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# Pragmatic accuracy of in-house loop-mediated isothermal amplification (LAMP) for diagnosis of pulmonary tuberculosis in a Thai community hospital --Manuscript Draft--

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Article Type:	Research Article
Full Title:	Pragmatic accuracy of للسرك buse loop-mediated isothermal amplification (LAMP) for diagnosis of pulmonary tuberculosis in a Thai community hospital
Short Title:	Diagnostic accuracy of in-house LAMP for pulmonary TB
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Keywords:	pulmonary tuberculosis; LAMP; Diagnosis; sensitivity; Specificity
Abstract:	Background improve the quality of diagnosing pulmonary tuberculosis (TB), WHO recommends the use of rapid molecular testing as an alternative to conventional microscopic methods. Loop-mediated isothermal amplification assay (LAMP test) is a practical and cost-effection ucleic amplification technique. We evaluated the pragmatic accuracy of the house LAMP assay for the diagnosis of TB in a remote health care setting where an advanced rapid molecular test is not available. Methods: A prospective diagnostic accuracy study was conducted. Patients with clinical symptoms suggestive of TB were consecutively enrolled from April to August 2016. Sputum samples were collected from each patient and were sent for microscopic examination (both acid-fast stain and fluorescence stain), in-house LAMP test, and TB culture. Results: One hundred and seven patients with TB symptoms were used in the final analysis. This included 50 (46.7%) culture-positive TB patients and 57 (53.3%) culture-negative patients. The overall sensitivity of the in-house LAMP base on culture positive was 88.8% (95%CI 81.2,94.1). The sensitivity was 0.0% CI 04.64.1) for smear-negative, culture-positive patients, and was 16.7% CI 0.4,64.1) for smear-negative, culture-positive patients. The overall sensitivity and accuracy of the in-house LAMP test compared to smear microscopy methods were not significantly different (p=0.375 and p=1.000, respectively). The specificity of the in-house LAMP based on non-TB patients (smear-negative, culture-negative) was 94.7% (95%CI 85.4,98.9). Conclusions: The diagnostic accuracy of the in-house LAMP test in a community hospital was comparable to other previous reports in terms of specificity. The sensitivity of the in-house assay could be improved with better sputum processing and DNA extraction method.
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Response to Reviewers:	Responses to Reviewers' comments Pragmatic accuracy of loop-mediated isothermal amplification (LAMP) for diagnosis of pulmonary tuberculosis in a Thai community hospital Reviewer #1: 1.The study aims to evaluate usefulness of a LAMP method in a practical setting in Thailand. The LAMP method is now available as an only commercial kit TB-LAMP assay (Loopamp™MTBC Detection Kit, Eiken Chemical Company Ltd., Japan) as endorsed by WHO in 2016. It seems that the method used in this study is a unique

system at least partially. So, it is important to state explicitly that the target to be evaluated was an in-house LAMP and not one commercially available LAMP recommended by WHO.

oThe LAMP test in our study was a non-commercial, in-house LAMP. oWe re-wrote the manuscript and emphasized that the test used was in-house LAMP. 2.In evaluating the sensitivity of the method, the authors used culture negative (clinically defined) cases, as well as bacteriologically confirmed cases, as a gold standard of the cases of TB. It may be difficult to admit the clinical diagnosis as a diagnostic basis for such a study as this, apart from clinical practice. Vice versa, the definition of the gold (conventional) standard for specificity (non-cases) should be reconsidered. The following paper may be of use in revising the paper; Kaku et al: Accuracy of LAMP-TB Method for Diagnosing Tuberculosis in Haiti. Jpn. J. Infect. Dis., 69, 488–492, 2016.

oWe modified the inclusion criteria for analysis as suggested by both reviewers. oAs the analysis was done in a per-patient fashion, patients with smear-positive and culture-negative results would be excluded, as these patients were considered as probable TB cases. Therefore, the evaluation of sensitivity would include patients with both smear positive and smear negative with positive culture results. In contrast, the evaluation of specificity would include only patients with smear-negative and culturenegative results.

#### Reviewer #2:

1.Abstract/Background: "proven diagnostic performance" – this is both vague and too specific at the same time, "most of the results were validated" – the results aren't validated, the assay is validated

oWe rewrote the abstract and introduction part as suggested.

2. The language surrounding people with possible TB needs to be updated throughout the paper - avoid the use of terms like "TB suspects" that increase the stigma surrounding this disease.

http://www.stoptb.org/assets/documents/resources/publications/acsm/LanguageGuide\_ ForWeb20131110.pdf

oWe rewrote the abstract and introduction part as suggested.

3. The paper states repeatedly that there is little work published from resourcechallenged settings, but this claim is not supported. Even the references given cite studies in such decentralized settings. Maybe it just hasn't been done in Thailand? A better summary of the literature needs to be included. How does this compare to other studies? How is the TB LAMP test performed in this study compare to the TB LAMP tests in other published literature? A better focus on properly relating the current study to the body of work in the literature rather than trying to claim it is quite novel would actually strengthen the paper. There is merit in replication or demonstrating an important diagnostic in a new geographical area.

oWe rewrote the abstract and introduction part as suggested.

4.In-house vs commercialized kit is mentioned but not explained. And the position of this paper (what LAMP testing approach is used) is not properly placed in the context of what other papers are using and the potential impact on sensitivity/specificity. oWe rewrote the abstract and introduction part as suggested.

5. The sensitivity/specificity of LAMP in other papers, settings, etc needs to be stated with numbers and not just alluded to. A proper, specific summary of the literature is lacking.

oWe rewrote the abstract and introduction part as suggested.

6."In 2016, WHO suggested the use of LAMP assay for the diagnosis of pulmonary tuberculosis" – this is not quite right, WHO recommendations are very specific and it is important to get that right. From the abstract of the citation provided: "WHO recommends that TB-LAMP can be used as a replacement for microscopy for the diagnosis of pulmonary TB in adults with signs and symptoms of TB". This needs to be stated correctly. Also, given the paper has mentioned in-house vs commercialized kits, it needs to be clarified that the WHO guidance refers only to the Eiken LAMP kit. oWe rewrote the abstract and introduction part as suggested.

7."LAMP assay has a low cost per test, does not required advanced technological facilities, and can be routinely practiced in general hospital laboratories [3]." Reference 3 doesn't support this statement – it doesn't say anywhere that the LAMP assay has a low cost per test. It says "Costs can be kept to a minimum if testing is limited to specimens from the most high-risk patients based on proper clinical assessments and national testing algorithms based on public health policies." There are other

publications on the cost of the LAMP assay for TB diagnosis. The authors might explain better the infrastructure/training needed for LAMP based on this reference and others.

oWe rewrote the abstract and introduction part as suggested.

oWe changed the references to the statement as follow: Sohn H. Cost, affordability, and cost-effectiveness of TB-LAMP assay. In: Report to WHO Guideline Development Group Meeting on TB-LAMP Assay. Edn. Geneva: World Health Organization; 2016 and Shete PB, Farr K, Strnad L, Gray CM, Cattamanchi A. Diagnostic accuracy of TB-LAMP for pulmonary tuberculosis: a systematic review and meta-analysis. BMC Infect Dis. 2019;19(1):268. Published 2019 Mar 19. doi:10.1186/s12879-019-3881-y 8.Reference 5 doesn't appear to really relate to the sentences it comes after. Reference 3 would make a lot more sense as it is a detailed overview of TB diagnostics including many molecular diagnostics.

oWe rewrote the abstract and introduction part as suggested.

#### Setting

1. The paper needs to do more to state what sets this setting apart from (or ties it to) other studies. See the methods section describing setting in reference 22 for how attributes of the specific site can be expressed in the context of the needs of LAMP. oWe elaborated the character of our setting as suggested:

oLevel of health system: rural

oDistance to reference laboratory: 0 km

oMedian LAMP test workload: 6 (4-10)

oElectricity and backup power: infrequent power outages, power generator (350 Kw) and UPS (2.7 Kw)

oBiosafety cabinet infrastructure: BSC class II

oLaboratory staff: 4 lab technicians, 1 lab assistant

2.Study Design: This is not a cross-sectional design; it is a prospective design. The plan was to prospectively enroll 120 patients.

oWe changed the type of design to prospective diagnostic accuracy study as suggested.

oWe would like to make a constructive argument on this point, as the diagnostic accuracy research is actually cross-sectional study in design. The cross-sectional design is only the type of membership condition, single component of study base, and cross-sectional design can therefore be collected prospectively or retrospectively. We would like to ask you to kindly refer to this reference: Assessment of the accuracy of diagnostic tests: the cross-sectional study by Knottnerus JA, 2003.

Link: https://www.ncbi.nlm.nih.gov/pubmed/14615003 3."New patients who were clinically suspected of 109 pulmonary TB (coughing for

more than two weeks with or without hemoptysis), aged more than 18 years old were consecutively invited into the study regardless of nation status." Suggest re-writing to something more like: 'Adults more than 18yrs of age with symptoms indicative of pulmonary TB (coughing...) and no history of TB were consecutively enrolled regardless of national status.' If patients were 'invited' but not enrolled, we need numbers on how many declined.

oWe re-wrote the sentence as suggested: Adult patients aged more than 15 years old with symptoms indicative of pulmonary TB (coughing for more than two weeks with or without hemoptysis) and no history of TB were consecutively enrolled regardless of national status.

4. "Samples with contaminated culture results or samples from patients who were previously documented as TB cases were excluded." Were the patients excluded or the samples?

oPatients with previously documented TB cases were excluded.

oPatients with two contaminated or missing culture results were excluded. Methods

1.A map of which samples were used for what tests would be quite helpful. Highlight if any of the reference tests (smear, LJ culture, MGIT culture) were performed on the same sputum as LAMP.

oConventional macroscopy, LAMP test, and culture were conducted as routinely done. oAll patients were given three sealed containers for the collection of morning sputum specimens. Of all containers sent to the laboratory, only the one with seemingly adequate sputum, containing both mucoid or mucopurulent characters with a sample volume more than 3 ml, was used for the whole investigation procedures as routinely done. Specimens were sent for smear microscopy with conventional acid-fast bacilli (AFB) staining with Ziehl-Neelsen technique and fluorescence acid-fast staining with Auramine O solution.

2.Make it clear somewhere that smear-negative refers to AFB smear-negative. oWe added detail on the smear-negative status as suggested.

oAccording to WHO definitions, any patient with at least two AFB smears of scanty grade or one or more smears of 1+ or more was defined as smear-positive case. Smear-negative case was conversely defined.

3.Study size estimation

This has no purpose here – the study is done. Sample size estimation is for study planning purposes, for securing funding and making sure the plan has statistical validity.

oThe study size estimation part was removed as suggested.

4. Statistical analysis. The first four sentences are unnecessary.

oThe first four sentences were removed as suggested.

5. The authors need to state what method was used to obtain the 95% CI for the sens/spec/PPV/NPV/LR+. It is clear from my testing that the Clopper Pearson binomial exact test was used, the authors should include the reference (usually found in the software documentation).

oThe 95% confidence intervals were calculated using the Clopper Pearson binomial exact method.

oWe added this statement in the statistical section and added the citation as suggested.

6.Kappa statistics are for inter-reader reliability, not for comparison of correlations between tests. It includes the concept that agreement may happen by chance when two people are guessing. However, it is not appropriate for comparison of diagnostic results because there isn't guessing – the samples should not agree by chance but because they are or are not TB and the sensitivities of tests objectively vary.

Spearman's correlation can be used, but I think what you actually want is McNemar's test. The desire is to compare the diagnostic performance (i.e. accuracy) between tests – McNemar's test will do that. Alternatively, Spearman's correlation can look at the [objective] agreement between tests.

oSpearman's rank correlation was inserted into the manuscript to represent the objective agreement between tests as suggested.

oThe agreement of LAMP test with smear microscopy methods was analyzed with Kappa's statistics and Spearman's rank correlation.

oWe still presented the value of Kappa's statistic many of the previous studies on LAMP assay and other diagnostic tests had don 2-3].

#### Results

1. Table 1 is dedicated to showing the patient clinical characteristics by culture status. The p-values shown test whether these characteristics differ significantly dependent on culture status. It is expected that gender, nationality, and age should not differ. Whereas it is also expected that chest x-rays and sputum quality would differ. The baseline demographic data between culture188 positive and negative patients were comparable except for the presence of cavitary lesions on 189 chest radiographs and the character of collected sputum (Table 1). Age, nationality, and gender are demographic data. Chest x-ray and sputum quality are clinical characteristics. oWe reanalyzed all the data after exclusion of patients with probable TB (LAMP test positive and AFB smear positive patients with negative culture). oAll the baseline demographic and clinical characteristics data were reanalyzed and presented in Table 1. oThe statements in the results section were re-written as suggested. 2. Table 2 - re-check the NPV for parallel testing oWe reanalyzed all the data after exclusion of patients with probable TB (LAMP test positive and AFB smear positive patients with negative culture). oAll the data on Table 2 were checked for any error as suggested. 3. There are a lot of LAMP-positive and AFB smear-positive patients with negative culture. Especially given that the tests are done on different sputum samples, these should be considered patients with probable TB and not used in assessing sensitivity and specificity. oWe reanalyzed all the data after exclusion of patients with probable TB (LAMP test

positive and AFB smear positive patients with negative culture).

oThe final study size for analysis of LAMP test diagnostic accuracy was therefore 107 patients. (8 patients were excluded, 6 patients with both LAMP test and AFB smear-

positive and culture negative, 1 patient with AFB positive and culture negative, and 1 patient with fluorescence stain positive and culture negative)

4. There are too few smear-negative, culture-positive patients to assess sensitivity. Specificity should not be stratified by smear status, only sensitivity. For the reason above (that smear-positive, culture-negative patients shouldn't be included in estimations of sensitivity/specificity of LAMP), what the paper is calling 'smear-negative

specificity' should in fact be reported as the actual specificity of LAMP. oWe exclude smear-positive, culture negative patients from the analysis as suggested.

oWe reported the actual specificity of LAMP test without stratification.

oWe acknowledged that our there are too few smear negative, culture positive patients to assess sensitivity in the discussion part.

5.Table 2 – the p-values shown have no real meaning! If you want to compare accuracy of tests, you cannot do a p-value over the final accuracy measures among a bunch of tests. You need to compare tests 1 against another by using 2x2 grids and McNemar's test. So, if you want to compare the accuracy of LAMP to the accuracy of AFB stain, you use the grid in Table 3 and McNemar's test:

oThe comparison of diagnostic indices between LAMP test and AFB, fluorescence stain was re-analyzed using McNemar's exact probability test as suggested. We presented the result of the pairwise tests separately and reformatted Table 2. oPairwise testing was not performed to compare the specificity between the LAMP test and the smear microscopy methods as the specificity of the latter was affected by incorporation bias and would not be comparable to the in-house LAMP. oTable 3 was also reformatted.

oSpearman's rank correlation was used as suggested.

#### Discussion

1."This study had demonstrated the pragmatic performance of the LAMP test, which was comparable to that of the conventional smear microscopy and the fluorescence microscopy." Not true, the performance of LAMP as evaluated in this study was below that of smear microscopy.

oWe rewrote the discussion part as suggested.

o"This study had demonstrated the pragmatic diagnostic performance of the in-house LAMP assay in a remote hospital of a high TB burden country. It was revealed that the overall sensitivity of the in-house LAMP in our study was lower than the numbers reported in the majority of the previous in-house LAMP studies. Nonetheless, the specificity was comparable to other figures reported in literature. In comparison to microscopy methods, the AFB and fluorescence stain, the in-house LAMP was found to be inferior in terms of overall sensitivity (82.0% vs. 88.0%, p=0.375) and accuracy (88.8% vs. 94.4%, p=1.000); however, the comparative statistical test revealed non-significant results. Based on the result of our study, we suggest that the in-house LAMP should not be a substitute to conventional smear methods, but should be done in parallel, which would result in a higher sensitivity with fewer false-negative TB cases."

2."Although the sensitivity and specificity of the LAMP test were lower than that of the acid-fast stain and the fluorescence stain, the comparative statistical test revealed non-significant results" This is still true when McNemar's test is performed, but the right statistical tests need to be used in the paper. Furthermore, a non-significant result doesn't mean no difference, it means the difference is likely smaller than the power of the study to detect.

oWe rewrote the discussion part as suggested.

oWe reanalyzed our data using McNemar's exact probability test as suggested. 3.Put PPV/NPV in the context of the local prevalence of disease! State from the literature or reliable source what the prevalence of TB is in the hospital's area of Thailand. I would suggest giving the readers an example: Given that prevalence and a group of 1000 patients, state how many would be true positives, false positive, true negatives, and false negatives. You can therefore assess what burden the different accuracies will place on the hospital. I.e. if the specificity is quite low and the sensitivity is higher, is that better? If the sensitivity is high and the specificity is lower, is that better? Relate this to the LR+.

oWe would like to make a constructive argument to this question as follow: The prevalence of culture-positive TB in this study was 46.7%. As this was a "consecutive recruitment of patients with sign and symptoms suggestive of pulmonary TB" or "patients with higher pre-test probability that the general prevalence" or the "person

that the in-house LAMP test was intended to be used", the calculation of positive predictive values could be directly calculated and reported from the study data as in the other study [1]. Moreover, both the in-house LAMP assay and acid-fast stain were not intended to be used as screening tests in the general population. For this reason, we did not include this part in our manuscript; however, we provide the answer to the question in this response paper.

oThe latest Maesot's population figures from the Health Data Center (HDC), the ministry of public health, Thailand, was 115,108 in 2019. The prevalence of pulmonary tuberculosis was 351 per 100,000 or 35 per 10,000. TB caseNon-TB caseTotal

LAMP positive29528557PPV 29/557=5.2%

LAMP negative69,4379,443NPV 9437/9443=94.9%

Total359,96510,000Prevalence=0.0035

4. "In the clinical context of TB diagnosis, both the LAMP test and the smear microscopy are considered as a diagnostic test which would normally be done in TB suspects with high pre-test probability [14]" – this is not what the reference says. oThe reference states "The TB LAMP assay is usually applied for TB-suspected patients and is rarely used for screening purpose. To rule-in the TB diagnosis, specificity is more important than sensitivity."

oWhat we're trying to imply from this statement was that the LAMP test was developed to be applied for patients who were suspicious of having TB with "higher pre-test probability than average person". As the LAMP test was not for screening purpose, specificity is more important and should be more focused than sensitivity. oAfter we re-analyzed the data with the exclusion of probable TB cases, our specificity

oAfter we re-analyzed the data with the exclusion of probable TB cases, our specificity increased to comparable level with previous studies. The parallel and serial testing was omitted from our analysis as the test accuracy of combination of the in-house LAMP with other smear microscopy methods would be seriously affected by incorporation bias (smear-positive, culture-negative patients were all excluded.

5. "Therefore, a serial test relying on both the result from the LAMP test and the acidfast stain would be more appropriate for use as a rule-in test as it carried higher specificity and positive likelihood ratio than other methods." Authors should define 'rule-in' test and what is generally expected of such a test. Should note the increased cost of such an approach.

oAfter we re-analyzed the data with the exclusion of probable TB cases, our specificity increased to comparable level with previous studies. The parallel and serial testing was omitted from our analysis as the test accuracy of combination of the in-house LAMP with other smear microscopy methods would be seriously affected by incorporation bias (smear-positive, culture-negative patients were all excluded.

6. The effect of a gold standard which is not itself perfect should be discussed. Also the variability between sputum samples should be discussed.

oThe use of routine TB culture as a reference standard might be inadequate, as some TB patients could be classified as not having TB [6]. Different culture media and techniques could be used in composite to achieve different performance characteristics[4]. With a higher quality reference standard, the sensitivity of the inhouse LAMP should be increased when a portion of three remaining false-positive

nouse LAMP should be increased when a portion of three remaining faise-positive cases was re-classified as true-positive cases.

oThis study had a higher proportion of salivary sputum than mucous sputum. This could affect the diagnostic performance of both the index and the reference test[5]. The percentage of culture-positive TB cases was lower in salivary samples than in mucous samples (35.8% vs. 65.0%, p=0.005). Both the quality and quantity of sputum specimens were associated with positivity of smear, molecular testing methods (Xpert MTB/RIF and PCR), and TB culture [6,7]. Thus, it was possible that some patients with pulmonary TB might be classified as smear-negative, LAMP-negative, or even culture-negative cases. Interestingly, it was revealed from our data that the proportion of smear-positive, LAMP-positive results was also significantly lower in salivary sputum than in mucous sputum (31.3% vs 57.5%, p=0.009 and 29.9% vs. 60.0%, p=0.003, respectively). Therefore, the sensitivity and accuracy of all tests, including LAMP, might be underestimated. Previous studies reported that by improving the sputum quality, TB diagnostic yield increased[8,9]. Therefore, high-quality sputum collection must be encouraged both in practice and studies.

7.A better look at the differences between this study and others with better test performance needs to be done.

oln this study, the sensitivity of the in-house LAMP test was 82.0% (95%Cl 68.6-91.4) in culture-positive TB patients, respectively. In the past, several studies had reported a

	higher sensitivity of the in-house LAMP test, which ranges from 90.0 to 100.0%. Most of these studies were either University hospital, TB-specialized centers or hospitals, or national TB-specialized laboratory, which were generally equipped with highly-trained personnel and adequate infrastructural supports. The overall sensitivity of our in-house LAMP was consistent with two previous studies from India and Zambia, which was 79.5% (95%CI 64.0-89.0) and 81.4% (95%CI 71.6-89.0), respectively. Although both studies were performed in University hospitals, the LAMP procedures were modified to suit local conditions, and sputum processing and DNA extraction was done with commercial kits. The higher sensitivity of the acid-fast stain and the fluorescnce stain in our study could be explained by the high prevalence of TB, the absence of HIV patient or a smaller number of patients with paucibacillary sputum, and the availability of skilled technicians 8. "Currently, the WHO only supported the use of two rapid molecular tests for the diagnosis of 294 pulmonary tuberculosis, which were Xpert MTB/RIF and the LAMP test" – as the concept of LAMP test from a kit and other LAMP tests has been raised, and the variability of accuracy depending, it needs to be clear that the WHO recommendation is only for the Eiken LAMP test kit! oWe edited the statement as follow: "Currently, the WHO only supported the use of two rapid molecular tests for the diagnosis of pulmonary tuberculosis, which were Xpert MTB/RIF and the commercialized TB-LAMP assay". References 1. George G, Mony P, Kenneth J. Comparison of the Efficacies of Loop-Mediated Isothermal Amplification, Fluorescence Smear Microscopy and Culture for the Diagnosis of Tuberculosis. PLoS ONE. 2011;6. doi:10.1371/journal.pone.0021007 2. Phestuskiri B, Rudeeaneksin J, Srisungnam S, Bunchoo S, Klayut W, Nakajima C, et al. Comparison of Loop-Mediated Isothermal Amplification, Microscopy, Culture, and PCR for Diagnosis of Pulmonary Tuberculosis. Jpn J Infect Dis. 2020;adypub. doi:10.7883/
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#### Competing Interests

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inter an ethics statement for this	Hospital, The Ministry of Public Health (serial number 37/2015) and The Human Research Ethics Committee of Thammasat University, Faculty of Medicine (COA
ubmission. This statement is required if	number 081/2016). The clinical samples used in this study were collected from all
ne study involved:	patients as routinely done. Informed consent was obtained from all patients prior to
Human participants	inclusion.
Human specimens or tissue	
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1	Pragmatic accuracy of in-house loop-mediated isothermal amplification
2	(LAMP) for diagnosis of pulmonary tuberculosis in a Thai community
3	hospital
4	
5	Short title: Diagnostic accuracy of in-house LAMP for pulmonary TB
6	
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20	<sup>¶</sup> These authors contributed equally to this work.

## 22 Abstract

23 Background: To improve the quality of diagnosing pulmonary tuberculosis (TB), WHO recommends the use of rapid molecular testing as an alternative to conventional microscopic 24 methods. Loop-mediated isothermal amplification assay (LAMP test) is a practical and cost-25 26 effective nucleic amplification technique. We evaluated the pragmatic accuracy of the in-27 house LAMP assay for the diagnosis of TB in a remote health care setting where an advanced rapid molecular test is not available. 28 Methods: A prospective diagnostic accuracy study was conducted. Patients with clinical 29 symptoms suggestive of TB were consecutively enrolled from April to August 2016. Sputum 30 31 samples were collected from each patient and were sent for microscopic examination (both acid-fast stain and fluorescence stain), in-house LAMP test, and TB culture. 32 **Results:** One hundred and seven patients with TB symptoms were used in the final analysis. 33 34 This included 50 (46.7%) culture-positive TB patients and 57 (53.3%) culture-negative patients. The overall sensitivity of the in-house LAMP based on culture positivity was 88.8% 35 (95%CI 81.2,94.1). The sensitivity was 90.9% (95%CI 78.3,97.5) for smear-positive, culture-36 positive patients, and was 16.7% (95%CI 0.4,64.1) for smear-negative, culture-positive 37 patients. The overall sensitivity and accuracy of the in-house LAMP test compared to smear 38 39 microscopy methods were not significantly different (p=0.375 and p=1.000, respectively). The specificity of the in-house LAMP based on non-TB patients (smear-negative, culture-40 negative) was 94.7% (95%CI 85.4,98.9). 41 42 **Conclusions:** The diagnostic accuracy of the in-house LAMP test in a community hospital was comparable to other previous reports in terms of specificity. The sensitivity of the in-43 house assay could be improved with better sputum processing and DNA extraction method. 44 45 Keywords: Pulmonary Tuberculosis, in-house LAMP, Diagnosis, Sensitivity, Specificity

# 47 Introduction

Tuberculosis (TB), an airborne communicable disease, has long been considered a significant 48 threat to global public health. According to The World Health Organization (WHO), 10 49 million people were newly infected with TB in 2018. Although the incidence and prevalence 50 51 of TB vary greatly across the globe, 87% of total cases resided within 30 countries with high TB burden, including Thailand, where the incidence rate was 153 cases per 100,000 52 population in 2018  $\bigcirc$  Early diagnosis and timely treatment is an essential component of The 53 End TB Strategy endorsed by the WHO, aiming to end the global TB epidemic by the year 54 2035 [2]. However, TB is still underdiagnosed and undertreated, especially in resource-55 limitin Duntries due to the lack of highly sensitive and specific diagnostic tools which are 56 usually expensive and require adequate infrastructure [1,3]. Novel diagnostic methods with 57 enough simplicity and cost-effectiveness  $\mathcal{P}$  therefore necessary to improve accurate 58 59 identification of TB patients in these particular settings [3,4].

60

Molecular testing methods such as polymerase chain reaction (PCR) or Xpert MTB/RIF have 61 been widely acknowledged as alternative tools the diagnosis of TB patients [3,5]. These 62 nucleic amplification techniques were known for yielding rapid and accurate TB diagnosis, 63 64 which would overcome the limitations of classical methods, insensitivity for smear microscopy, and lengthy incubation period for TB culture. However, several obstacles remain 65 for the application of these molecular tests as point-of-care testing in community settings 66 because of their complexity in executio 2nd substantial requirements for financial and 67 personnel resources [3,6]. Loop-mediated isothermal amplification (LAMP) assay is another 68 recently developed nucleic acid amplification technique. Unlike PCR, where the 69 70 amplification of DNA fragment occurs in temperature-dependent steps, the reaction of LAMP assay functions in isothermal or constant temperature conditions [7,8]. In 2016, WHO 71

sugge the use of commercial TB-LAMP assay (Eiken Chemical Co., Tokyo, Japan) as a
replacement for smear microscopy for the diagnosis of TB in patients with symptoms
suggestive of TB [9]. TB-LAMP assay has a low cost per test, does not required advanced
technological facilities, and can be routinely practiced in general hospital laboratories [6,10].

77 As financial resources are usually limited in countries with high TB prevalence, setting up an infrastructure to support the commercial TB-LAMP could still be unattainable. A more 78 affordable in-house LAMP was developed in 2008 [11]. The main advantage of the in-house 79 assay was that it could be implied on the readily-available infrastructure of any laboratory, 80 even in the decentralized  $\overline{\mathcal{D}}$  However, it did require extra-training and skill of technicians 81 82 to process the clinical specimens. In the past decades, several clinical studies and metaanalyses had evaluated the diagnostic accuracy of the in-house LAMP  $\bigcirc$  for the diagnosis 83 of pulmonary TB [12–14] (S1 Table). From the latest meta-analysis, the overall sensitivity 84 and specificity of the in-house LAMP 93.0% (95% CI 88.9-95.7) and 91.8% (95% CI 85 86.4-95.1), respectively [14]. One recent study in Thailand reported the sensitivity and the 86 specificity of the in-house LAMP at 94.4% (95%CI 88.9-97.7) and 94.3% (95%CI 87.2-87 98.1), respectively [15]. However, the reported accuracy could be overestimated if being 88 assessed in qualified laboratories with highly skilled technicians and sufficient resources 89 90 where molecular tests usually are available [14]. Therefore, this study aimed to evaluate the 91 pragmatic accuracy of the in-house LAMP assay for the diagnosis of pulmonary TB in a peripheral community hospital of a developing country with a high TB burden. 92

## 94 Materials and Methods

95

## 96 Ethics Statement

- 97 This study was approved by the Research Ethics Committee of Maesot General Hospital, The
- 98 Ministry of Public Health (serial number 37/2015) and The Human Research Ethics
- 99 Committee of Thammasat University, Faculty of Medicine (COA number 081/2016). The
- 100 clinical samples used in this study were collected from all patients as routinely done.
- 101 Informed consent was obtained from all patients prior to inclusion.
- 102

## 103 Setting

The study was settle Maesot General Hospital, a large-sized community hospital with 365 104 in-patient beds. The hospital is located in Maesot district in Tak (province), which shares the 105 106 border with Myanmar and provides standard health care to both Thai and non-Thai patients (Burmese immigrants and ethnic minorities). According to the Health Data Center, the 107 ministry of public health, pailand, the incidence rate of pulmonary TB in Maesot was 351 108 per 100,000 in 2019. The level of  $\mathbb{D}^2$  lth care system of the hospital is considered rural. 109 Maesot hospital has its own reference laboratory with biosafety cabinet infrastructure, BSC 110 class II. There are four lab technicians and one lab assistant within each working shift. Power 111 generator (350 kW) and UPS (2.7 kW) were available in case of power outages, which was 112 infrequent. Median LAMP test workload per day was 6 (range 4-10). 113

114

## 115 Study Design

116 This prospective diagnostic accuracy research was conducted from April to August 2016.

117 Adult patients aged more than 15 years old with symptoms indicative of pulmonary TB

(coughing for more than two weeks with or without hemoptysis) and no history of TB were
consecutively enrolled regardless of nationality status. Patients with previously documented
TB history or patients with two contaminated or missing cultures were excluded from the
study.

122

## 123 Methods

All patients were given three sealed containers for the collection of morning sputum 124 specimens. Of all containers sent to the laboratory, only the one the seemingly adequate 125 sputum containing both mucoid or mucopurulent characters with a sample volume of more 126 127 than 3 ml, was used for the whole investigation procedures as routinely done. Specimens were sent for smear microscopy with conventional acid-fast bacilli (AFB) staining with 128 Ziehl-Neelsen technique and fluorescence acid-fast staining with Auramine O solution. 129 130 Smear-positive case was defined according to WHO definitions as the presence of at least two smears of scanty grade or one or more smears of 1+ or more. A smear negative case or 131 AFB smear-negative was conversely defined 132

133

### 134 Sputum decontamination and culture examination

For the sputum decontamination process, the collected samples and 2% N-Acetyl-L-cysteine
(NALC) NaOH were poured into a 50 ml sterile centrifuge tube in an equal proportion and
were subsequently mixed by vortexing for 30 seconds and left at room temperature (20-25 °C)
for 15 minutes. Then, the test tubes were filled with phosphate buffer saline (pH 6.8) until the
volume reached the level of 50 ml. The samples were put in a high-speed refrigerated
centrifuge at 3,000 g for 20 minutes. Next, the supernatants were poured off, leaving the tube

with decontaminated sputum samples. Finally, a drop (1 ml) of phosphate buffer saline (pH6.8) was used for resuspension of the specimen.

143

For TB culture, the reference test, we performed both conventional culture method on L-J 144 (Lowenstein-Jensen) medium and BBL MGIT 960 (mycobacterial growth indicator tube) 145 culture method. The culture media were inoculated with processed sputum specimens and 146 incubated at 35 to 37 °C and monitored weekly for growth until 8 weeks. The sputum samples 147 were considered as "culture-positive" if growth was detected in either of L-J or MGIT 148 149 culture, regardless of the smear status. If growth was not detected in neither of the culture methods and both microscopy results were negative, the samples were considered as "culture-150 negative" or "non-TB patients". Patients with smear-positive and culture-negative, which 151 152 were generally considered as probable TB, were excluded from the analysis. Both smear 153 microscopy and culture methods were performed according to the standard protocols [16].

154

#### 155 In-house LAMP test

The LAMP test consists of three steps as follows: DNA extraction, isothermal amplification, 156 157 and visual interpretation with fluorescence. The National Institute of Health of Thailand had developed the TB Fast Amp technique (a modified LAMP procedure) to suite local practice 158 since 2009. The procedures were described as follow. Flexi Gene® DNA Kit (Qiagen co., 159 160 USA) and Protenase K Kit (Qiagen co., USA) were used for DNA extraction [17,18]. Four primers (MTB primers, MAV primers, MIN primers, and Muniv primers) were used for the 161 recognition of six distinct regions on the 16S ribosomal RNA gene of M. tuberculosis. Each 162 single LAMP reaction includes 12 µl of TB-Fast AMP mixture (FastAMP master mix 163 includes 2 µl 10Xbuffer, 4 µl 2mM dNTPs, 3.2 µl 5M betaine, 1.2 µl 100 mM MgSO<sub>4</sub>, 1.6 µl 164 165 primer mixture), 1 µl Bst DNA polymerase enzyme, 1 µl fluorescent detection reagent and 6

µl of extracted DNA samples. Amplification of reaction mixture was performed in the
heating blocks at 65 °C for 60 minutes, then examined directly by visual observation. The
LAMP assay was considered "positive" if the color of the reaction mixture changed from
orange to green or fluorescence was directly observed with the naked eyes. The test was
considered "negative" if the color of the mixture remained unchanged. For quality control,
positive control (test tube with M. *tuberculosis* genetic materials) and negative control (test
tube without M. *tuberculosis* genetic materials) were included in all runs.

173

## 174 Statistical Analysis

We used Fisher's exact probability test for comparison of differences in independent 175 proportions and Student's t-test for two independent means. The sensitivity, specificity, 176 positive predictive values (PPV), negative predictive values (NPV), and positive and negative 177 likelihood ratios were calculated and reported with its 95% confidence interval. The 95% 178 confidence interval were estimated using the Clopper Pearson binomial exact method. The 179 comparison of sensitivity, specificity, and overall test accuracy between the LAMP test and 180 181 smear microscopy methods was performed with McNemar's exact probability test. Pairwise 182 testing to compare the specificity between the LAMP test and the smear microscopy methods was not performed as the specificity of the latter was affected by incorporation bias and 183 would not be comparable to the in-house LAMP. The agreement of the LAMP test with 184 smear microscopy methods was analyzed with Kappa's statistics and Spearman's rank 185 correlation. P-values of less than 0.05 were considered statistically significant. All statistical 186 analyses were done using Stata version 16 (StataCorp, Texas). 187

## **Results**

190 A total of 120 patients to be evaluated for TB were consecutively included from April to

- 191 August 2016. Three patients with two contaminated cultures, two patients who subsequently
- 192 were detected as previously documented TB cases, and eight patients who had smear-positive
- and culture-positive results were excluded from the analysis; only 107 patients remained in
- the study (Fig. 1). Most of the included patients were male (60% vs. 40%) with a mean age of
- 47 years old. Fifty (46.7%) were culture-positive TB patients and 57 (53.3%) were culture-
- 196 negative patients. The baseline demographic data between culture-positive and culture-
- 197 negative patients were comparable. For clinical characteristics, the presence of cavitary
- 198 lesions on chest radiographs and the character of collected sputum was found to be
- significantly different (Table 1). Culture-positive TB patients had higher proportion of
- 200 cavitary lesions (14.0% vs. 1.8%, p=0.024) and mucous sputum specimen (52.0% vs 24.6%,
- 201 p=0.005) than patients with negative TB culture.

Characteristics	TB Culture Positive	TB Culture Negative	P-Value	
	(S+ or S-, C+)	(S-, C-)		
	n=50 (46.7%)	n=57 (53.3%)	_	
Gender				
Male	30 (60.0)	36 (63.2)	0.842	
Female	20 (40.0)	21 (36.8)		
Nationality				
Thai	28 (56.0)	21 (36.8)	0.054	
Non-Thai	22 (44.0)	36 (63.2)		
Age (year, mean±SD)	48.7±17.4	45.8±18.7	0.408	
Chest radiographs				
Without cavitary lesions	43 (86.0)	56 (98.2)	0.024	
With cavitary lesions	7 (14.0)	1 (1.8)		
Character of sputum				
Salivary	24(48.0)	43 (75.4)	0.005	
Mucous	26 (52.0)	14 (24.6)		

## 204 Table 1. Demographic and clinical characteristics of the patients by TB culture status

 205
 Abbreviations: TB, tuberculosis; C, culture (+ positive or – negative); S, smear microscopy (+ positive or – negative); SD,

standard deviation.

207

## 208 Fig. 1. Study flow diagram of patient enrollment and results of index and reference test

209 **based on culture result** 

210

212 The overall sensitivity of the LAMP test was 82.0% (95%CI 68.6-91.4), whereas the sensitivity in smear-positive, culture-positive patients and smear-negative, culture-positive 213 was 90.9% (95% CI 78.3-97.5) and 16.7% (95% CI 0.4-64.1), respectively. The overall 214 sensitivity of both the AFB and the fluorescence stain was slightly higher than that of the 215 LAMP test; however, the differences were non-significant (Table 2). The specificity, positive 216 predictive value, and negative predictive value of LAMP test was 94.7% (95%CI 85.4-98.9), 217 93.2% (95% CI 81.3-98.6), and 85.7% (95% CI 74.6-93.3), respectively. The positive and 218 negative likelihood ratios of the LAMP test was 15.6 (95%CI 4.47-82.12) and 0.19 (95%CI 219 220 0.08-0.44), respectively. Even though the accuracy measures for the diagnosis of TB cases were shown to vary across different test methods (LAMP test, AFB stain, and fluorescence 221 stain), the differences were without statistical significance (Table 2). 222 223 LAMP test results were highly correlated with those of AFB and fluorescence stain 224 (Spearman's rho 0.85, p<0.001) in the diagnosis of culture-positive TB cases (Table 3). The in-house LAMP also showed substantial to almost perfect agreement with both microscopy 225 methods in the diagnosis of culture-positive cases (Kappa 0.85, 95% CI 0.74,0.95) (Table 3). 226

## 228 Table 2. Diagnostic accuracy of the in-house LAMP test, AFB stain, and Fluorescence

#### 229 stain.

Method	Sensitivity% (95% CI), no. corrects			Specificity% (95%CI), no. corrects	Accurrents	PPV% (95%CI)	NPV (95%CI)	LR+ (95%CI)	LR- (95%CI)
	S+, C+ (n=44)	S-, C+ (n=6)	Any S, C+ (n=50)	S-, C- (n=57)	(n=107)	(507002)	(307002)	() ( ) ( ) ( )	()0/001)
	90.9	16.7	82.0	94.7	88.8	93.2	85.7	15.6	0.2
LAMP	(78.3,97.5), N=40	(0.4,64.1), n=1	(68.6,91.4), n=41	(85.4,98.9), n=54	(81.2,94.1),	(81.3,98.6)	(74.6,93.3)	(4.5,82.1)	(0.1,0.4)
	N=40	11=1	88.0	100.0	n=95 94.4	100.0	00.5		
AFB stain	-	-	(75.7,95.5),	(93.7,100.0),	(88.2,97.9),	100.0 (93.7,100.0)	90.5 (80.4,96.4)	-	-
			n=44 88.0	n=57 100.0	n=101 94.4				
Fluorescence stain	-	-	(75.7,95.5),	(93.7,100.0),	(88.2,97.9),	100.0 (93.7,100.0)	90.5 (80.4,96.4)	-	-
			n=44	n=57	n=101	(55.7,100.0)	(00.4,70.4)		
LAMP test vs. AFB stain			P=0.375*	P=0.250*	P=1.000*				
LAMP test vs.			P=0.375*	P=0.250*	P=1.000*				
Fluorescence stain			- 0.070	1 0.200					

230 \*P-values from McNemar's Exact probability test

231 Abbreviations: AFB, acid fast bacilli; C, culture (+ positive or – negative); CI, confidence interval; LAMP, loop-mediated

isothermal amplification; LR+, positive likelihood ratio; LR-, negative likelihood ratio; no. correct, number correctly

233 identified; NPV, negative predictive value; PPV, positive predictive value; S, smear microscopy (+ positive or – negative).

## 235 Table 3. Diagnostic agreement and correlation between the in-house LAMP test and

#### AFB Stain & LAMP Test Fluorescence stain Positive Total Negative 40 Positive 4 44 Negative 4 59 63 Total 44 63 107 Agreement (%) 92.5% 0.85 (0.74, p<0.001) Kappa (95%CI, p-value) 0.85 )01) Spearman's rho (p-value) 237 Abbreviations: LAMP, loop-mediated isothermal amplification; CI, confidence interval. 238

## 236 AFB stain-fluorescence stain.

239

## 241 **Discussion**

This study have monstrated the pragmatic diagnostic performance of the house LAMP 242 assay in a remote hospital of a high TB burden country. It was revealed the overall 243 sensitivity of the in-house LAMP in our study was lower than the numbers reported in the 244 245 majority of the previous in-house LAMP studies. Nonetheless, the specificity was comparable to other figures reported in the literature. In comparison to microscopy methods, 246 the AFB and fluorescence stain, the in-house LAMP was found to be inferior in terms of 247 overall sensitivity (82.0% vs. 88.0%, p=0.375) and accuracy (88.8% vs. 94.4%, p=1.000); 248 however, the comparative statistical test revealed no gnificant results. Based on the result 249 of our study, we suggest that the in-house LAMP should not be a substitute to conventional 250 smear methods, but should be done in parallel, which would result in a higher sensitivity with 251 fewer false-negative TB cases. 252

253

In this study, the sensitivity of the in-house LAMP test was 82.0% CI 68.6-91.4) in 254 culture-positive TB patients, resperted a higher 255 sensitivity of the in-house LAMP test, which ranges from 90.0 to 100.0% [11,15,19–24]. 256 Most of these studies were either University hospitals, TB-specialized centers or hospitals, or 257 258 national TB-specialized laboratories, which were generally equipped with highly-trained personnel and adequate infrastructural supports. The overall sensitivity of our in-house 259 LAMP was consistent with two previous studies from India and Zambia, which was 79.5% 260 (95%CI 64.0-89.0) and 81.4% (95%CI 71.6-89.0), respectively [12,25]. Although both 261 studies were performed in University hospitals, the LAMP procedures were modified to suit 262 local conditions, and sputum processing and DNA extraction was done with commercial kits. 263 The higher sensitivity of the acid-fast stain and the fluorescence stain in our study could be 264 explained by the high prevalence of TB, the absence of HIV patients or less photometers of the second secon 265

patients with paucibacillary sputum, and the availability of skilled technicians [12,26–28].
Besides, specimen decontamination with concentrated NaOH decreases the amount of viable
genetic materials for amplification, which could reduce the sensitivity of both the LAMP test
and TB cultures. A lower concentration of NaOH (1-1.5%) or NaOH free methods during
sample decontamination may be suggested [12,29]. The sensitivity of the LAMP test in
smear-negative specimens could not be accurately estimated in this study as there were too
few smear-negative, culture-positive patients.

273

274 The overall specificity of the LAMP test was 94.7% (95%CI 85.4-98.9) for non-TB patients, respectiv This was in concordance with a recent meta-analysis, which reported pooled 275 specificity of the in-ho LAMP at 91.8% (95%CI 86.4-95.1) [14]. However, it was 276 277 concluded that the specificity of the in-house assays was lower than that of the Loopamp 278 commercial kit, which was reported at 96.5% (95%CI 94.7-97.7). A false positive LAMP result in smear-positive cases was frequently encountered in routine practice, which could be 279 280 explained by multiple factors such as higher temperature, higher humidity, suboptimal reagents volume, and crossover contamination [14,30]. For in-house LAMP, an extensive 281 laboratory technician training and continuous quality assessment should be conducted to 282 lessen the risk of false-positive results. However, other potential factors might still account 283 284 for the low specificity, such as temperature controls and volume of reaction used. For 285 temperature, only available water bath was applied for temperature controls during LAMP procedures instead of a more stable dry heating block. A recent study suggested a high 286 reaction volume of 30-35 µl due to the risk of self-priming in concentrated reagents [30]. 287 288 Currently, the WHO only suppose the use of two rapid molecular tests for the diagnosis of

289 Currently, the WHO only support the use of two rapid molecular tests for the diagnosis of
290 pulmonary TB, which were Xpert MTB/RIF and the commercialized TB-LAMP assay [9].

291 According to previous studies, both had shown comparable performance in smear-positive samples, but higher sensitivity was shown in Xpert MTB/RIF than in the LAMP test [6,25]. 292 Xpert MTB/RIF has been endorsed for use in the diagnosis of TB in many countries, 293 294 including Thailand [4,31]. Nonetheless, Xpert MTB/RIF might not be suitable in peripheral regions with poor infrastructure as the instrument requires a stable electricity supply and an 295 appropriate environment. The device also requires high continuous maintenance costs leading 296 297 to a relatively high cost per test compared to the LAMP test. The LAMP test is readily available and can be done in any resource-poor settings with regular infrastructure and 298 299 technicians with adequate training. In Thailand, only a portion of patients, not including foreigners and ethnic minorities, could reimburse the cost for Xpert MTB/RIF due to the 300 301 regulation stated by The National Health Security Office (NHSO). To effectively prevent the 302 spread of TB, all patients to be evaluated for TB should have equal access to high-quality 303 diagnostic tools. Therefore, smear microscopy and the LAMP test may be more applicable in terms of accessibility and affordability, especially in the distant areas and the borderlands. 304 305

However, there may be some limitations to this study. First, the study size might not be 306 powered enough to confirm the statistical insignific  $\mathcal{P}$  e of the between-test comparison. 307 Second, no patients with HIV infection were included during the study period, as HIV status 308 could be influential to the diagnostic performance of both the smear microscopy and the 309 310 LAMP test, especially in areas with a high prevalence of TB-HIV coinfection. Third, this study had a higher proportion of salivary sputum than mucous sputum. This could affect the 311 diagnostic performance of both the index and the reference test [32]. The percentage of 312 313 culture-positive TB cases was lower in salivary samples than in mucous samples (35.8% vs. 65.0%, p=0.005). Both the quality and quantity of sputum specimens were associated with 314 315 the positivity of smear, molecular testing methods (Xpert MTB/RIF and PCR), and TB

316 culture [33,34]. Thus, it was possible that some patients with pulmonary TB might be classified as smear-negative, LAMP-negative, or even culture-negative cases. No previous 317 study had officially addressed the effect of sputum quality on the LAMP test. Moreover, the 318 319 character of sputum specimens was rarely reported. Interestingly, it was revealed from our data that the proportion of smear-positive, LAMP-positive results was also significantly 320 lower in salivary sputum than in mucous sputum (31.3% vs. 57.5%, p=0.009 and 29.9% vs. 321 60.0%, p=0.003, respectively). Therefore, the sensitivity and accuracy of all tests, including 322 LAMP, might be underestimated. Previous studies reported that by improving the sputum 323 324 quality, TB diagnostic yield increased [35,36]. Thus, high-quality sputum collection must be encouraged both in practice and studies. 325

326

327 Finally, the use of routine TB culture as a reference standard might be inadequate, as some 328 TB patients could be classified as not having TB [6]. With a higher quality reference standard, the sensitivity of the in-house LAMP should be increased when a portion of three 329 330 remaining false-positive cases was re-classified as true-positive cases. Different culture media and techniques could be used in composite to achieve different performance characteristics 331 [37]. In our study, two different culture techniques, L-J and MGIT, were used to increase the 332 diagnostic rate of TB[38]. We also applied a strict diagnostic definition in calculating 333 334 specificity by considering only patients with smear-negative and culture-negative results[39]. 335

# 336 **Conclusions**

In conclusion, the LAMP test is a practical and affordable nucleic amplification technique for
the diagnosis of pulmonary TB, which should be implemented in resource-limiting settings
where Xpert MTB/RIF is unavailable. The diagnostic accuracy of the in-hose LAMP was
similar to previous studies for specificity. Better sputum processing and DNA extraction

341 method should be identified to improve the test sensitivity. The overall accuracy of the in-

342 house LAMP test was comparable to that of conventional microscopy and fluorescence

343 microscopy with minimal inferiority in terms of sensitivity. Therefore, a parallel examination

of both smear microscopy and the in-house LAMP test is suggested to minimize the risk of

345 false-negative results, especially in an endemic area.

346

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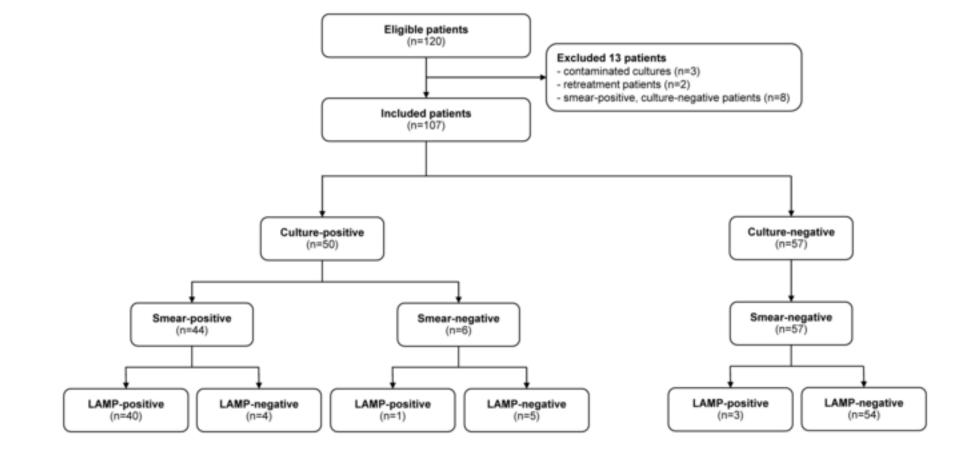
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# 477 Supporting information

- 478 S1 Table. Review on diagnostic accuracy of in-house LAMP assays for diagnosis of
- 479 pulmonary tuberculosis (DOCX)
- 480 S2 Table. LAMP minimal dataset (CSV)





Click here to access/download Supporting Information S2 LAMP dataset.csv Click here to access/download Supporting Information Table S1.docx

1	Pragmatic accuracy of in-house of-loop-mediated isothermal amplification ←	Formatted: Line spacing: Double
2	(LAMP)	
	for diagnosis of pulmonary tuberculosis in a Thai community hospital	
3	for diagnosis of pumonary tuberculosis in a rmar community hospital	
4		
5	Short title: Diagnostic accuracy of <u>in-house</u> LAMP for pulmonary TB	
6		
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8	Jayanton Patumanond <sup>3</sup> , Janisara Rudeeaneksin <sup>4¶</sup> , Wiphat Klayut <sup>4¶</sup>	
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20	<sup>¶</sup> These authors contributed equally to this work.	
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# 22 Abstract

- 24 Background: To improve the quality of diagnosing pulmonary tuberculosis (TB), WHO
- 25 recommends the use of rapid molecular testing as an alternative to conventional microscopic
- 26 methods. Loop-mediated isothermal amplification assay (LAMP test) is a practical and
- 27 recently developed<u>cost-effective</u> nucleic amplification technique<u>.</u> with proven diagnostic
- 28 performance over the past decades. However, most of the results were validated within large
- 29 centers with highly skilled personnel and adequate infrastructure. We evaluated the pragmatic
- 30 accuracy of the the in-house LAMP assay for the diagnosis of TB in a remote health care
- 31 setting where an advanced rapid molecular test is not available.
- 32 Methods: Diagnostic accuracy research using a cross-sectional designA prospective
- 33 diagnostic accuracy study was conducted. Clinically suspected TB patients Patients with
- 34 <u>clinical symptoms suggestive of TB</u> were consecutively <u>included enrolled</u> from April to
- 35 August 2016. Sputum samples were collected from each patient and were sent for
- 36 microscopic examination (both acid-fast stain and fluorescence stain), -in-house LAMP test,
- 37 and TB culture, and LAMP test.
- 38 **Results:** One hundred and fifteen seven <u>TB suspects patients with TB symptoms</u> were used in
- the final analysis. This included 50 (43.546.7%) culture-positive TB patients and 5765
- 40 (5<u>3.36-5</u>%) culture-negative patients. The sensitivity, specificity, positive predictive value,
- 41 and negative predictive value of the LAMP test compared to the reference TB culture were
- 42 <del>82.0% (68.6-91.4), 84.6% (73.5-92.4), 80.4% (66.9-90.2), and 85.9% (75.0-93.4),</del>
- 43 respectively. The overall sensitivity of the in-house LAMP based on culture positivity was
- 44 88.8% (95% CI 81.2,94.1). The sensitivity was 90.9% (95% CI 78.3,97.5) for smear-positive,
- 45 culture-positive patients, and was 16.7% (95%CI 0.4,64.1) for smear-negative, culture-

46	positive patientsThe overall diagnostic performansensitivity and accuracyee of the in-house
47	LAMP test compared to direct microscopic examination and fluorescence microscopsmear
48	microscopy methodsy were not significantly different (p=0.375 and p=1.000, respectively).
49	The specificity of the in-house LAMP based on non-TB patients (smear-negative, culture-
50	negative) was 94.7% (95%CI 85.4,98.9)
51	Conclusions: The diagnostic accuracy of the the in-house LAMP test in a community
52	hospital was comparable to other previous reports in terms of specificity. The sensitivity of
53	the in-house assay could be improved with better sputum processing and DNA extraction
54	methodcomparable to the conventional smear microscopy examination for the diagnosis of
55	TB in a remote hospital of high TB burden country. Serial testing of both tests may be
56	suggested to improve the overall accuracy of TB diagnosis.
57	
58	Keywords: Pulmonary Tuberculosis, in-house LAMP, Diagnosis, Sensitivity, Specificity

# 60 Introduction

61	Tuberculosis (TB), an airborne communicable disease, has long been considered as a	
62	significant threat to global public health. According to The World Health Organization	
63	(WHO), 10 million people were newly infected with TB in 2018. Although the incidence and	
64	prevalence of TB vary greatly across the globe, 87% of total cases resided within 30 countries	
65	with high TB burden, including Thailand, where the incidence rate was 153 cases per 100,000	
66	population in 2018 [1]. Early diagnosis and timely treatment is an essential component of The	Field
67	End TB Strategy endorsed by the WHO, aiming to end the global TB epidemic by the year	
68	2035 [2]. However, tuberculosis is still underdiagnosed and undertreated, especially in	Field
69	resource-limiting countries due to the lack of highly sensitive and specific diagnostic tools	
70	which are usually expensive and require adequate infrastructure [1,3]. Novel diagnostic	Field
71	methods with enough simplicity and cost-effectiveness are therefore necessary to improve	
72	accurate identification of tuberculosis patients in these particular settings [3,4][4,5].	Field
73		Forma Forma
74	Molecular testing methods such as polymerase chain reaction (PCR) or Xpert MTB/RIF have	
75	been widely acknowledged as alternative tools for the diagnosis of tuberculosis patients	
76	[3,5][5,6]. These nucleic amplification techniques were known for yielding rapid and accurate	Forma
77	TB diagnosis, which would elearly overcome the limitations of classical methods,	Forma Field
78	insensitivity for smear microscopy, and lengthy incubation period for TB culture. However,	
79	several obstacles remain for the application of these molecular tests as point-of-care testing in	
80	community settings because of their complexity in executions and substantial requirements	
81	for financial and personnel resources [3.6][3.7]. Loop-mediated isothermal amplification	Field
82	(LAMP) assay is another recently developed nucleic acid amplification technique. Unlike	Forma Forma
83	PCR <sub>a</sub> where the amplification of DNA fragment occurs in temperature-dependent steps, the	
84	reaction of LAMP assay functions in isothermal, or constant temperature, conditions	

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85	[7,8][8,9]. LAMP assay has a low cost per test, does not required advanced technological		Formatted: Font: (Default) Times New Roman, 12 pt
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86	facilities, and can be routinely practiced in general hospital laboratories [3]. In 2016, WHO		Field Code Changed
87	suggested the use of commercial TB-LAMP assay (Eiken Chemical Co., Tokyo, Japan) for		Field Code Changed
88	theas a replacement for smear microscopy for the diagnosis of pulmonary tuberculosis TB in		
89	patients with symptoms suggestive of TB [9][10]. TB-LAMP assay has a low cost per test.	_	Field Code Changed
90	does not required advanced technological facilities, and can be routinely practiced in general	$\overline{\ }$	Formatted: Font: (Default) Times New Roman, 12 pt
50	does not required advanced termological facilities, and can be fournerly practiced in general		Formatted: Font: 11 pt, Font color: Auto
91	hospital laboratories [6,10].		Field Code Changed
92			Formatted: Font: (Default) Times New Roman, 12 pt
93	As health care-financial resources are usually limited in countries with high TB prevalence,		
94	setting up an infrastructure to support the commercial TB-LAMP could still be unattainable.		
95	A more affordable in-house LAMP was developed in 2008 [11]. The main advantage of the		Field Code Changed
96	in-house assay was that it could be implicated on the readily-available infrastructure of any		Formatted: Font: (Default) Times New Roman, 12 pt
97	laboratory, even in the decentralized one. However, it did require extra-training and skill of		
98	technicians to process the clinical specimens. a simple and affordable molecular test would be		
99	suitable for achieving accurate TB diagnosis. In the past decades, several clinical studies and		
100	meta-analyses had evaluated the diagnostic accuracy of the in-house LAMP test for the		
101	diagnosis of pulmonary tuberculosis [12-14][7,11-14] (S1 Table). Overall, the LAMPFrom		Field Code Changed
102	the latest meta-analysis, the overall sensitivity and specificity of the in-house LAMP was		
103	93.0% (95%CI 88.9-95.7) and 91.8% (95%CI 86.4-95.1), respectively [14]. One recent study		Field Code Changed
104	in Thailand reported the sensitivity and the specificity of the in-house LAMP at 94.4%		Formatted: Font: (Default) Times New Roman, 12 pt
105	(95%CI 88.9-97.7) and 94.3% (95%CI 87.2-98.1), respectively [15]assay revealed high	<	Formatted: Font: (Default) Times New Roman, 12 pt
106	diagnostic performance especially in smear positive TB patients and had been suggested as		Field Code Changed
107	an alternative test for TB diagnosis, especially in resource-limiting areas where advanced		
108	molecular tests (e.g. PCR and Xpert MTB/RIF) are inaccessible [1,7]. However, the LAMP		Field Code Changed
109	procedures and types of assay used (in house or commercialized kit) varied across studies		Formatted: Font: 11 pt, Font color: Auto
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- 110 and yielded some discrepancies in results. Moreover<u>However</u>, the reported accuracy could be
- overestimated if being assessed in qualified laboratories with highly skilled technicians and
- sufficient resources where molecular tests usually are available [14][14]. Therefore, this study
- aimed to evaluate the pragmatic accuracy of the <u>in-house</u> LAMP assay for the diagnosis of
- 114 pulmonary tuberculosis in a peripheral community hospital of a developing country with <u>a</u>
- 115 high TB burden.

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# **Materials and Methods**

#### 118

### 119 **Ethics Statement**

120 This study was approved by the Research Ethics Committee of Maesot General Hospital, The

121 Ministry of Public Health (serial number 37/2015) and The Human Research Ethics

122 Committee of Thammasat University, Faculty of Medicine (COA number 081/2016). The

123 clinical samples used in this study were collected from all patients as routinely done.

124 Informed consent was obtained from all patients prior to inclusion.

## 125

## 126 Setting

- 127 The study was settled in Maesot General Hospital, a large-large-sized community hospital
- 128 with 365 in-patient beds. The hospital is located in Maesot district in Tak (province), which
- 129 shares the border with Myanmar and provides standard health care to both Thai and non-Thai
- 130 patients (Burmese immigrants and ethnic minorities). According to the Health Data Center,
- 131 the ministry of public health, Thailand, the incidence rate of pulmonary TB in Maesot was
- 132 <u>351 per 100,000 in 2019. The level of health care system of the hospital is considered rural.</u>
- 133 Maesot hospital has its own reference laboratory with biosafety cabinet infrastructure, BSC
- 134 class II. There are four lab technicians and one lab assistant within each working shift. Power
- 135 generator (350 kW) and UPS (2.7 kW) were available in case of power outages, which was
- 136 <u>infrequent.</u>
- 137

## 138 Study Design

- 139 This <u>prospective</u> diagnostic accuracy research with a population analog cross sectional
- 140 design was conducted from April to August 2016. New patients who were clinically

141	suspected of pulmonary TB (coughing for more than two weeks with or without hemoptysis),
142	aged more than 18 years old were consecutively invited into the study regardless of nation
143	status. Adult patients aged more than 15 years old with symptoms indicative of pulmonary TB
144	(coughing for more than two weeks with or without hemoptysis) and no history of TB were
145	consecutively enrolled regardless of nationality status. Samples with contaminated culture
146	results or samples from patients who were previously documented as TB cases were
147	excluded.Patients with previously documented TB history or patients with two contaminated
148	or missing cultures were excluded from the study.
149	

150	
151	Methods
152	All patients were given three sealed containers for the collection of morning sputum
153	specimens. Of all containers sent to the laboratory, only the one with seemingly adequate
154	sputum containing both mucoid or mucopurulent characters with a sample volume of more
155	than 3 ml, was used for the whole investigation procedures as routinely done. Specimens
156	were sent for smear microscopy with conventional AFB-acid-fast bacilli (AFB) staining with
157	Ziehl-Neelsen technique and fluorescence acid-fast staining with Auramine O solution.
158	Smear-positive case was defined according to WHO definitions as the presence of at least
159	two smears of scanty grade or one or more smears of 1+ or more. A smear negative case or
160	AFB smear-negative was conversely defined. For TB culture, the reference test, we
161	performed both conventional culture method on L-J (Lowenstein Jensen) medium and BBL
162	MGIT (mycobacterial growth indicator tube) culture method.
163	
164	Sputum decontamination and <u>culture</u> examination
165	For the sputum decontamination process, the collected samples and 2% N-Acetyl-L-cysteine
166	(NALC) NaOH were poured into a 50 ml sterile centrifuge tube in an equal proportion and
167	were subsequently mixed by vortexing for 30 seconds and left at room temperature (20-25 °C)
168	for 15 minutes. Then, the test tubes were filled with phosphate buffer saline (pH 6.8) until the
169	volume reached the level of 50 ml. The samples were put in a high-speed refrigerated
170	centrifuge at 3,000 g for 20 minutes. Next, the supernatants were poured off, leaving the tube
171	with decontaminated sputum samples. Finally, a drop (1 ml) of phosphate buffer saline (pH
172	6.8) was used for resuspension of the specimen.
173	

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17	74	For TB culture, the reference test, we performed both conventional culture method on L-J
17	75	(Lowenstein-Jensen) medium and BBL MGIT 960 (mycobacterial growth indicator tube)
17	76	culture method. The cCulture media were inoculated with processed sputum specimens and
17	77	incubated at 35 to 37 °C and monitored weekly for growth until 8 weeks. The sputum samples
17	78	were considered as "culture-positive" if growth was detected in either of L-J or MGIT
17	79	culture, regardless of the smear status. If growth was not detected in neither of the culture
18	30	methods and both microscopy results were negative, the samples were considered as "culture-
18	31	negative" or "non-TB patients". Patients with smear-positive and culture-negative, which
18	32	were generally considered as probable TB, were excluded from the analysis. Both smear
18	33	microscopy and culture methods were performed according to the standard protocols [16][15].
18	34	
18	25	In-house LAMP test

186 The LAMP test consists of three steps as follows: DNA extraction, isothermal amplification, 187 and visual interpretation with fluorescence. The National Institute of Health of Thailand had developed the TB Fast Amp technique (a modified LAMP procedure) to suite local practice 188 since 2009. The procedures were described as follow. Flexi Gene® DNA Kit (Qiagen co., 189 190 USA) and Protenase K Kit (Qiagen co., USA) were used for DNA extraction [17,18][16,17]. 191 Four primers (MTB primers, MAV primers, MIN primers, and Muniv primers) were used for the recognition of six distinct regions on the 16S ribosomal RNA gene of M. tuberculosis. 192 193 Each single LAMP reaction includes 12 µl of TB-Fast AMP mixture (FastAMP master mix includes 2  $\mu l$  10X buffer, 4  $\mu l$  2mM dNTPs, 3.2  $\mu l$  5M betaine, 1.2  $\mu l$  100 mM MgSO4, 1.6  $\mu l$ 194 195 primer mixture), 1 µl Bst DNA polymerase enzyme, 1 µl fluorescent detection reagent and 6 µl of extracted DNA samples. Amplification of reaction mixture was performed in the 196 heating blocks at 65 °C for 60 minutes, then examined directly by visual observation. The 197 LAMP assay was considered "positive" if the color of the reaction mixture changed from 198

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199	orange to green or fluorescence was directly observed with the naked eyes. The test was	
200	considered "negative" if the color of the mixture remained unchanged. For quality control,	
201	positive control (test tube with M. tuberculosis genetic materials) and negative control (test	
202	tube without M. tuberculosis genetic materials) were included in all runs.	
203		
204		
205	Study size estimation	
206	Pandey et al. reported the sensitivity and specificity of in-house LAMP assay for MTB	
207	detection at 97% and 94%, respectively [18]. Based on the hypothesis that the sensitivity of	Field
208	the LAMP test in this study would not differ from that previously reported by more than	
209	10%, the study size was estimated (using one-sample comparison of proportion to	
210	hypothesized value), yielding a total number of 60 culture positive TB cases. From a	
211	retrospective review of Maesot General Hospital data, the prevalence of culture positive TB	
212	cases was 50% of all patients who were TB suspects. A total of 120 patients were therefore	
213	planned to be included in our study.	
214		
215	Statistical Analysis	
216	Frequency and percentage were used for the description of categorical data. For continuous	
217	data, visualization of data distribution was done with histogram. For normally distributed	
218	data, mean and standard deviation was reported. For non-normally distributed data, median	
219	and interquartile range was reported. We used Fisher's exact probability test for comparison	
220	of differences in independent proportions and Student's t-test for two independent means.	
221	The sensitivity, specificity, positive predictive values (PPV), negative predictive values	
222	(NPV), and positive and negative likelihood ratios (LHR+) of all testing methods were	
223	calculated and reported with its 95% confidence interval. The 95% confidence interval were	
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- 224 estimated using the Clopper Pearson binomial exact method. The comparison of sensitivity,
- 225 specificity, and overall test accuracy between the LAMP test and smear microscopy methods
- 226 was performed with McNemar's exact probability test. Pairwise testing to compare the
- 227 <u>specificity between the LAMP test and the smear microscopy methods was not performed as</u>
- 228 the specificity of the latter was affected by incorporation bias and would not be comparable
- 229 to the in-house LAMP. The agreement of the LAMP test with smear microscopy methods
- 230 was analyzed with <u>Kappa's statistics and Kappa statistics</u>Spearman's rank correlation. The
- 231 subgroup analysis of LAMP test accuracy in smear-negative, culture positive TB patients was
- 232 pre-specified. P-values of less than 0.05 were considered statistically significant. <u>All</u>
- 233 <u>statistical analyses were done using Stata version 16 (StataCorp, Texas).</u>

# **Results**

236	A total of 120 <del>clinically suspected cases of TBpatients to be evaluated for TB</del> were
237	consecutively included from April to August 2016. Three patients with two_contaminated
238	cultures, -and two patients who subsequently were detected as previously documented TB
239	cases, and eight patients who had smear-positive and culture-positive results were excluded
240	from the analysis; only 115-107 samples patients remained in the study (Fig. 1). Most of the
241	included patients were male (60% vs. 40%) with a mean age of 47 years old. Fifty
242	(4346.75%) were culture-positive TB patients and $65-57$ ( $56.553.3%$ ) were culture-negative
243	patients. The baseline demographic data between culture-positive and <u>culture-negative</u>
244	patients were comparableFor clinical characteristics, except for the presence of cavitary
245	lesions on chest radiographs and the character of collected sputum was found to be
246	significantly different (Table 1). Culture-positive TB patients had higher proportion of
247	cavitary lesions (14.0% vs. $1.\frac{58}{20}$ %, p= $0.\frac{020024}{20024}$ ) and mucous sputum specimen (52.0% vs
248	24.6%, p=0.003005) than patients with negative TB culture.
249	
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# 250

#### 251 Table 1. Baseline clinical characteristics of the patients by culture status

Characteristics	TB Culture Positive	TB Culture Negative	P-Value	
	n=50 (43,5%)	<del>n=65 (56,5%)</del>	- •	Formatted: Line spacing: Double
Gender			•	Formatted: Line spacing: Double
Male	<del>30(60.0)</del>	<del>39(60.0)</del>	<del>1:000</del> •	Formatted: Line spacing: Double
Female	<del>20(40.0)</del>	<del>26(40.0)</del>	•	Formatted: Line spacing: Double
Nationality			•	Formatted: Line spacing: Double
Thai	<del>22(44.0)</del>	<del>38(58.5)</del>	<del>0,136</del> •	Formatted: Line spacing: Double
<del>Non-Thai</del>	<del>28(56.0)</del>	<del>27(41.5)</del>	•	Formatted: Line spacing: Double
Age (year, mean±SD)	4 <del>8.7±17.</del> 4	45.6±18.5	<del>0.362</del> ←	Formatted: Line spacing: Double
Chest radiographs			•	Formatted: Line spacing: Double
without cavitary lesions	<del>43(86.0)</del>	<del>64(98.5)</del>	0.020	Formatted: Line spacing: Double
with cavitary lesions	<del>#(14.0)</del>	+ <del>(1.5)</del>	•	Formatted: Line spacing: Double
Character of sputum			•	Formatted: Line spacing: Double
Salivary	24(48.0)	4 <del>9(75.4)</del>	<del>0.003</del> •	Formatted: Line spacing: Double
Mucous	<del>26(52.0)</del>	<del>16(24.6)</del>	•	Formatted: Line spacing: Double
Table 1. Demographic and	d clinical characteristics of	f the patients by TB cult	ure status	Formatted: Font: 12 pt
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				Formatted: Line spacing: Double
Characteristics	TB Culture Positive	TB Culture Negative	P-Value	Formatted: Font: 12 pt
	<u>(S+ or S-, C+)</u>	<u>(S-, C-)</u>		
	<u>n=50 ((46.7%))</u>	<u>n=57 ((53.3%))</u>	-	Formatted: Line spacing: Double
Gender				Formatted: Line spacing: Double
Male	<u>30 (60.0)</u>	<u>36 (63.2)</u>	0.842	Formatted: Line spacing: Double
Female	<u>20 (40.0)</u>	<u>21 (36.84)</u>	4	Formatted: Line spacing: Double
<u>Nationality</u>			4	Formatted: Line spacing: Double
<u>Thai</u>	<u>28 (56.0)</u>	<u>21 (36.8)</u>	0.054	Formatted: Line spacing: Double
Non-Thai	22 (44.0)	<u>36 (63.2)</u>	4	Formatted: Line spacing: Double
Age (year, mean±SD)	48.7±17.4	45.8±18.7	0.408	Formatted: Line spacing: Double

1	Chest radiographs			<b>4</b> -00	 Formatted: Line spacing: Double
	Wwithout cavitary lesions	43 (86.0)	<u>56 (98.2)</u>	<u>₀.₀₂</u> 0.02 <u>4</u> ←	Formatted: Line spacing: Double
	Wwith cavitary lesions	7 (14.0)	1 (1.8)	•	Formatted: Line spacing: Double
		<u> </u>			Formatted: Line spacing: Double
	Character of sputum			4-	Formatted: Line spacing: Double
	Salivary	24(48.0)	<u>43 (75.4)</u>	<u>0.005</u>	Formatted: Line spacing: Double
	Mucous	26 (52.0)	<u>14 (24.6)</u>	4	Formatted: Line spacing: Double
253	Abbreviations: TB, tuberculosis; C, c	culture (+ positive or - negative); S, si	mear microscopy (+ positive or	– negative); SD, 🔸	Formatted: Line spacing: Double
254	standard deviation.				
255	Abbreviations: TB, tubercul	losis; SD, standard deviation	<del>L.</del>		
256					

Fig. 1. Study flow diagram of patient enrollment and results of index and reference test

# 258 based on <del>conventional smear microscopy<u>culture</u> result</del>

259

261	The overall sensitivity of the LAMP test was 82.0% (95% CI 68.6-91.4), whereas the
262	sensitivity in smear-positive, culture-positive patients and smear-negative, culture-positive
263	was 90.9% (95% CI 78.3-97.5) and 16.7% (95% CI 0.4-64.1), respectively. The overall
264	sensitivity of both the AFB and the fluorescence stain was slightly higher than that of the
265	LAMP test; however, the differences were non-significant (Table 2). The sensitivity,
266	specificity, positive predictive value, and negative predictive value of LAMP test compared
267	to the reference TB culture was were 82.0% (68:6-91:4), 84:694.7% ((95%CI 73:5-92:485.4-
268	<u>98.9)</u> , <del>80.4</del> 93.2% ((95% CI 66.9-90.281.3-98.6)), and <del>85.985.7</del> % ((95% CI 75.0-93.474.6-93.3)),
269	respectively. The diagnostic accuracy of both the AFB and the fluorescence stain was slightly
270	higher than that of the LAMP test; however, the differences were non-significant (Table 2).
271	The positive and negative likelihood ratios of the LAMP test was 15.6 (95%CI 4.47-82.12)
272	and 0.19 (95%CI 0.08-0.44), respectivelyall tests were depicted in table 2. Even though the
273	accuracy measures for the diagnosis of tuberculosis cases were shown to vary across different
274	test methods (LAMP test, AFB stain, and fluorescence stain), the differences were without
275	statistical significance (Table 2).
276	LAMP test results showed substantial to almost perfect agreement were highly correlated with
277	both-those of AFB-(Kappa 0.82, 95%CI: 0.64-1.01, p<0.001) and fluorescence stain (Kappa 0.84,
278	95% <del>CI: 9.66-1.03, p&lt;0.001</del> Spearman's rho 0.85, p<0.001) in the diagnosis of culture-positive TB
279	cases (Table 3). The in-house LAMP also showed substantial to almost perfect agreement
280	with both microscopy methods in the diagnosis of culture-positive cases (Kappa 0.85, 95%CI
281	<u>0.74,0.95) (Table 3).</u>
282	

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# 283 Table 2. Diagnostic accuracy of LAMP test, AFB stain, Fluorescence stain, parallel and

### 284

serial testing of LAMP test and AFB stain.

		TB-Culture		Sensitivity	Specificity	PPV %	NPV %	LHR+		
•	Positive	Negative	Total	<del>%</del>	%-	<del>(95%CI)</del>	<del>(95%CI)</del>	<del>(95%CI)</del>		
	<del>(n=50)</del>	<del>(n=65)</del>	<del>(n=115)</del>	<del>(95%CI)</del>	<del>(95%CI)</del>					
LAMP Tes	sŧ								Fc	ormatted: Line spacing: Double
Positive	41	<del>10</del>	<del>51</del>	<del>82.0</del>	<del>84.6</del>	<del>80</del> .4	<del>85.9</del>	5.3	• Fo	ormatted: Line spacing: Double
Negative	9	<del>55</del>	<del>64</del>	( <del>68.6-91.4)</del>	( <del>73.5-92.4)</del>	( <del>66.9-90.2)</del>	(7 <del>5,0-93</del> ,4)	( <del>3.0-9.6)</del>	Fr	ormatted: Line spacing: Double
AFB stain	t.								+ Fr	ormatted: Line spacing: Double
Positive	44	7	<del>51</del>	<del>88.0</del>	<del>89,2</del>	<del>86.3</del>	<del>90.6</del>	<del>8.2</del>	• Fc	ormatted: Line spacing: Double
Negative	6	<del>58</del>	<del>64</del>	(75.7-95.5)	(79.1-95.6)	<del>(73.7-94.3)</del>	( <del>80.7-96.5)</del>	(4.0-16.6)	Fr	ormatted: Line spacing: Double
Fluorescen	<del>ice stain</del>								F	ormatted: Line spacing: Double
Positive	44	8	<del>52</del>	<del>88.0</del>	<del>87.7</del>	<del>84,6</del>	<del>90.5</del>	7.2	Fc	ormatted: Line spacing: Double
Negative	6	<del>57</del>	<del>63</del>	( <del>75.7-95.5)</del>	<del>(77.2.94.5)</del>	(71.9-93.1)	<del>(80.4-96.4)</del>	(3.7-13.8)	Fc	ormatted: Line spacing: Double
Parallel tes	sting (LAN	MP or AFB)							Fr	ormatted: Line spacing: Double
Positive	<del>45</del>	#	<del>56</del>	<del>90.0</del>	<del>83.1</del>	<del>80.4</del>	<del>91.5</del>	<del>5.3</del>	Fc	ormatted: Line spacing: Double
Negative	5	55	<del>59</del>	<del>(78.2-96.7)</del>	(71.7-91.2)	<del>(67.6-</del>	<del>(81.3-</del>	<del>(3.1-9.2)</del>	• E	ormatted: Line spacing: Double
1.0guilt	-			(, 0.2 , 0,	(,,,,,	<del>89.9)</del>	<del>97.2)</del>	(0.1 ,		Smatteu. Luie spacuig. Double
Serial testi	ing (LAMI	P and AFB)							F	ormatted: Line spacing: Double
Positive	<del>40</del>	<del>6</del>	<del>46</del>	<del>80.0</del>	<del>90.8</del>	<del>87.0</del>	<del>85.5</del>	<del>8.67</del>	F	ormatted: Line spacing: Double
						<del>(73.7-</del>	<del>(75.0-</del>	<del>(3.99-</del>	_	
Negative	<del>10</del>	<del>59</del>	<del>69</del>	<del>(66.3-90.0)</del>	<del>(81.0-96.5)</del>	<del>95.1)</del>	<del>92.8)</del>	<del>18.8)</del>	Fc	ormatted: Line spacing: Double
P-value				<del>0.59</del> 4	<del>0.702</del>	<del>0.840</del>	<del>0.738</del>		Fc	ormatted: Line spacing: Double
Table 2. I	Diagnost	t <mark>ic accur</mark> a	cy of the	e in-house L	AMP test,	AFB stain.	, and Fluo	rescence	- Fr	ormatted: Font: 12 pt
									Fc	ormatted: Line spacing: Double
stain.									Fc	ormatted: Font: 12 pt
									Fc	ormatted: Font: 12 pt
Method	Sensiti	vity% (95% CI)	), no. corrects	<u>Specificity%</u> s (95%CI),	<u>Accuracy%</u> (95%CI),	<u>PPV%</u>	<u>NPV</u>		<u>LK-</u>	ormatted: Font: 12 pt
						<u>(95%CI)</u>	<u>(95%CI)</u> (	(95%CI) (95	5% <u>CI)</u>	

		<u>S+, C+</u>	<u>S-, C+</u>	Any S, C+	<u>S-, C-</u>	<u>(n=107)</u>				-	_
		<u>(n=44)</u>	<u>(n=6)</u>	<u>(n=50)</u>	<u>(n=57)</u>						
		<u>90.9</u>	<u>16.7</u>	<u>82.0</u>	<u>94.7</u>	<u>88.8</u>	93.2	85.7	15.6	<u>0.2</u>	
LAMP		<u>(78.3,97.5),</u>	<u>(0.4,64.1),</u>	<u>(68.6,91.4),</u>	<u>(85.4,98.9),</u>	<u>(81.2,94.1).</u>	(81.3,98.6)	(74.6,93.3)	(4.5,82.1)	(0.1,0.4)	Formatted: Line spacing: Double
		<u>N=40</u>	<u>n=1</u>	<u>n=41</u>	<u>n=54</u>	<u>n=95</u>	(01.5,76.0)	(14.0,75.5)	(4.5,62.1)	(0.1,0.4)	<u>1</u>
				<u>88.0</u>	100.0	<u>94.4</u>	100.0	90.5			
AFB stain		=	=	<u>(75.7,95.5),</u>	<u>(93.7,100.0),</u>	<u>(88.2,97.9),</u>	(93.7,100.0)	<u>90.5</u> (80.4,96.4)	=	<u>+</u>	Formatted: Line spacing: Double
				<u>n=44</u>	<u>n=57</u>	<u>n=101</u>	(95.7,100.0)	(80.4,90.4)			
				<u>88.0</u>	<u>100.0</u>	<u>94.4</u>	100.0	<u>90.5</u>			
Fluorescenc	<u>ce stain</u>	=	=	<u>(75.7,95.5),</u>	<u>(93.7,100.0),</u>	<u>(88.2,97.9),</u>	(93.7,100.0)	(80.4,96.4)	=	- ±	Formatted: Line spacing: Double
				<u>n=44</u>	<u>n=57</u>	<u>n=101</u>	(93.7,100.0)	(80.4,90.4)			
LAMP test	VS.			P=0.375*	P=0.250*	P=1.000*				•	Formatted: Line spacing: Double
AFB stain				1-0.375	1-0.250	1-1.000					
LAMP test	VS.			P=0.375*	P=0.250*	P=1.000*				•	Formatted: Line spacing: Double
Fluorescent	ce stain			1-0.575	1-0.200						
87 <u>*P-va</u>	ulues from	n McNemar's	s Exact proba	ability test						•	Formatted: Line spacing: Double
88 <u>Abbre</u>	eviations	: AFB, acid fa	ast bacilli; C	, culture (+ po	ositive or – neg	gative); CI, co	onfidence inter	val; LAMP,	loop-mediat	ed	
.89 <u>isothe</u>	ermal am	plification; L	R+, positive	likelihood rat	tio; LR-, negat	ive likelihood	ratio; no. cor	rect, number	correctly		

290 identified; NPV, negative predictive value; PPV, positive predictive value; S, smear microscopy (+ positive or – negative).

able 3. Diagnostic a							11-	
FB stain-fluorescer	nce stain.							Formatted: Font: 12 pt
								Formatted: Line spacing: Double
		AFB Stain	&					Formatted: Font: 12 pt
LAMP Test		Fluorescence	stain					
	Positive	Negative	-	<u>Fotal</u>				
Positive	<u>40</u>	<u>4</u>		<u>44</u>			•	Formatted: Line spacing: Double
legative	<u>4</u>	<u>59</u>		<u>63</u>			•	Formatted: Line spacing: Double
otal	<u>44</u>	<u>63</u>		<u>107</u>			•	Formatted: Line spacing: Double
greement (%)		<u>92.5%</u>					•	Formatted Table
appa (95%CI, p-value)		0.85 (0.74,0.95, 1	n<0.001)					Formatted: Line spacing: Double
								Formatted: Line spacing: Double
spearman's rho (p-value)		<u>0.85 (p&lt;0.0</u>	<u>101)</u>					Formatted: Line spacing: Double
bbreviations: LAMP, loop-	mediated isotherm	al amplification;	CI, confidend	e interval.			•	Formatted: Line spacing: Double
ubbreviations: TB, tu alue; LHR+, positive sothermal amplificati	<del>: likelihood ra</del>	tio; CI, confie			<b>č</b>			
alue; LHR+, positive	e likelihood ra on; AFB, acic agreement be	tio; CI, confic l fast bacilli. <b>tween LAM</b> I	dence into	erval; LAMP	, loop-mediate	<del>d</del> stain.		
<del>ilue; LHR+, positive</del> othermal amplificati <mark>able 3. Diagnostic a</mark>	e likelihood ra on; AFB, acic agreement be	tio; CI, confic <del>l fast bacilli.</del>	dence into	erval; LAMP	, loop-mediate	<del>d</del> stain.	-	
i <del>lue; LHR+, positive</del> othermal amplificati able 3. Diagnostic a	e likelihood ra on; AFB, acic agreement be	tio; CI, confic l fast bacilli. <b>tween LAM</b> I	dence into	erval; LAMP	, loop-mediate	<del>d</del> stain.	-	
alue; LHR+, positive oothermal amplificati	e likelihood ra on; AFB, acic agreement be	tio; CI, confit I fast bacilli. I tween LAMI	<del>dence inte</del> P test and	<del>erval; LAMP</del> I AFB stain Flue	, loop-mediate fluorescence (	s <mark>tain.</mark>	-	Formatted: Line spacing: Double
alue; LHR+, positive othermal amplificati <b>able 3. Diagnostic a</b> _AMP Test	e likelihood ra on; AFB, acic agreement be z Positive	tio; CI, confit I fast bacilli. <b>tween LAMI</b> AFB Stain Negative	dence into P test and Total	erval; LAMP LAFB stain Flue Positive	fluorescence of the state of th	<del>stain.</del> Total	-	Formatted: Line spacing: Double
lue; LHR+, positive othermal amplificati able 3. Diagnostic a AMP Test Positive	e likelihood ra on; AFB, acid agreement be z Positive 46	tio; CI, confit I fast bacilli. I fast bacilli	dence into P test and Total 51	erval; LAMP	fluorescence ( prescence stain Negative 4	stain. Total	-	
ilue; LHR+, positive othermal amplificati able 3. Diagnostic a _AMP Test Positive	e likelihood ra on; AFB, acid agreement be Positive 46 5	tio; CI, confit I fast bacilli. I fast bacilli	dence into P test and Total 51 64	erval; LAMP	fluorescence ( prescence stain Negative 4 59	<del>stain.</del> <del>Total</del> 51 64	-	Formatted: Line spacing: Double
lue; LHR+, positive othermal amplificati able 3. Diagnostic a _AMP Test Positive Vegative	e likelihood ra on; AFB, acid agreement be Positive 46 5 51	tio; CI, confit I fast bacilli. I fast bacilli	dence into P test and Total 51 64	erval; LAMP	fluorescence ( prescence stain Negative 4 59 63	<del>stain.</del> <del>Total</del> 51 64		Formatted: Line spacing: Double

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301

Abbreviations: LAMP, loop-mediated isothermal amplification; CI, confidence interval.

302	
303	When parallel testing of LAMP and AFB stain was done, the sensitivity raised to 90.0%
304	(78.2-96.7) while the specificity dropped to 83.1% (71.7-91.2). Serial testing of LAMP and
305	AFB stain yielded higher specificity at 90.8% (81.0-96.5) with relatively lower sensitivity at
306	80.0% (66.3-90.0). Even though the accuracy measures for the diagnosis of tuberculosis cases
307	were shown to vary across different test methods (LAMP test, AFB-stain, fluorescence stain,
308	parallel testing and serial testing of both LAMP and AFB stain), the differences were without
309	statistical significance (Table 2).
310	

311	Of 50 culture-positive TB cases, six were smear-negative. The sensitivity, specificity,
312	positive predictive value, and negative predictive value of LAMP test in smear negative,
313	culture positive TB patients was 16.7% (0.4-64.1), 93.1% (83.3-98.1), 20.0% (0.5-71.6), and
314	91.5% (81.3-97.2), respectively. In smear positive, culture positive TB patients, the
315	sensitivity, specificity, positive predictive value, and negative predictive value of LAMP test
316	was 90.9% (78.3 97.5), 84.5% (74.0 92.0), 78.4% (64.7 88.7), and 93.8% (84.8 98.3),
317	<del>respectively.</del>
318	
319	Discussion
320	This study had demonstrated the pragmatic <u>diagnostic performance performance</u> of the <u>in-</u>
321	house LAMP testassay in a remote hospital of a high TB burden country. It was revealed that
322	the overall sensitivity of the in-house LAMP in our study was lower than the numbers
323	reported in the majority of the previous in-house LAMP studies. Nonetheless, the specificity

324 was comparable to other figures reported in the literature. In comparison to microscopy

325 methods, the AFB and fluorescence stain, the in-house LAMP, which was found to be

326 comparable inferior to that of the conventional smear microscopy and the fluorescence

327 microscopy in terms of overall sensitivity (82.0% vs. 88.0%, p=0.375) and accuracy (88.8%

328 vs. 94.4%, p=1.000-); however, Although the sensitivity and specificity of the LAMP test

329 were lower than that of the acid fast stain and the fluorescence stain, the comparative

330 statistical test revealed non-significant results. <u>Based on the result of our study, we suggest</u>

that the in-house LAMP should not be a substitute to conventional smear methods, but should

332 be done in parallel, which Using the LAMP test and the acid fast stain in parallel might

333 increase the sensitivity but lower the specificity in the diagnosis of tuberculosis patients. For

334 sereening purposes, parallel testing with high sensitivity would result in a higher sensitivity

335	with fewer false-negative TB cases. However, the relative reduction in specificity would		
336	increase the number of false positives where some patients might be subject to unnecessary		
337	treatment with serious side effects and risk of drug resistance. In the clinical context of TB		
338	diagnosis, both the LAMP test and the smear microscopy are considered as a diagnostic test		
339	which would normally be done in TB suspects with high pre-test probability [14]. Therefore,		Formatted: Font: 11 pt
340	a serial test relying on both the result from the LAMP test and the acid-fast stain would be		Field Code Changed
341	more appropriate for use as a rule in test as it carried higher specificity and positive		
342	likelihood ratio than other methods.		
343	۸		Formatted: English (United States)
344			
345	In this study, the sensitivity of the <u>in-house</u> LAMP test was 82.0% (95%CI 68.6-91.4) in		
346	culture-positive and 16.7% in smear positive and smear negative-TB patients, respectively.		
347	Unlike most of the previous studies which reported higher sensitivity of the LAMP test		
348	compared to conventional microscopic examination [7,14], the sensitivity of the LAMP test in	<	Field Code Changed
349	our study was just comparable to lower than the smear microscopy. In the past, several		Formatted: Font: 11 pt
350	studies had reported a higher sensitivity of the in-house LAMP test, which ranges from 90.0		
351	to 100.0% [11,15,19–24]. Most of these studies were either University hospitals, TB-		Field Code Changed
352	specialized centers or hospitals, or national TB-specialized laboratories, which were generally		
353	equipped with highly-trained personnel and adequate infrastructural supports. The overall		
354	sensitivity of our in-house LAMP was consistent with two previous studies from India and		
355	Zambia, which was 79.5% (95%CI 64.0-89.0) and 81.4% (95%CI 71.6-89.0), respectively		
356	[12,25]. Although both studies were performed in University hospitals, the LAMP procedures		Formatted: Font: (Default) Times New Roman, 12 pt
357	were modified to suit local conditions, and sputum processing and DNA extraction was done		Field Code Changed
358	with commercial kits. The higher sensitivity of the acid-fast stain and the fluorescence stain		
359	in our study could be explained by the high prevalence of TB, the absence of HIV patients or		
1			

360	less number of patients with paucibacillary sputum, and the availability of skilled technicians		
361	[12,26–28][11,18-20]. Besides, specimen decontamination with concentrated NaOH		Field Code Changed
362	decreases the amount of viable genetic materials for amplification, which could reduce the		
363	sensitivity of both the LAMP test and TB cultures. A lower concentration of NaOH (1-1.5%)		
364	or NaOH free methods during sample decontamination may be suggested [12.29][11,21], The		Formatted: Font: 11 pt
365	sensitivity of the LAMP test in smear-negative specimens could not be accurately estimated	$\square$	Field Code Changed
			Formatted: Font: (Default) Times New Roman
366	in this study as there were too few smear-negative, culture-positive patients.		Formatted: Font: (Default) Times New Roman
367			
368	The overall specificity of the LAMP test was 84.6% and 93.1% in smear-positive and smear-		
369	negative94.7% (95%CI 85.4-98.9) for nonTB patients, respectively. A positive LAMP		
370	result in a smear-positive patient is ,therefore, at high risk of false-positive, whereas a		
371	positive result in a smear-negative patient would significantly increase the probability of TB		
372	diagnosis [14]. This was discordant in concordance with a recent meta-analyses analysis,		Field Code Changed
373	which reported higher-pooled specificity ranging from 94.0-98.1% of the in-house LAMP at		Formatted: Font: 11 pt
374	91.8% (95%CI 86.4-95.1) for smear-positive patients and 97.7-98.6% for smear-negative		
375	patients [14][7,14]. However, it was concluded that the specificity of the in-house assays was		Field Code Changed
270	have the state of the Leasen commercial bit which mercane to a to 0.50% (0.50% CL 0.4.7		<b>Formatted:</b> Font: (Default) Times New Roman
376	lower than that of the Loopamp commercial kit, which was reported at 96.5% (95%CI 94.7-		Formatted: Font: 11 pt
377	97.7). This was due to the higher occurrence of false-positive cases in this study. A false		
378	positive LAMP result in smear-positive cases was frequently encountered in routine practice,		
379	which could usually be explained by multiple factors such as higher temperature, higher		
380	humidity, suboptimal reagents volume, and crossover contamination [14.30][14.22]. For in-		Field Code Changed
381	house LAMP, aAn extensive laboratory technician training and continuous quality	$\overline{\ }$	Formatted: Font: (Default) Times New Roman
501	<u>nouse Ervin , u</u> in extensive habitatory technician danning and <u>continuous</u> quarty		Formatted: Font: 11 pt
382	assessment should be conducted to lessen the risk of false-positive results. However, other		
383	potential factors might still account for the low specificity, such as temperature controls and		
384	volume of reaction used. For temperature, only available water bath was applied for		
1			

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Field Code Changed
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385	temperature controls during LAMP procedures instead of a more stable dry heating block. A		
386	recent study suggested a high reaction volume of 30-35 $\mu$ l due to the risk of self-priming in		
387	concentrated reagents [30][22]. The volume of reaction in our study was lower at 20 µl which		For
388	was based on the previous study of the in house LAMP by The National Institute of Health,		For Fiel
389	The Ministry of Public Health of Thailand [16].		Fiel
390			For
391 392	The diagnostic accuracy of the LAMP test in smear-negative specimens was consistent with previous literature. However, the sensitivity was much lower in our study, which could result		
393	from the low number of TB cases in smear-negative samples. This information supports the		
394	use of LAMP as a rule in test in smear-negative adult patients. In smear-positive samples, a		
395	serial test of both acid fast stain and LAMP test would likely result in a more accurate		
396	diagnosis of TB than each in isolation. The WHO had made a conditional recommendation		
397	based on a piece of very low-quality evidence that the LAMP test may be used as an		
398	alternative test for sputum direct microscopic examination to diagnose TB suspects [10].		Fiel
399	Based on the result of this study, we suggest that both the smear microscopic method and the		For
400	LAMP test should be tested in serial to maximize the diagnostic specificity. As the LAMP		
401	test had shown different diagnostic abilities on different smear status [23], the interpretation		Fiel
402	of the LAMP test in practice should also rely on the result of smear microscopy and thus		For
403	should not be done independently.		
404			
405	Currently, the WHO only supported the use of two rapid molecular tests for the diagnosis of		
406	pulmonary tuberculosis, which were Xpert MTB/RIF and the commercialized TB-LAMP test		
407	assay [9][10]. According to previous studies, both had shown comparable performance in	$\langle$	Fiel
408	smear-positive samples, but higher sensitivity was shown in Xpert MTB/RIF than in the		For
			Fiel
409	LAMP test [6,25][7,24]. Xpert MTB/RIF has been endorsed for use in the diagnosis of TB in	<	For
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410	many countries, including Thailand [4,31][4,5]. Nonetheless, Xpert MTB/RIF might not be
411	suitable in peripheral regions with poor infrastructure as the instrument requires a stable
412	electricity supply and an appropriate environment. The device also requires high continuous
413	maintenance costs leading to a relatively high cost per test compared to the LAMP test. In
414	contrast, the The LAMP test is readily available and can be done in any resource-poor settings
415	with regular infrastructure and technicians with adequate training. In Thailand, only a portion
416	of patients, not including foreigners and ethnic minorities, could reimburse the cost for Xpert
417	MTB/RIF due to the regulation stated by The National Health Security Office (NHSO). To
418	effectively prevent the spread of TB, all suspected patients to be evaluated for TB should
419	have equal access to high-quality diagnostic tools. Therefore, smear microscopy and the
420	LAMP test may be more applicable in terms of accessibility and affordability, especially in
421	the distant areas and the borderlands.
422	
423	However, there may be some limitations to this study. First, the study size was-might not be
424	powered enough to confirm the statistical insignificance of the between-test comparison.
425	Second, there were no new suspected TB cases with HIV infection during study
426	recruitmentsno patients with HIV infection were included during the study period, as HIV
427	status could be influential to the diagnostic performance of both the smear microscopy and
428	the LAMP test, especially in areas with a high prevalence of TB-HIV coinfection. Third, this
429	study had a higher proportion of salivary sputum than mucous sputum. This could affect the
430	diagnostic performance of both the index and the reference test [32]. The percentage of
431	culture-positive TB cases was lower in salivary samples than in mucous samples (35.8% vs.
432	65.0%, p=0.005). Both the quality and quantity of sputum specimens were associated with
433	the positivity of smear, molecular testing methods (Xpert MTB/RIF and PCR), and TB
434	culture [33,34]. Thus, it was possible that some patients with pulmonary TB might be

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435	classified as smear-negative, LAMP-negative, or even culture-negative cases. No previous		
436	study had officially addressed the effect of sputum quality on the LAMP test. Moreover, the		
437	character of sputum specimens was rarely reported. Interestingly, it was revealed from our		
438	data that the proportion of smear-positive, LAMP-positive results was also significantly		
439	lower in salivary sputum than in mucous sputum (31.3% vs. 57.5%, p=0.009 and 29.9% vs.		
440	60.0%, p=0.003, respectively). Therefore, the sensitivity and accuracy of all tests, including		
441	LAMP, might be underestimated. Previous studies reported that by improving the sputum		
442	guality, TB diagnostic yield increased [35,36]. Thus, high-quality sputum collection must be	$\langle$	Formatte
443	encouraged both in practice and studies.		Field Co
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445	Third, this study had a higher proportion of salivary sputum than mucous sputum. This could		
446	affect the diagnostic performance of both the index and the reference test $[25]$ . The	$\langle$	Formatte
447	percentage of culture-positive TB cases was lower in salivary samples than in mucous		Field Co
448	samples (32.9% vs. 61.9%, p=0.003). Thus, it was possible that some TB patients might be		
449	classified as culture negative or false negative cases. Finally, the use of routine TB culture as		
450	a reference standard might be inadequate, as some TB patients could be classified as not		
451	having TB [6][7]. With a higher quality reference standard, the sensitivity of TB-the in-house	$\langle$	Field Co
452	LAMP should be increased when a portion of 10-three remaining false-positive cases was re-		Formatte Formatte
453	classified as true-positive cases. Different culture media and techniques could be used in		Formatte
454	composite to achieve different performance characteristics [37]. In our study, two different	$\langle$	Field Co
455	culture techniques, L-J and MGIT, were used to increase the diagnostic rate of TB[38]. We		Formatte
456	also applied a strict diagnostic definition in calculating specificity by considering only		Formatte
457	patients with smear-negative and culture-negative results[39].	$\langle$	Field Co
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459 **Conclusions** 

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460	In conclusion, the LAMP test is a practical and affordable nucleic amplification technique for
461	the diagnosis of pulmonary tuberculosis, which should be implemented in resource-limiting
462	settings where Xpert MTB/RIF is unavailable. The diagnostic accuracy of the in-hose LAMP
463	was similar to previous studies for specificity. Better sputum processing and DNA extraction
464	method should be identified to improve the test sensitivityThe pragmatic diagnosticoverall
465	accuracy of the in-house LAMP test was comparable to that of conventional microscopy and
466	fluorescence microscopy with minimal inferiority in terms of sensitivity. To rule in TB
467	diagnosis <u>Therefore</u> , a serial-parallel examination of both smear microscopy and the in-house
468	LAMP test is suggested to minimize the risk of false-positive negative results, especially in
469	an endemic area.
470	

# 471 Acknowledgements

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680	Outperforms Mycobacterial Culture in Detecting Mycobacterium tuberculosis from
681	Salivary Sputum. BioMed Res Int. 2018;2018. doi:10.1155/2018/1514381
682	

683	Supporting information		/	Formatted: Font: 18 pt, Bold
684	S1 Table. Review on diagnostic accuracy of in-house LAMP assays for diagnosis of			
685	pulmonary tuberculosis (DOCX)			
686	S2 Table. LAMP minimal dataset (CSV)	•		Formatted: Font: Bold
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# **Responses to Reviewers' comments**

# Pragmatic accuracy of loop-mediated isothermal amplification (LAMP) for diagnosis of pulmonary tuberculosis in a Thai community hospital

#### **Reviewer #1:**

- The study aims to evaluate usefulness of a LAMP method in a practical setting in Thailand. The LAMP method is now available as an only commercial kit TB-LAMP assay (Loopamp<sup>™</sup>MTBC Detection Kit, Eiken Chemical Company Ltd., Japan) as endorsed by WHO in 2016. It seems that the method used in this study is a unique system at least partially. So, it is important to state explicitly that the target to be evaluated was an in-house LAMP and not one commercially available LAMP recommended by WHO.
  - The LAMP test in our study was a non-commercial, in-house LAMP.
  - We re-wrote the manuscript and emphasized that the test used was in-house LAMP.
- 2. In evaluating the sensitivity of the method, the authors used culture negative (clinically defined) cases, as well as bacteriologically confirmed cases, as a gold standard of the cases of TB. It may be difficult to admit the clinical diagnosis as a diagnostic basis for such a study as this, apart from clinical practice. Vice versa, the definition of the gold (conventional) standard for specificity (non-cases) should be reconsidered. The following paper may be of use in revising the paper; Kaku et al: Accuracy of LAMP-TB Method for Diagnosing Tuberculosis in Haiti. Jpn. J. Infect. Dis., 69, 488–492, 2016.
  - We modified the inclusion criteria for analysis as suggested by both reviewers.
  - As the analysis was done in a per-patient fashion, patients with smear-positive and culture-negative results would be excluded, as these patients were considered as probable TB cases. Therefore, the evaluation of sensitivity would include patients with both smear positive and smear negative with positive culture results. In contrast, the evaluation of specificity would include only patients with smear-negative and culture-negative results.

# **Reviewer #2:**

- 1. Abstract/Background: "proven diagnostic performance" this is both vague and too specific at the same time, "most of the results were validated" the results aren't validated, the assay is validated
  - We rewrote the abstract and introduction part as suggested.
- The language surrounding people with possible TB needs to be updated throughout the paper

   avoid the use of terms like "TB suspects" that increase the stigma surrounding this disease.
   <u>http://www.stoptb.org/assets/documents/resources/publications/acsm/LanguageGuide\_ForWeb20131110.pdf</u>
  - We rewrote the abstract and introduction part as suggested.
- 3. The paper states repeatedly that there is little work published from resource-challenged settings, but this claim is not supported. Even the references given cite studies in such decentralized settings. Maybe it just hasn't been done in Thailand? A better summary of the literature needs to be included. How does this compare to other studies? How is the TB LAMP test performed in this study compare to the TB LAMP tests in other published literature? A better focus on properly relating the current study to the body of work in the literature rather than trying to claim it is quite novel would actually strengthen the paper. There is merit in replication or demonstrating an important diagnostic in a new geographical area.
  - $\circ$  We rewrote the abstract and introduction part as suggested.
- 4. In-house vs commercialized kit is mentioned but not explained. And the position of this paper (what LAMP testing approach is used) is not properly placed in the context of what other papers are using and the potential impact on sensitivity/specificity.
  - We rewrote the abstract and introduction part as suggested.
- 5. The sensitivity/specificity of LAMP in other papers, settings, etc needs to be stated with numbers and not just alluded to. A proper, specific summary of the literature is lacking.
  - $\circ$   $\,$  We rewrote the abstract and introduction part as suggested.
- 6. "In 2016, WHO suggested the use of LAMP assay for the diagnosis of pulmonary tuberculosis" this is not quite right, WHO recommendations are very specific and it is important to get that right. From the abstract of the citation provided: "WHO recommends that TB-LAMP can be used as a replacement for microscopy for the diagnosis of pulmonary TB in adults with signs and symptoms of TB". This needs to be stated correctly. Also, given the paper has mentioned in-house vs commercialized kits, it needs to be clarified that the WHO guidance refers only to the Eiken LAMP kit.
  - We rewrote the abstract and introduction part as suggested.
- 7. "LAMP assay has a low cost per test, does not required advanced technological facilities, and can be routinely practiced in general hospital laboratories [3]." Reference 3 doesn't support this statement it doesn't say anywhere that the LAMP assay has a low cost per test. It says "Costs can be kept to a minimum if testing is limited to specimens from the most high-risk patients based on proper clinical assessments and national testing algorithms based on public health policies." There are other publications on the cost of the LAMP assay for TB diagnosis. The authors might explain better the infrastructure/training needed for LAMP based on this reference and others.
  - We rewrote the abstract and introduction part as suggested.
  - We changed the references to the statement as follow: Sohn H. Cost, affordability, and cost-effectiveness of TB-LAMP assay. In: Report to WHO Guideline Development Group Meeting on TB-LAMP Assay. Edn. Geneva: World Health Organization; 2016 and Shete PB, Farr K, Strnad L, Gray CM, Cattamanchi A. Diagnostic accuracy of TB-LAMP for pulmonary tuberculosis: a systematic review

and meta-analysis. BMC Infect Dis. 2019;19(1):268. Published 2019 Mar 19. doi:10.1186/s12879-019-3881-y

- 8. Reference 5 doesn't appear to really relate to the sentences it comes after. Reference 3 would make a lot more sense as it is a detailed overview of TB diagnostics including many molecular diagnostics.
  - We rewrote the abstract and introduction part as suggested.

#### Setting

- 1. The paper needs to do more to state what sets this setting apart from (or ties it to) other studies. See the methods section describing setting in reference 22 for how attributes of the specific site can be expressed in the context of the needs of LAMP.
  - We elaborated the character of our setting as suggested:
  - Level of health system: rural
  - Distance to reference laboratory: 0 km
  - Median LAMP test workload: 6 (4-10)
  - Electricity and backup power: infrequent power outages, power generator (350 Kw) and UPS (2.7 Kw)
  - Biosafety cabinet infrastructure: BSC class II
  - Laboratory staff: 4 lab technicians, 1 lab assistant
- 2. Study Design: This is not a cross-sectional design; it is a prospective design. The plan was to prospectively enroll 120 patients.
  - We changed the type of design to prospective diagnostic accuracy study as suggested.
  - We would like to make a constructive argument on this point, as the diagnostic accuracy research is actually cross-sectional study in design. The cross-sectional design is only the type of membership condition, single component of study base, and cross-sectional design can therefore be collected prospectively or retrospectively. We would like to ask you to kindly refer to this reference: Assessment of the accuracy of diagnostic tests: the cross-sectional study by Knottnerus JA, 2003. Link: https://www.ncbi.nlm.nih.gov/pubmed/14615003
- 3. "New patients who were clinically suspected of 109 pulmonary TB (coughing for more than two weeks with or without hemoptysis), aged more than 18 years old were consecutively invited into the study regardless of nation status." Suggest re-writing to something more like: 'Adults more than 18yrs of age with symptoms indicative of pulmonary TB (coughing...) and no history of TB were consecutively enrolled regardless of national status.' If patients were 'invited' but not enrolled, we need numbers on how many declined.
  - We re-wrote the sentence as suggested: Adult patients aged more than 15 years old with symptoms indicative of pulmonary TB (coughing for more than two weeks with or without hemoptysis) and no history of TB were consecutively enrolled regardless of national status.
- 4. "Samples with contaminated culture results or samples from patients who were previously documented as TB cases were excluded." Were the patients excluded or the samples?
  - o Patients with previously documented TB cases were excluded.
  - Patients with two contaminated or missing culture results were excluded.

## Methods

- 1. A map of which samples were used for what tests would be quite helpful. Highlight if any of the reference tests (smear, LJ culture, MGIT culture) were performed on the same sputum as LAMP.
  - Conventional macroscopy, LAMP test, and culture were conducted as routinely done.

- All patients were given three sealed containers for the collection of morning sputum specimens. Of all containers sent to the laboratory, only the one with seemingly adequate sputum, containing both mucoid or mucopurulent characters with a sample volume more than 3 ml, was used for the whole investigation procedures as routinely done. Specimens were sent for smear microscopy with conventional acid-fast bacilli (AFB) staining with Ziehl-Neelsen technique and fluorescence acid-fast staining with Auramine O solution.
- 2. Make it clear somewhere that smear-negative refers to AFB smear-negative.
  - $\circ$  We added detail on the smear-negative status as suggested.
  - According to WHO definitions, any patient with at least two AFB smears of scanty grade or one or more smears of 1+ or more was defined as smear-positive case. Smear-negative case was conversely defined.
- 3. Study size estimation

This has no purpose here – the study is done. Sample size estimation is for study planning purposes, for securing funding and making sure the plan has statistical validity.

- $\circ$   $\;$  The study size estimation part was removed as suggested.
- 4. Statistical analysis. The first four sentences are unnecessary.
  - The first four sentences were removed as suggested.
- 5. The authors need to state what method was used to obtain the 95% CI for the sens/spec/PPV/NPV/LR+. It is clear from my testing that the Clopper Pearson binomial exact test was used, the authors should include the reference (usually found in the software documentation).
  - $\circ~$  The 95% confidence intervals were calculated using the Clopper Pearson binomial exact method.
  - $\circ$  We added this statement in the statistical section and added the citation as suggested.
- 6. Kappa statistics are for inter-reader reliability, not for comparison of correlations between tests. It includes the concept that agreement may happen by chance when two people are guessing. However, it is not appropriate for comparison of diagnostic results because there isn't guessing the samples should not agree by chance but because they are or are not TB and the sensitivities of tests objectively vary. Spearman's correlation can be used, but I think what you actually want is McNemar's test. The desire is to compare the diagnostic performance (i.e. accuracy) between tests McNemar's test will do that. Alternatively, Spearman's correlation can look at the [objective] agreement between tests.
  - Spearman's rank correlation was inserted into the manuscript to represent the objective agreement between tests as suggested.
  - The agreement of LAMP test with smear microscopy methods was analyzed with Kappa's statistics and Spearman's rank correlation.
  - We still presented the value of Kappa's statistics as many of the previous studies on LAMP assay and other diagnostic tests had done [1–3].

# Results

- Table 1 is dedicated to showing the patient clinical characteristics by culture status. The p-values shown test whether these characteristics differ significantly dependent on culture status. It is expected that gender, nationality, and age should not differ. Whereas it is also expected that chest x-rays and sputum quality would differ. The baseline demographic data between culture188 positive and negative patients were comparable except for the presence of cavitary lesions on 189 chest radiographs and the character of collected sputum (Table 1). Age, nationality, and gender are demographic data. Chest x-ray and sputum quality are clinical characteristics.
  - We reanalyzed all the data after exclusion of patients with probable TB (LAMP test positive and AFB smear positive patients with negative culture).
  - All the baseline demographic and clinical characteristics data were reanalyzed and presented in Table 1.
  - The statements in the results section were re-written as suggested.
- 2. Table 2 re-check the NPV for parallel testing
  - We reanalyzed all the data after exclusion of patients with probable TB (LAMP test positive and AFB smear positive patients with negative culture).
  - All the data on Table 2 were checked for any error as suggested.
- 3. There are a lot of LAMP-positive and AFB smear-positive patients with negative culture. Especially given that the tests are done on different sputum samples, these should be considered patients with probable TB and not used in assessing sensitivity and specificity.
  - We reanalyzed all the data after exclusion of patients with probable TB (LAMP test positive and AFB smear positive patients with negative culture).
  - The final study size for analysis of LAMP test diagnostic accuracy was therefore 107 patients. (8 patients were excluded, 6 patients with both LAMP test and AFB smear-positive and culture negative, 1 patient with AFB positive and culture negative, and 1 patient with fluorescence stain positive and culture negative)
- 4. There are too few smear-negative, culture-positive patients to assess sensitivity. Specificity should not be stratified by smear status, only sensitivity. For the reason above (that smear-positive, culture-negative patients shouldn't be included in estimations of sensitivity/specificity of LAMP), what the paper is calling 'smear-negative specificity' should in fact be reported as the actual specificity of LAMP.
  - We exclude smear-positive, culture negative patients from the analysis as suggested.
  - We reported the actual specificity of LAMP test without stratification.
  - We acknowledged that our there are too few smear negative, culture positive patients to assess sensitivity in the discussion part.
- 5. Table 2 the p-values shown have no real meaning! If you want to compare accuracy of tests, you cannot do a p-value over the final accuracy measures among a bunch of tests. You need to compare tests 1 against another by using 2x2 grids and McNemar's test. So, if you want to compare the accuracy of LAMP to the accuracy of AFB stain, you use the grid in Table 3 and McNemar's test:
  - The comparison of diagnostic indices between LAMP test and AFB, fluorescence stain was re-analyzed using McNemar's exact probability test as suggested. We presented the result of the pairwise tests separately and reformatted Table 2.
  - Pairwise testing was not performed to compare the specificity between the LAMP test and the smear microscopy methods as the specificity of the latter was affected by incorporation bias and would not be comparable to the in-house LAMP.
  - Table 3 was also reformatted.
  - Spearman's rank correlation was used as suggested.

## Discussion

- "This study had demonstrated the pragmatic performance of the LAMP test, which was comparable to that of the conventional smear microscopy and the fluorescence microscopy." Not true, the performance of LAMP as evaluated in this study was below that of smear microscopy.
  - We rewrote the discussion part as suggested.
  - "This study had demonstrated the pragmatic diagnostic performance of the in-house LAMP assay in a remote hospital of a high TB burden country. It was revealed that the overall sensitivity of the in-house LAMP in our study was lower than the numbers reported in the majority of the previous in-house LAMP studies. Nonetheless, the specificity was comparable to other figures reported in literature. In comparison to microscopy methods, the AFB and fluorescence stain, the in-house LAMP was found to be inferior in terms of overall sensitivity (82.0% vs. 88.0%, p=0.375) and accuracy (88.8% vs. 94.4%, p=1.000); however, the comparative statistical test revealed non-significant results. Based on the result of our study, we suggest that the in-house LAMP should not be a substitute to conventional smear methods, but should be done in parallel, which would result in a higher sensitivity with fewer false-negative TB cases."
- 2. "Although the sensitivity and specificity of the LAMP test were lower than that of the acidfast stain and the fluorescence stain, the comparative statistical test revealed non-significant results" This is still true when McNemar's test is performed, but the right statistical tests need to be used in the paper. Furthermore, a non-significant result doesn't mean no difference, it means the difference is likely smaller than the power of the study to detect.
  - We rewrote the discussion part as suggested.
  - We reanalyzed our data using McNemar's exact probability test as suggested.
- 3. Put PPV/NPV in the context of the local prevalence of disease! State from the literature or reliable source what the prevalence of TB is in the hospital's area of Thailand. I would suggest giving the readers an example: Given that prevalence and a group of 1000 patients, state how many would be true positives, false positive, true negatives, and false negatives. You can therefore assess what burden the different accuracies will place on the hospital. I.e. if the specificity is quite low and the sensitivity is higher, is that better? If the sensitivity is high and the specificity is lower, is that better? Relate this to the LR+.
  - We would like to make a constructive argument to this question as follow: The prevalence of culture-positive TB in this study was 46.7%. As this was a "consecutive recruitment of patients with sign and symptoms suggestive of pulmonary TB" or "patients with higher pre-test probability that the general prevalence" or the "person that the in-house LAMP test was intended to be used", the calculation of positive predictive values could be directly calculated and reported from the study data as in the other study [1]. Moreover, both the in-house LAMP assay and acid-fast stain were not intended to be used as screening tests in the general population. For this reason, we did not include this part in our manuscript; however, we provide the answer to the question in this response paper.
  - The latest Maesot's population figures from the Health Data Center (HDC), the ministry of public health, Thailand, was 115,108 in 2019. The prevalence of pulmonary tuberculosis was 351 per 100,000 or 35 per 10,000.

	TB case	Non-TB case	Total	
LAMP positive	29	528	557	PPV 29/557=5.2%
LAMP negative	6	9,437	9,443	NPV 9437/9443=94.9%

Total 35	9,965 10,000	Prevalence=0.0035
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- 4. "In the clinical context of TB diagnosis, both the LAMP test and the smear microscopy are considered as a diagnostic test which would normally be done in TB suspects with high pretest probability [14]" this is not what the reference says.
  - The reference states "The TB LAMP assay is usually applied for TB-suspected patients and is rarely used for screening purpose. To rule-in the TB diagnosis, specificity is more important than sensitivity."
  - What we're trying to imply from this statement was that the LAMP test was developed to be applied for patients who were suspicious of having TB with "higher pre-test probability than average person". As the LAMP test was not for screening purpose, specificity is more important and should be more focused than sensitivity.
  - After we re-analyzed the data with the exclusion of probable TB cases, our specificity increased to comparable level with previous studies. The parallel and serial testing was omitted from our analysis as the test accuracy of combination of the in-house LAMP with other smear microscopy methods would be seriously affected by incorporation bias (smear-positive, culture-negative patients were all excluded.
- 5. "Therefore, a serial test relying on both the result from the LAMP test and the acid-fast stain would be more appropriate for use as a rule-in test as it carried higher specificity and positive likelihood ratio than other methods." Authors should define 'rule-in' test and what is generally expected of such a test. Should note the increased cost of such an approach.
  - After we re-analyzed the data with the exclusion of probable TB cases, our specificity increased to comparable level with previous studies. The parallel and serial testing was omitted from our analysis as the test accuracy of combination of the in-house LAMP with other smear microscopy methods would be seriously affected by incorporation bias (smear-positive, culture-negative patients were all excluded.
- 6. The effect of a gold standard which is not itself perfect should be discussed. Also the variability between sputum samples should be discussed.
  - The use of routine TB culture as a reference standard might be inadequate, as some TB patients could be classified as not having TB [6]. Different culture media and techniques could be used in composite to achieve different performance characteristics[4]. With a higher quality reference standard, the sensitivity of the inhouse LAMP should be increased when a portion of three remaining false-positive cases was re-classified as true-positive cases.
  - This study had a higher proportion of salivary sputum than mucous sputum. This could affect the diagnostic performance of both the index and the reference test[5]. The percentage of culture-positive TB cases was lower in salivary samples than in mucous samples (35.8% vs. 65.0%, p=0.005). Both the quality and quantity of sputum specimens were associated with positivity of smear, molecular testing methods (Xpert MTB/RIF and PCR), and TB culture [6,7]. Thus, it was possible that some patients with pulmonary TB might be classified as smear-negative, LAMP-negative, or even culture-negative cases. Interestingly, it was revealed from our data that the proportion of smear-positive, LAMP-positive results was also significantly lower in salivary sputum than in mucous sputum (31.3% vs 57.5%, p=0.009 and 29.9% vs. 60.0%, p=0.003, respectively). Therefore, the sensitivity and accuracy of all tests, including LAMP, might be underestimated. Previous studies reported that by improving the sputum quality, TB diagnostic yield increased[8,9]. Therefore, high-quality sputum collection must be encouraged both in practice and studies.
- 7. A better look at the differences between this study and others with better test performance needs to be done.
  - In this study, the sensitivity of the in-house LAMP test was 82.0% (95%CI 68.6-91.4) in culture-positive TB patients, respectively. In the past, several studies had reported

a higher sensitivity of the in-house LAMP test, which ranges from 90.0 to 100.0%. Most of these studies were either University hospital, TB-specialized centers or hospitals, or national TB-specialized laboratory, which were generally equipped with highly-trained personnel and adequate infrastructural supports. The overall sensitivity of our in-house LAMP was consistent with two previous studies from India and Zambia, which was 79.5% (95%CI 64.0-89.0) and 81.4% (95%CI 71.6-89.0), respectively. Although both studies were performed in University hospitals, the LAMP procedures were modified to suit local conditions, and sputum processing and DNA extraction was done with commercial kits. The higher sensitivity of the acid-fast stain and the fluorescence stain in our study could be explained by the high prevalence of TB, the absence of HIV patient or a smaller number of patients with paucibacillary sputum, and the availability of skilled technicians

- 8. "Currently, the WHO only supported the use of two rapid molecular tests for the diagnosis of 294 pulmonary tuberculosis, which were Xpert MTB/RIF and the LAMP test" as the concept of LAMP test from a kit and other LAMP tests has been raised, and the variability of accuracy depending, it needs to be clear that the WHO recommendation is only for the Eiken LAMP test kit!
  - We edited the statement as follow: "Currently, the WHO only supported the use of two rapid molecular tests for the diagnosis of pulmonary tuberculosis, which were Xpert MTB/RIF and the commercialized TB-LAMP assay".

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