Evaluation of Visual LAMP detection of *Plasmodium falciparum* and non-*Plasmodium falciparum*

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Research

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Abstract

Background: To detect the DNA of the malaria using Loop-mediated isothermal amplification (LAMP) and evaluate the effect.

Method; According to the 18s rRNA sequence of the malaria, the LAMP primer of Plasmodium falciparum and non-Plasmodium falciparum were designed using Primer Explorer V5 software. The method of Visual LAMP detecting malaria was formation. The sensitivity, specificity and amplification efficiency were tested, compared with the Nest-PCR.

Result: The filter papers of blood from 47 patients with Plasmodium falciparum and 49 with non-Plasmodium falciparum were detected using Visual LAMP. The patients with Plasmodium falciparum were all positive. In the patients with non-Plasmodium falciparum , false negative rate was 2.1%. In the 10 patients with Leishmaniasis, 8 with Schistosomiasis japonicum, 38 healthy human, none was positive. The results using visual LAMP were consistent with Nest-PCR (Kappa value = 0.956). All of the Tt in Visual LAMP are less than 60 min, especially the Tt of Plasmodium falciparum is 18.2 min. When adding calcium pyridoxine indicator during the Visual LAMP detection, the progress of the reaction to different substes of malaria delayed of about 9-23 min.

Conclusion: LAMP is efficient and visible, deserved the prospect of application in the on-site and primary medical institutions.

Background

Malaria is one of the most important parasitic infectious diseases which threaten human life safety[1]. There are 4 kinds of malaria parasites, of which *Plasmodium falciparum* is the most harmful, toxic, and with the highest fatality rate[2,3]. According to The World Malaria Report 2018, $3.1 billion was invested in control and eradication of malaria in 2017, with a total of 219 million malaria cases worldwide and 435,000 deaths. In Africa, South-East Asia, the Eastern Mediterranean, the Western Pacific and China, *Plasmodium falciparum* accounted for 99.7%, 62.8%, 69%, 71.9% and 63.7% in all malaria cases, respectively[4,5]. Malaria control in China is at the elimination phase. Importation of malaria remains widespread due to increased travel and population movements to malaria-endemic areas. To reduce malaria complications, fatality and transmission, it is important to diagnose malaria correctly [1]. The common detection methods of malaria parasites are mainly optical microscope examination and RDT. In the chronic infection stage, due to the malaria parasite emias always maintain low density, optical microscope examination and RDT leak detection [3,6]. The RDT is insensitive to *Plasmodium vivax and Plasmodium ovale.* The patients with asymptomatic *Plasmodium falciparum* infection may be malaria-infected reservoir[7,8]. Therefore, a high sensitivity diagnostic methods able to detect low-density malaria parasite emias for the elimination of malaria is critical[6,9].There is urgent need for extremely sensitive and field-based diagnostic system [10]. Molecular diagnosis is a highly sensitive method for detecting malaria infection [7,9,11]. For example, PCR is one of the most sensitive and reliable methods for detecting

DNA of malaria, but it requires expensive equipment [7]. The sensitivity of the ring-mediated so-mediated temperature amplification (LAMP) method is close to or exceeds the NEST-PCR [6,7,12,13]. LAMP method does not require expensive equipment and results can be judged by visual observation. The main purpose of this study is to evaluate the diagnostic performance of LAMP and compare it with the results of detection using microscope and PCR.

Material And Method

1. Reagents and instruments: Bst DNA polymerase (large fragment, Biolabs, USA), dNTPs (TANGEN, China), calcium-yellow chlorophyll (Calcein, Sigma, USA), manganese tetrachloral chloride (MnCl2·4H2O; Sigma, USA), DNA Extraction Kit (OMEGA, USA), LA–500 Real-Time Turbidity Detection System (Loopamp, Japan), PTC–200 Peltier Thermal Cycler Gradient PCR (Bio-Rad, USA), Genius Bio-Imaging System Gel Imaging System (Syngene, UK).

2. Sample: This study was certified by the Ethics Committee of the Wuhan Center for Disease Control and Prevention. The informed consent of all patients and healthy people was obtained.

2.1 Malaria patients: Blood samples from malaria patients were clinically diagnosed in 2013–2018. The patients' earlobe blood was collected, filter paper of blood spots was made(about 20μl), naturally dried, saved at 4℃.

2.2 Leishmaniasis patients: blood on glass, provided by the China Center for Disease Control and Prevention of Parasitic Diseases.

2.3 Schistosomiasis japonicum samples: bloodfrom rabbitinfected with *Schistosomiasis japonicum,* provided by the Hubei Provincial Center for Disease Control and Prevention schistosomiasis Research.

2.4 Healthy people: blood from the healthy volunteers were collected, filter paper blood spots were made, naturally dried, saved at 4℃.

3. DNA extraction: filter paper with dried blood were droplets by 3mmx3mm, according with The OMEGA D3096 micro-DNA extraction kit operation, extract DNA of malaria (50 μl), saved at –20℃.

4. Primer Synthesis: 3 groups of LAMP Primer was designed with Primer Explorer V5, including *Plasmodium falciparum* (AJ276844) and non-*Plasmodium falciparum* (NP.f, KY923424, LT594520, MF693428). For detection of malaria, 1 group of LAMP primers with high sensitivity, specific and amplification efficiency was screened by experimental. The Nest-PCR primer sequence (18S rRNA gene) is synthesized according to the references [6,14,15,16], by Bioengineering Co., Ltd(Shanghai).

5. Visual LAMP: 10X Thermo pol Buffer 2.5μl, 10 mM dNTPs 3.5μl, 100 mM MgSO4 1.5μl, 40μM FIP, 40μl BIP, 5μM F3, 5μM B3, 10μM LpF, 10 m LpB each 1 sl, Bst DNA polymerase 1μl, 25μM Calcein 1μl, DNA extract 2μl, adding H_2O to 25μl, 65℃ 60–90 min, observation.

6. Nest-PCR: reaction system and conditions and operation according to the reference[14,15,16].

7. Results judge: LAMP results observed by eyes: green fluorescence is positive, orange is negative; To identified the subsets of malaria, the PCR amplification product was analysised by 1% agar sugar gel electrophoresis, and the Purpose Stripe of *Plasmodium falciparum (*P.f), *Plasmodium vivax* (P.v), *Plasmodium malaria (*P.m). *and Plasmodium ovale curtisi*P.oc) and *Plasmodium ovale wallikeri* (P.ow) are 205bp, 120bp, 144bp, 800bp and 780 bp respectively.

8. Statistic: Database was established using Excel 2003 software. The comparison between groups was analyzed with the test of χ^2 and the test level is $\alpha = 0.05$

Results

1. Judgment of Visual LAMP and Nest-PCR results: Adding calcium-yellow toludoline indicator, LAMP reaction liquid was observed by eyes. Changing into green as positive, unchanging (orange) as negative (Figure 1); The Nest-PCR amplification gel Imaging showed 205bp、120bp、144 bp、800 bp、780 bp bands , which are P.f, P.v, P.m, P.oc and P.ow positive each(Figure 2).

2. Visual LAMP and Nest-PCR : 96 filter papers of blood from patients infected with malaria confirmed in 2013-2018 were tested by Visual LAMP and Nest-PCR simultaneously. 93 of them were positive (Table 1).

Table 1 Visual LAMP and Nest-PCR test in malaria patients

Method	P.f	P.v	P.o	P.m	P.f+NP.f	Total
Microscope	47	27	18	4	0	96
Nest-PCR	46	25	17	4	1	93
LAMP	45	25	16	4	3	93

P=0.449

3. Consistency of Visual LAMP and Nest-PCR： 96 filter papers of blood from patients infected with malaria and 38 blood sample from healthy human were tested by Visual LAMP and Nest-PCR. The consistent of the two method were analyzed. Kappa = 0.956 (Table 2).

Table 2 The consistent of Visual LAMP and Nest PCR

Method	Classification	LAMP				Total
		P.f	NP.f	P.f+NP.f	Negative	
Nest PCR	P.f	45	0	1	0	46
	NP.f	0	44	1	1	46
	P.f+NP.f	0	0	1	0	1
	Negative	0	1	0	40	41
Total		45	45	3	41	134

Kappa = 0.956

4. The specificity of the method: 10 patients with *Leishmaniasis,* 8 with *Schistosomiasis japonicum,* 38 healthy human were tested by Visual LAMP and Nest PCR , none was positive.

5. The effect of calcium-yellow-green indicator on LAMP reaction: DNA from blood of 25000 P.f/μl, 3000 P.v/μl, 4000 P.o/μl, 2000 P.m/μl was extracted as template. The Tt of turbidity peak time are 18.2 min, 35.6min, 50.8min, 33.1min when the LAMP reaction fluid was without calcium-yellow-green indicator (A) (Figure 3-6). Tt plus are 29.1 min, 44.1min, 74.1min, 74.1min, 47.8min when calcium-yellow green oxalate indicator were added(B) (Figure 3-6). No turbidity showed in 90min when pure water is used as a template.

Discussion

Malaria is an infectious disease caused by different types of *Plasmodium,* which live in red blood cells (RBC) and destroy host erythrocyte, leading to fever and/or anemia, or even kidney failure, brain coma and death. Accurate diagnosis of malaria infection is critical. Microscope examination of blood smears is still considered the gold standard for the diagnosis of malaria, which was labor-intensive, time-consuming[7,10,16], with detection limit of 100 malaria parasites/μl [10,16]. It was usually unable to detect low-density and mixed infections, the sensitivity and specificity of which depends on the skill of the technician, quality and staining quality of blood smear, and so on [3,17]. Compared with molecular diagnostic methods, in malaria endemic areas, only half of malaria patients were correctly diagnosed. The WHO external assessment of the malaria parasite's mirror test capacity in China showed that the correct rates of negative, *Plasmodium falciparum (P.f),*, *Plasmodium vivax* (*P.v)* were 92.5%, 78.3%, 70.8% respectively[20]. RDT is facilitate and fast, with detection limit of 100 plasmids/μl[19,21]. It shows high sensitivity to plasmid infections of more than 100 plasmids/μl(median 94.3%), sensitivity of which

decrease significantly with a density of less than 100 plasmids/μl (median 74.1%) [21]. Meantime it shows limited sensitivity when detecting low-density infection [16,22], and less sensitivity to non-*P.f*、*P.v as* 66.0–88.0%, *P.o as* 5.5–86.7%, *P.m as* 21.4–45.2% [19]. PCR is one of the most sensitive and reliable methods for detecting DNA of malaria, with a high sensitivity and specificity limit to 1–10 malaria parasites/μl [10,11]. However, it requires technical expertise and infrastructure which limit its wide range of applications. LAMP has been used in clinical diagnosis of a variety of infectious diseases, including malaria. It is considered to be faster, more operational and cost-effective than PCR. At the same time, it has been reported that in testing malaria, LAMP own a similar sensitivity and specificity to PCR [7,10,11,23,,24]. It is capable of identifying low-density malaria infections by LAMP, which cannot be detected by the microscope[7]. Sensitivity of LAMP is several orders of magnitude higher than by microscope and RDT [21].

At present, detecting malaria by LAMP is mainly to detect the *genus malaria* and *Plasmodium falciparum,* the primer is depend on 18s rRNA region of the malaria parasite species. In this study, 3 groups of LAMP Primer was designed including *P.f* and non-*P.f.* For detection of malaria, 1 group of LAMP primers with high sensitivity, specific and amplification efficiency was screened by experimental. By Visual LAMP, filter paper of blood from 47 cases of *P.f* and 49 non-*P.f* patients was detected. There was no significant difference with by Nest-PCR or by microscope examination ($P > 0.05$), of which 2 non-*P.f* patients were both negative tested by Visual LAMP and Nest-PCR. It may because the blood on filter paper was kept too long, and the poor extraction quality of DNA or not extracted at all. 1 *P.v* case by Nest-PCR was positive, but by Visual LAMP was negative. 1 *P.f* case by visual LAMP was positive, but by Nest-PCR was negative. The false negative rate of visual LAMP of non-*P.f* is 2.1%. 47 cases of *P.f* were all positive by Visual LAMP and Nest-PCR, of which 2 cases of *P.f* and non-*P.f* mixture infection were found by Visual LAMP, only 1 case of mixture infection by Nest-PCR, and 1 case of *P.o* by microscope examination. It may be due to the false positive or the more sensitivity of Visual LAMP, that the *P.f* and non-*P.f* mixture infection could be discovered by Visual LAMP but microscope examination.Detected by Visual LAMP and Nest PCR simultaneously, the results of blood in filter paper from 96 malaria patient and 38 healthy men showed extremely consistent (Kappa = 0.956). In the results of 10 patients with *Leishmaniasis,* 8 with *Schistosomiasis japonicum,* 38 healthy human, none was positive，which indicated that the Visual LAMP primer in this study had high specificity. Visual LAMP is a method that require no expensive instruments or special laboratories, with simple and convenient operation, suitable for on-site inspection. In LAMP of this study, LA–500 real-time turbidity detection system were used and DNA from blood of 25000 P.f/μl, 3000 P.v/μl, 4000 P.o/μl, 2000 P.m/μl was extracted as template. When the LAMP reaction was not added with calcium-yellow green indicator, the turbidity peak time(Tt) is 18.2 min, 35.6 min, 50.8min, 33.1min. Mean Tt was less than 60min, which represented a higher amplification efficiency. LAMP Tt of *P.v* is 18.2min in particular, the amplification efficiency of which was very high. It could meet the requirements of LAMP. When adding the calcium-yellow green indicator in LAMP, Tt is 29.1min, 44.1 min, 74.1 min, 47.8min. The delay effect of adding calcite-yellow-green-oxidant in malaria subsets was different, the mean of which was about 9–23min. It is convenient for the visual observation of the results when adding calcium-yellow green indicator, suitable for on-site application.

As worldwide countries set targets of malaria elimination, accurate diagnosis, optimal treatment, stop of transmission, and reducing mortality of malaria are becoming critical [25,26]. As a result of the sudden decline in local patients and the gradual elimination of malaria, there are a trend of degradation in the clinical diagnosis and screening capacity of malaria. The prevalence of infrequent diagnosis, with some cases ranging from onset to the determination of diagnosis for more than 30 days. At the same time, due to increasing travel and population movements to malaria-endemic areas, imported malaria is still widespread [21]. Particularly, asymptomatic malaria infection and low-density malaria parasite emias are hard to be detected by microscope examination and RDT due to below the detection threshold [11,24], causing misdiagnosis or delayed diagnosis[3,21]. But they could maintaining transmission of malaria as infected intermediate host[24,26]. It has been reported that subclinical malaria accounts for 70–80% of all malaria infections[11]. Therefore, it is a challenge to detect the asymptomatic malaria parasite infection and low density malaria parasite emias in malaria prevention and control [8,24]. Stopping transmission of malaria requires detecting methods with high sensitive. The LAMP shows high sensitivity and specificity, by which asymptomatic and asymptomatic malaria infections could be diagnosed accurately, and low-density infections that cannot be detected by conventional diagnosis methods could be identified [26]. At the same time, LAMP is fast, easy to operate and effective which provides the possibility for clinical diagnosis and monitoring of malaria.

Conclusion

Visual LAMP is highly sensitive and specific to the diagnosis of malaria,and the results using visual LAMP are consistent with Nest-PCR.During the malaria elimination stage,visual LAMP provides a portable, sensitive and specific method for on-site inspection.

Declarations

Ethics approval and consent to participate

This study was certified by the Ethics Committee of the Wuhan Center for Disease Control and Prevention. The informed consent of all patients and healthy people was obtained.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZHOU Shui-mao and JIA Xi-shuai conceived the study. ZHOU Shui-mao,TU Zu-wu and JIA Xi-shuai undertook statistical analysis,and drafted the manuscript. YANG Yan,TANG Wei-feng, CHEN Fang undertook epidemiological investigations and sample collection.All authors contributed to the writing of the manuscript and approved the submitted version of themanuscript.

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Not applicable

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Figures

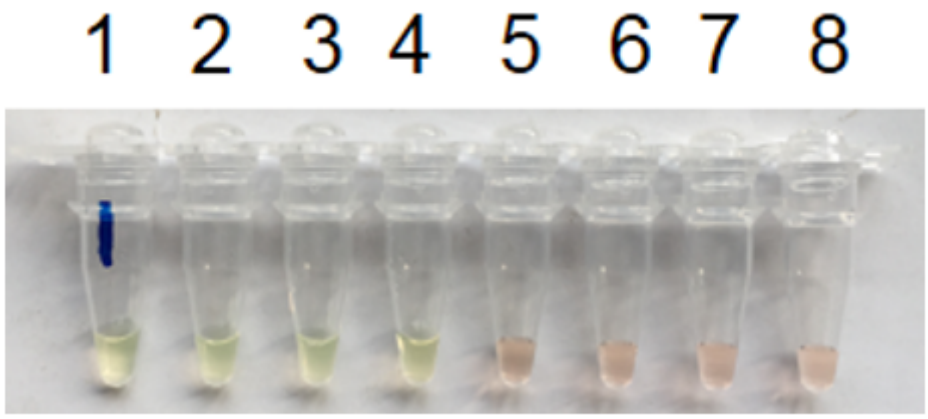

Figure 1

LAMP reaction 1:P.f, 2:P.v, 3:P.m, 4:P.o, 5:Leishmaniasis, 6:S.j, 7:Healthy Man, 8:Blank Control

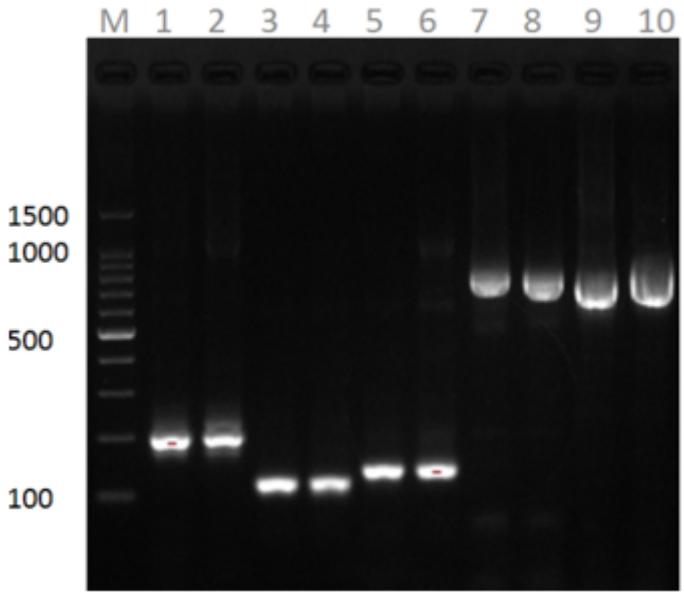

Figure 2

PCR Amplifier Gel Imaging 1,2:P.f, 3,4:P.v, 5,6:P.m, 7,8:P.oc, 9,10:P.ow, M:Marker

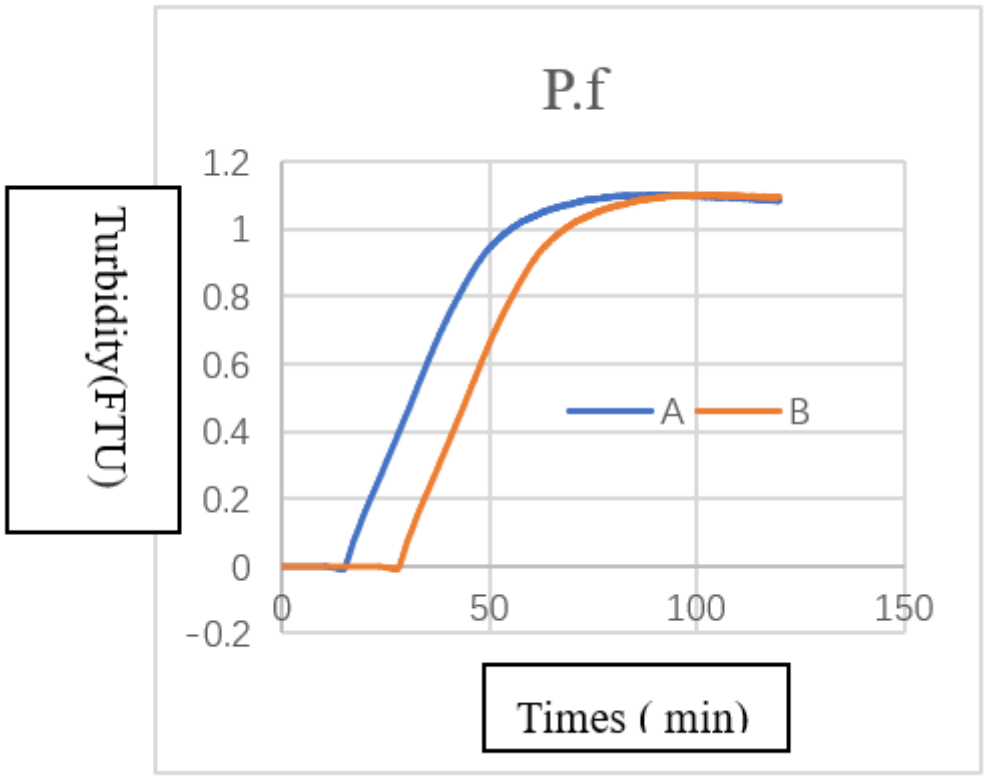

Figure 3

Effect of the indicator on the LAMP of P.f

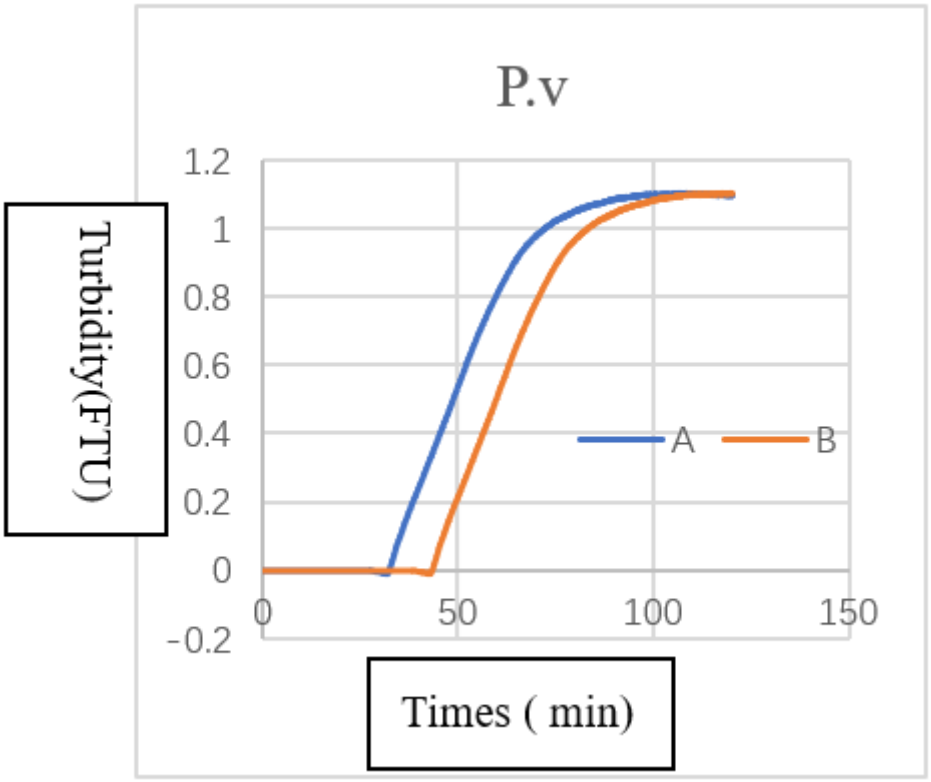

Figure 4

Effect of the indicator on the LAMP of P.v

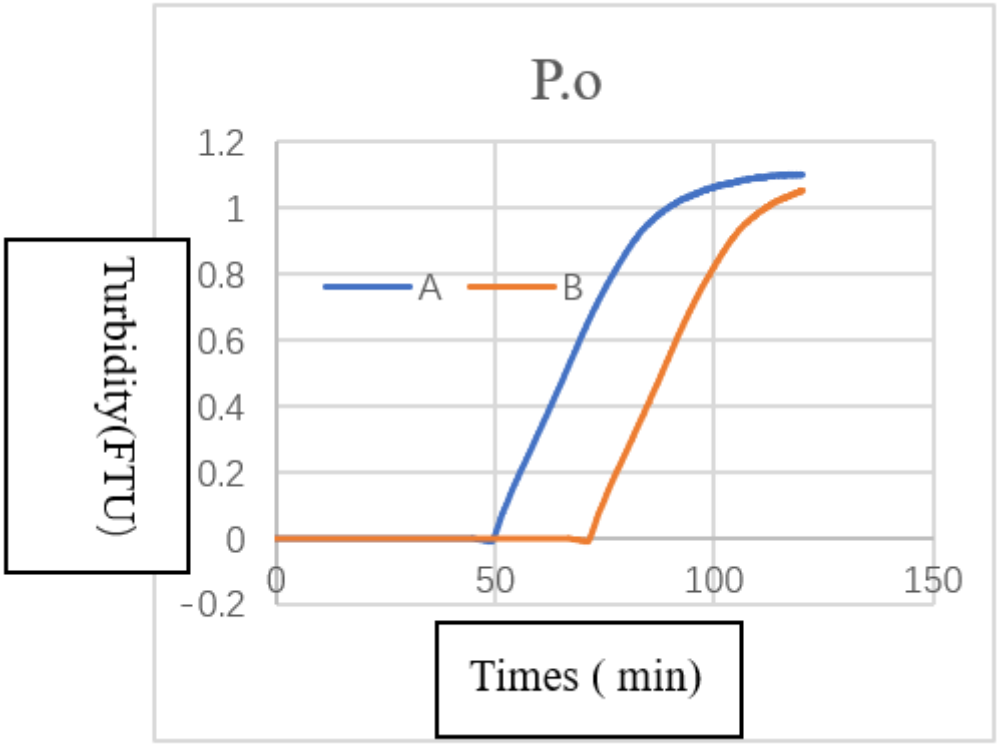

Figure 5

Effect of the indicator on the LAMP of P.o

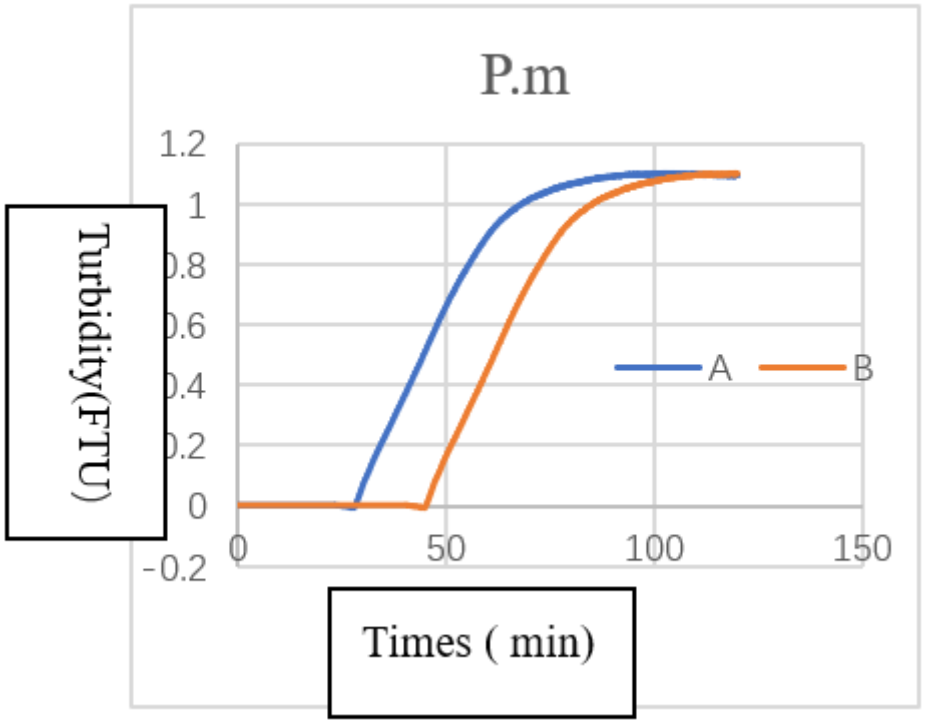

Figure 6

Effect of the indicator on the LAMP of P.m