the field set up. moreover, incorporating some quicker DNA extraction methods would ease in field application.

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APPENDIX

Results of malaria diagnostic tests for archived extracted DNA of blood samples collected from Busia, western Kenya.

Serial no.	Archived Sample No.	Day sample collected	Microscopy Parasitemia	Nested PCR	HT LAMP
	001	Day 0	66720	+	+
	002	Day 0	12000	+	+
	003	Day 0	2440	+	+
	004	Day 0	1200	+	+
	005	Day 0	20400	+	+
	006	Day 0	2240	+	+
	007	Day 0	49440	+	+
	008	Day 0	12440	+	+
	009	Day 0	2840	+	+
	010	Day 0	29760	+	+
	011	Day 0	55040	+	+
	012	Day 0	4040	+	+

	013	Day 0	43540	+	+
	002	Day 1	400	+	+
	003	Day 1	200	+	+
	004	Day 1	480	+	+
	005	Day 1	356	+	+
	006	Day 1	1080	+	+
	009	Day 1	0	-	-
	012	Day 1	0	-	-
	018	Day 1	0	-	-
	021	Day 1	0	-	-
	022	Day 1	3160	+	+
	002	Day 2	0	-	-
	003	Day 2	0	-	-
	004	Day 2	0	-	-
	005	Day 2	0	+	+
	006	Day 2	0	-	-
	007	Day 2	0	-	-
	008	Day 2	0	-	-
	009	Day 2	0	-	-
	010	Day 2	0	-	-
	011	Day 2	0	-	-
	012	Day 2	0	-	-
	013	Day 2	0	-	-
	014	Day 2	0	-	-
	015	Day 2	0	-	-
	016	Day 2	0	-	-
	017	Day 2	0	-	-
	018	Day 2	0	-	-
	019	Day 2	0	-	-
	020	Day 2	0	-	-
	021	Day 2	0	-	-
	022	Day 2	0	-	-
	023	Day 2	0	-	-
	024	Day 2	0	-	-
	025	Day 2	0	-	-
	026	Day 2	0	-	-
	027	Day 2	0	-	-
	028	Day 2	0	-	-
	029	Day 2	0	-	-
	030	Day 2	0	-	-
	031	Day 2	0	-	-
	032	Day 2	0	-	-
	033	Day 2	0	-	-
	034	Day 2	0	-	-
	035	Day 2	0	+	+
	036	Day 2	0	-	-
	037	Day 2	0	-	-
	038	Day 2	0	+	+
	039	Day 2	0	+	+
	040	Day 2	0	+	+
	041	Day 2	0	+	+
	042	Day 2	0	+	+
	043	Day 2	0	+	+
	044	Day 2	0	-	-
	053	Day 1	1560	+	+
	054	Day 1	1120	+	+
	055	Day 1	1400	+	+
	056	Day 1	1200	+	+
	057	Day 1	0	-	-
	058	Day 1	0	-	-
	059	Day 1	0	-	-
	045	Day 2	0	-	-
	046	Day 2	0	-	-
	047	Day 2	0	-	-
	048	Day 2	0	+	-
	049	Day 2	0	+	+

	050	Day 2	0	-	-
	051	Day 2	0	-	-
	052	Day 2	0	-	-
	053	Day 2	0	-	-
	054	Day 2	0	+	-
	055	Day 2	0	-	-
	056	Day 2	0	-	-
	057	Day 2	0	+	-
	058	Day 2	0	-	-
	078	Day 2	2240	+	+
	059	Day 2	0	-	-
	060	Day 2	0	-	-
	061	Day 2	0	-	-
	062	Day 2	0	-	-
	063	Day 2	0	-	-
	064	Day 2	0	-	-
	065	Day 2	0	-	-
	066	Day 2	0	-	-
	067	Day 2	0	-	-
	068	Day 2	0	-	-
	059	Day 1	1000	+	+
	060	Day 1	1500	+	+
	061	Day 1	1640	+	+
	062	Day 1	1469	+	+
	063	Day 1	1600	+	+
	064	Day 1	1160	+	+
	065	Day 1	1320	+	+
	066	Day 1	2040	+	+
	069	Day 2	0	-	-
	070	Day 2	0	-	-
	071	Day 2	0	-	-
	072	Day 2	0	-	-
	073	Day 2	0	-	-
	074	Day 2	780	+	+
	075	Day 2	0	+	+
	076	Day 2	0	-	-
	077	Day 2	0	-	-
	078	Day 2	0	-	-
	079	Day 2	0	-	-
	080	Day 2	0	-	-
	081	Day 2	0	-	-
	082	Day 2	0	-	-
	083	Day 2	0	-	-
	084	Day 2	0	-	-
	085	Day 2	0	-	-
	086	Day 2	0	-	-
	087	Day 2	0	-	-
	088	Day 2	0	-	-
	089	Day 2	0	-	-
	090	Day 2	0	-	-
	091	Day 2	0	-	-
	092	Day 2	0	-	-
	093	Day 2	0	-	-
	094	Day 2	0	-	-
	095	Day 2	0	-	-
	096	Day 2	0	-	-

COLOUR CHART

1. Yellow: Indicated repeated samples in day 1 and day 2
2. Red: Indicated samples that were negative with microscopy and positive in both HT LAMP and Nested PCR
3. Green: Indicated samples Negative in HT LAMP and positive in Nested PCR

No colour: These are results that agreed in all the diagnostic tests