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

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Full Title:	Pragmatic accuracy of an in-house loop-mediated isothermal amplification (LAMP) for diagnosis of pulmonary tuberculosis in a Thai community hospital
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Corresponding Author:	Phichayut Phinyo Chiang Mai University Chiang Mai, Chiang Mai THAILAND
Keywords:	Pulmonary Tuberculosis, In-House LAMP, Diagnosis, Sensitivity, Specificity
Abstract:	Background: To 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-effective nucleic amplification technique. We evaluated the pragmatic accuracy of an in-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 based on culture positivity was 88.8% (95/107) with a 95%CI of 81.2-94.1. The sensitivity was 90.9% (40/44) with a 95%CI of 78.3-97.5 for smear-positive, culture-positive patients, and was 16.7% (1/6) with a 95%CI of 0.4-64.1 for smear-negative, culture-positive patients. The overall sensitivity of the in-house LAMP test compared to smear microscopy methods were not significantly different (p=0.375). The specificity of the in-house LAMP based on non-TB patients (smear-negative, culture-negative) was 94.7% (54/57) with a 95%CI of 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:	Response to Reviewers We want to thank both the editor and the reviewers for granting the opportunity to revise our manuscript for publication in the PLOS one journal. We hope that our responses and revisions would substantially improve the quality of our manuscript and would be qualified for publication in the journal. If there were any further questions or minor points to be addressed or elaborated, please let us know. We would be more than eager to make any further revision. Editor's comments Specific comments:

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1.Abstract and Tables: Change the format 95%CI to be consistent with the rest of the manuscript, for example "95%CI 78.3,97.5" should be "95%CI 78.3-97.5".
•Changed as suggested.

2.Line 80, implicated has negative meaning, suggest changing to implemented.
•We modified the first two sentences as "As financial resources are usually limited in countries with high TB prevalence, a commercial TB-LAMP could still be unattainable. More affordable in-house LAMP assays were later developed and applied in several centers".

3.Line 162 change M. tuberculosis to Mycobacterium tuberculosis since this is the first time you mentioned the bacteria. Also, all the "M." need to be italicized in M. tuberculosis throughout the manuscript.
•Corrected as suggested.

4.Line 192 – 193, should the "smear-positive and culture-positive results" be "smear-positive and culture-negative results"?
•Corrected as suggested.

5.Line 243 – 246: These sentences need reference(s)
•We inserted some references to the two sentences as suggested.
Reviewer's comments
Thank you to the authors for the revisions made. This is a much better paper to present what is important work. However, I still have a few concerns. These focus on clarification of the 'in-house assay' and the discussion. Additionally, I think a review of the paper by a medical writer or any strong English editor would boost the communication of the results enormously.
1.The paper needs to be reviewed in detail for grammar and English. Other than general tidiness, in a number of places, the intent of what the authors are saying is lost due to odd grammar choices. For the best readability and better reach for the research contained, a review of the writing is recommended. I have made a few notes and suggestions in specific places.
a.We corrected all of your English suggestions.
b.We also modified and re-written some of the sentences in the manuscript to improve the readability.
2.The difference in assays still needs to be clearer. An 'in-house assay' is one that is not performed from a kit. You refer to 'the in-house' LAMP assay a lot as if there is only one, which is not the case. There are many papers out there with different 'in-house' LAMP assays. From the introduction, it sounds like you are presenting the findings from an in-house assay you developed following the protocol presented in Pandey et al. If so, this needs to be stated very clearly. However, from the methods section, it does not necessarily sound like you are not following that protocol and that this is a unique in-house assay. Please clarify in the paper.
a.We made the modification and improved the clarity of our in-house LAMP method as suggested.
3.When discussing previous results and meta-analyses, it needs to be clear that these refer to 'in-house LAMP assays' and not 'the in-house LAMP assay' as they are not uniform.
a.Corrected as suggested.
4.Inclusion of 'Accuracy' in Table 2 is a bit odd, but it can be kept if it is defined in the statistical methods section.
a.It was pre-specified in the methods section.
5.The discussion has a lengthy discourse on the costs of Xpert vs LAMP. But there is no referencing of the studies that have costed these two in order to make a proper comparison. It feels quite unsupported.
a.We removed unsupported statements from the paragraph and make the paragraph more concise.
6.In the discussion, the authors state 'No previous study had officially addressed the effect of sputum quality on the LAMP test'. I'm not sure this is true and would caution the authors not to make such a sweeping statement.
a.We removed the sentence out of the discussion section as suggested.
7.In the discussion, "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,

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	respectively). Therefore, the sensitivity and accuracy of all tests, including LAMP, might be underestimated." 1) Do not present new results in the discussion – these need to be included in the Results section first. 3) are these sensivity? Specificity? Accuracy? 3) This is not an interpretation that makes sense. The sensitivity/specificity is reported based on the best sputum sample available from the patients – quality samples are difficult to obtain. You can instead interpret it as 'Sensitivity and specificity would be improved if higher quality sputum is obtained'. a.We modified the content as suggested. b.We moved the findings to the results section. 8.In general, the discussion needs to be revised to make only statements supported by the literature, the study, or a comparison of the two. Much of the discussion feels like the authors musings. a.We modified the whole discussion sections to be as objective as possible. 9.In the discussion, I would suggest focusing on sensitivity and specificity and not accuracy as accuracy is not a common way of discussing or assessing diagnostic tests due to its difficulty of interpretation. a.Corrected as suggested. 10.The references as displayed in the reference section aren't quite right. In reference #1, instead of Lancet, the Journal is listed as Lancet Lond Engl which is not correct. This inclusion of a city occurs in reference #7 as well. a.Corrected as suggested.
Additional Information:	
Question	**Response**
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Competing Interests Use the instructions below to enter a competing interest statement for this submission. On behalf of all authors, disclose any competing interests that could be perceived to bias this work—acknowledging all financial support and any other relevant financial or non-financial competing interests. This statement **will appear in the published article** if the submission is accepted. Please make sure it is accurate. View published research articles from *PLOS ONE* for specific examples.	The authors have declared that no competing interests exist.

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NO authors have competing interests Enter: *The authors have declared that no competing interests exist.* **Authors with competing interests** Enter competing interest details beginning with this statement: *I have read the journal's policy and the authors of this manuscript have the following competing interests: [insert competing interests here]* * typeset	
Ethics Statement Enter an ethics statement for this submission. This statement is required if the study involved: • Human participants • Human specimens or tissue • Vertebrate animals or cephalopods • Vertebrate embryos or tissues • Field research Write "N/A" if the submission does not require an ethics statement. General guidance is provided below. Consult the submission guidelines for detailed instructions. **Make sure that all information entered here is included in the Methods section of the manuscript.**	This study was approved by the Research Ethics Committee of Maesot General Hospital, The Ministry of Public Health (serial number 37/2015) and The Human Research Ethics Committee of Thammasat University, Faculty of Medicine (COA number 081/2016). The clinical samples used in this study were collected from all patients as routinely done. Informed consent was obtained from all patients prior to inclusion.

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Format for specific study types **Human Subject Research (involving human participants and/or tissue)** • Give the name of the institutional review board or ethics committee that approved the study • Include the approval number and/or a statement indicating approval of this research • Indicate the form of consent obtained (written/oral) or the reason that consent was not obtained (e.g. the data were analyzed anonymously) **Animal Research (involving vertebrate animals, embryos or tissues)** • Provide the name of the Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board that reviewed the study protocol, and indicate whether they approved this research or granted a formal waiver of ethical approval • Include an approval number if one was obtained • If the study involved *non-human primates*, add *additional details* about animal welfare and steps taken to ameliorate suffering • If anesthesia, euthanasia, or any kind of animal sacrifice is part of the study, include briefly which substances and/or methods were applied **Field Research** Include the following details if this study involves the collection of plant, animal, or other materials from a natural setting: • Field permit number • Name of the institution or relevant body that granted permission	
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1 # Pragmatic accuracy of an 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 an in-house LAMP for pulmonary TB

6

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21

Abstract

Background: To 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-effective nucleic amplification technique. We evaluated the pragmatic accuracy of an in-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 based on culture positivity was 88.8% (95/107) with a 95%CI of 81.2-94.1. The sensitivity was 90.9% (40/44) with a 95%CI of 78.3-97.5 for smear-positive, culture-positive patients, and was 16.7% (1/6) with a 95%CI of 0.4-64.1 for smear-negative, culture-positive patients. The overall sensitivity of the in-house LAMP test compared to smear microscopy methods were not significantly different ($p=0.375$). The specificity of the in-house LAMP based on non-TB patients (smear-negative, culture-negative) was 94.7% (54/57) with a 95%CI of 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.

Keywords: Pulmonary Tuberculosis, In-House LAMP, Diagnosis, Sensitivity, Specificity

47 # Introduction

48 Tuberculosis (TB), an airborne communicable disease, has long been considered a significant
49 threat to global public health. According to The World Health Organization (WHO), 10
50 million people were newly infected with TB in 2018 [1]. The incidence and prevalence of TB
51 vary greatly across the globe, 87% of total cases resided within 30 countries with a high TB
52 burden. In Thailand, the incidence rate was 153 cases per 100,000 population in 2018. Early
53 diagnosis and timely treatment is an essential component of The End TB Strategy endorsed
54 by the WHO, aiming to end the global TB epidemic by the year 2035 [2]. However, TB is
55 still underdiagnosed and undertreated, especially in resource-limited countries, due to the
56 lack of highly sensitive and specific diagnostic tools which are usually expensive and require
57 adequate infrastructure [1,3]. Novel diagnostic methods with enough simplicity and cost-
58 effectiveness are therefore necessary to improve the accurate identification of TB patients in
59 those resource-limited settings [3,4].
60
61 Molecular testing methods such as polymerase chain reaction (PCR) or Xpert MTB/RIF have
62 been widely acknowledged as alternative tools to TB culture for the diagnosis of TB patients
63 [3,5]. These nucleic amplification techniques were known for yielding rapid and accurate TB
64 diagnosis. This would overcome the limitations of classical methods, insensitivity for smear
65 microscopy, and lengthy incubation period for TB culture. However, several obstacles remain
66 for the application of these molecular tests as point-of-care testing in community settings.
67 This is because of their complexity to execute and substantial requirements for financial and
68 personnel resources [3,6]. Loop-mediated isothermal amplification (LAMP) assay is another
69 recently developed nucleic acid amplification technique. Unlike PCR, where the
70 amplification of DNA fragment occurs in temperature-dependent steps, the reaction of LAMP

71 assay functions in isothermal or constant temperature conditions [7,8]. In 2016, WHO
72 endorsed the use of commercial TB-LAMP assay (Eiken Chemical Co., Tokyo, Japan) as a
73 replacement for smear microscopy for the diagnosis of TB [9]. TB-LAMP assay has a low
74 cost per test, does not required advanced technological facilities, and can be routinely
75 practiced in general hospital laboratories [6,10].
76
77 As financial resources are usually limited in countries with high TB prevalence, a commercial
78 TB-LAMP could still be unattainable. More affordable in-house LAMP assays were later
79 developed and applied in several centers [11–15]. However, it did require extra-training and
80 skill of technicians to process the clinical specimens. In the past decades, several clinical
81 studies and meta-analyses had evaluated the diagnostic accuracy of in-house LAMP tests for
82 the diagnosis of pulmonary TB [14,16,17] (S1 Table). From the latest meta-analysis, the
83 overall sensitivity and specificity of in-house LAMP was 93.0% (95%CI 88.9-95.7) and
84 91.8% (95%CI 86.4-95.1), respectively [17]. One recent study in Thailand reported the
85 sensitivity and the specificity of the in-house LAMP at 94.4% (95%CI 88.9-97.7) and 94.3%
86 (95%CI 87.2-98.1), respectively [15]. However, the reported accuracy could be
87 overestimated if it is assessed in qualified laboratories with highly skilled technicians and
88 sufficient resources where molecular tests are usually available [17]. Therefore, this study
89 aimed to evaluate the pragmatic accuracy of the in-house LAMP assay for the diagnosis of
90 pulmonary TB in a community hospital of a developing country with a high TB burden.
91

Materials and Methods

Ethics Statement

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

Setting

The study was performed in Maesot General Hospital, a large-sized community hospital with 365 in-patient beds. It is located in Maesot District, Tak Province, which shares the border with Myanmar. The hospital provides standard health care to both Thai and non-Thai patients (Burmese immigrants and ethnic minorities). According to the Health Data Center, the Ministry of Public Health, in Thailand, the incidence rate of pulmonary TB in Maesot was 351 per 100,000 in 2019. The health care system of the hospital is considered rural. Maesot hospital has its reference laboratory with biosafety cabinet infrastructure, BSC class II. There are four lab technicians and one lab assistant within each working shift. Power generator (350 kW) and UPS (2.7 kW) were available in case of power outages, which was infrequent. The median LAMP test workload per day was 6 (range 4-10).

Study Design

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

115 TB history or patients with two contaminated cultures or missing cultures were excluded

116 from the study.

117 Methods

118 All patients were given three sealed containers for the collection of morning sputum

119 specimens. Only one sputum specimen with adequate sputum containing both mucoid or

120 mucopurulent characters and a sample volume of more than 3 ml was selected in all

121 investigation procedures. Specimens were sent for smear microscopy with conventional acid-

122 fast bacilli (AFB) staining with Ziehl-Neelsen technique and fluorescence acid-fast staining

123 with Auramine O solution. The smear-positive case was defined according to WHO

124 definitions as the presence of at least two smears of scanty grade, or one or more smears of

125 1+ or more. A smear negative case was conversely defined.

126 Sputum decontamination and culture examination

127 For the sputum decontamination process, the collected samples and 2% N-Acetyl-L-cysteine

128 (NALC) NaOH were poured into a 50 ml sterile centrifuge tube in an equal proportion. The

129 specimens were subsequently mixed by vortexing for 30 seconds and left at room

130 temperature (20-25℃) for 15 minutes. Then, the test tubes were filled with phosphate buffer

131 saline (pH 6.8) until the volume reached the level of 50 ml. The samples were put in a high-

132 speed refrigerated centrifuge at 3,000 g for 20 minutes. Next, the supernatants were poured

133 off, leaving the tube with decontaminated sputum samples. Finally, a drop (1 ml) of

134 phosphate buffer saline (pH 6.8) was used for resuspension of the specimens.

135 For TB culture, the reference test, we performed both conventional culture method on L-J

136 (Lowenstein-Jensen) medium and BBL MGIT 960 (mycobacterial growth indicator tube)

137 culture method. The culture media were inoculated with processed sputum specimens and

138 incubated at 35 to 37℃ and monitored weekly for growth until 8 weeks. The sputum samples

were considered as "culture-positive" if growth was detected in either of L-J or MGIT 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-negative" or "non-TB patients". Patients with smear-positive and culture-negative, which were generally considered as probable TB, were excluded from the analysis. Both smear microscopy and culture methods were performed according to the standard protocols [18].

In-house LAMP test

The LAMP test consists of three steps as follows: DNA extraction, isothermal amplification, and visual interpretation with fluorescence. In this study, we followed the TB Fast AMP technique, which was developed by the National Institute of Health of Thailand and was described in our previous studies [13,15,19]. The procedures were described as follow. Flexi Gene® DNA Kit (Qiagen co., USA) and Protenase K Kit (Qiagen co., USA) were used for DNA extraction. Six primers were used for the recognition of eight distinct regions on the 16S ribosomal RNA gene of M. *tuberculosis*. Each single LAMP reaction includes 12 µl of TB-Fast AMP mixture (FastAMP master mix includes 2 µl 10Xbuffer, 4 µl 2mM dNTPs, 3.2 µl 5M betaine, 1.2 µl 100 mM $MgSO_4$, 1.6 µl primer mixture), 1 µl *Bst* DNA polymerase enzyme (New England Biolabs, Ipswich MA, USA), 1 µl fluorescent detection reagent (FDR; Eiken Chemical Tokyo, Japan) and 6 µl of extracted DNA samples. Amplification of reaction mixture was performed in the heating blocks at 65℃ 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 *Mycobacterium tuberculosis* genetic materials) and negative control (test tube without *M. tuberculosis* genetic materials) were included in all runs.

164 ## Statistical Analysis

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

Results

A total of 120 patients to be evaluated for TB were consecutively included from April to August 2016. Three patients with two contaminated cultures, two patients who subsequently were detected as previously documented TB cases, and eight patients who had smear-positive and culture-negative 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. Fifty (46.7%) were culture-positive TB patients and 57 (53.3%) were culture-negative patients. The baseline demographic data between culture-positive and culture-negative patients were comparable (Table 1). For clinical characteristics, the presence of cavitary lesions on chest radiographs and the character of collected sputum was statistically different. Culture-positive TB patients had higher proportion of cavitary lesions (14.0% vs. 1.8%, p=0.024) and mucous sputum specimen (52.0% vs 24.6%, p=0.005) than those with negative TB culture. The proportion of patients with salivary sputum was significantly lower than mucous sputum in both smear-positive and LAMP-positive results (31.3% vs. 57.5%, p=0.009 and 29.9% vs. 60.0%, p=0.003, respectively).

196

197 **Table 1. Demographic and clinical characteristics of the patients by TB culture status**

Characteristics	TB Culture Positive (S+ or S-, C+)	TB Culture Negative (S-, C-)	P-Value
	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)	

198 Abbreviations: TB, tuberculosis; C, culture (+ positive or – negative); S, smear microscopy (+ positive or – negative); SD,
199 standard deviation.

200

201 **Fig. 1. Study flow diagram of patient enrollment and results of index and reference test**
202 **based on culture result**

203

204

205 The overall sensitivity of the LAMP test was 82.0% (95%CI 68.6-91.4), whereas the
206 sensitivity in smear-positive, culture-positive patients and smear-negative, culture-positive
207 was 90.9% (95%CI 78.3-97.5) and 16.7% (95%CI 0.4-64.1), respectively. The overall
208 sensitivity of both the AFB and the fluorescence stain was slightly higher than that of the
209 LAMP test; however, the differences were non-significant (Table 2). The specificity, positive
210 predictive value, and negative predictive value of the LAMP test was 94.7% (95%CI 85.4-
211 98.9), 93.2% (95%CI 81.3-98.6), and 85.7% (95%CI 74.6-93.3), respectively. The positive
212 and negative likelihood ratios of the LAMP test was 15.6 (95%CI 4.47-82.12) and 0.19
213 (95%CI 0.08-0.44), respectively. The accuracy measures for the diagnosis of TB cases were
214 shown to vary across different test methods (LAMP test, AFB stain, and fluorescence stain),
215 the differences were without statistical significance (Table 2).
216 LAMP test results were highly correlated with those of AFB and fluorescence stain
217 (Spearman's rho 0.85, 95%CI 0.74-0.95, $p<0.001$) in the diagnosis of culture-positive TB
218 cases (Table 3). The in-house LAMP also showed substantial to an almost perfect agreement
219 with both microscopy methods in the diagnosis of culture-positive cases (Kappa 0.85, 95%CI
220 0.74-0.95, $p<0.001$) (Table 3).
221

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

223 **stain.**

Method	Sensitivity% (95% CI), no. corrects			Specificity% (95%CI), no. corrects	Accuracy% (95%CI), no. corrects (n=107)	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)					
LAMP	90.9 (78.3,97.5), N=40	16.7 (0.4,64.1), n=1	82.0 (68.6,91.4), n=41	94.7 (85.4,98.9), n=54	88.8 (81.2,94.1), n=95	93.2 (81.3,98.6)	85.7 (74.6,93.3)	15.6 (4.5,82.1)	0.2 (0.1,0.4)
AFB stain	-	-	88.0 (75.7,95.5), n=44	100.0 (93.7,100.0), n=57	94.4 (88.2,97.9), n=101	100.0 (93.7,100.0)	90.5 (80.4,96.4)	-	-
Fluorescence stain	-	-	88.0 (75.7,95.5), n=44	100.0 (93.7,100.0), n=57	94.4 (88.2,97.9), n=101	100.0 (93.7,100.0)	90.5 (80.4,96.4)	-	-
LAMP test vs. AFB stain			P=0.375*	P=0.250*	P=1.000*				
LAMP test vs. Fluorescence stain			P=0.375*	P=0.250*	P=1.000*				

224 *P-values from McNemar's Exact probability test

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

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

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

228

229 **Table 3. Inter-rater reliability and diagnostic agreement between an in-house LAMP**

230 **test and AFB stain-fluorescence stain.**

LAMP Test	AFB Stain & Fluorescence stain		
	Positive	Negative	Total
Positive	40	4	44
Negative	4	59	63
Total	44	63	107
Agreement (%)	92.5%		
Kappa (95%CI, p-value)	0.85 (0.74-0.95, p<0.001)		
Spearman's rho (p-value)	0.85 (0.74-0.95, p<0.001)		

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

232

233

234

Discussion

This study has demonstrated the pragmatic diagnostic performance of our in-house LAMP assay in a remote hospital of a high TB burden country. The overall sensitivity was lower than the majority of the previous in-house LAMP studies [11,15,20–23]. Nonetheless, the specificity was comparable to other figures reported in the literature [11,12,15,21,22]. In comparison to microscopy methods (AFB and fluorescence stain), the in-house LAMP was inferior in terms of overall sensitivity. 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.

In the past, several studies reported a higher sensitivity of in-house LAMP tests, ranging from 90.0 to 100.0% [11,15,20–25]. Most of these studies were reported from either university hospitals, TB-specialized centers or hospitals, or national TB-specialized laboratories, which were generally equipped with highly trained personnel and adequate infrastructural supports [17]. 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 [12,16]. Although both studies were performed in university hospitals, the LAMP procedures were modified to suit local conditions, and sputum processing and DNA extraction were 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 patients or fewer patients with paucibacillary sputum, and the availability of skilled technicians [16,26–28]. 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

260 decontamination may be suggested [16,29]. The sensitivity of the LAMP test in smear-
261 negative specimens could not be accurately estimated in this study as there were too few
262 smear-negative, culture-positive patients.

263 The overall specificity of the LAMP test was 94.7% (95%CI 85.4-98.9) for non-TB
264 patients. This was in concordance with a recent meta-analysis, which reported pooled
265 specificity of in-house LAMP tests of 91.8% (95%CI 86.4-95.1) [17]. However, the
266 specificity of the in-house assays was lower than that of the Loopamp commercial kit, which
267 was reported at 96.5% (95%CI 94.7-97.7). A false positive LAMP result in smear-positive
268 cases was frequently encountered in routine practice, which could be explained by multiple
269 factors such as higher temperature, higher humidity, suboptimal reagents volume, and
270 crossover contamination [17,30]. For temperature, only available water bath was applied for
271 temperature controls during LAMP procedures instead of a more stable dry heating block. A
272 recent study suggested a high reaction volume of 30-35 µl due to the risk of self-priming in
273 concentrated reagents [30].

274 Currently, the WHO only endorses the use of two rapid molecular tests for the
275 diagnosis of pulmonary TB, which were Xpert MTB/RIF and the commercialized TB-LAMP
276 assay [9]. According to previous studies, both had shown comparable performance in smear-
277 positive samples, but higher sensitivity was shown in Xpert MTB/RIF than in the LAMP test
278 [6,12]. Xpert MTB/RIF has been endorsed for use in the diagnosis of TB in many countries,
279 including Thailand [4,31]. However, only a portion of patients, excluding foreigners and
280 ethnic minorities, could reimburse the cost for Xpert MTB/RIF due to the regulation stated by
281 The National Health Security Office (NHSO). To better control the spread of TB, access to
282 rapid diagnostic tools should be provided to all patients with symptoms suggestive of TB [3].
283 Thus, a LAMP assay may be more applicable in terms of accessibility and affordability,
284 especially in the decentralized areas [4,32].

However, there were some limitations to this study. First, the study size may not be substantial enough to provide the power required to detect a statistically significant difference between tests. Second, no patients with HIV infection were included during the study period, as HIV status could be influential to the diagnostic performance of both the smear microscopy and the LAMP test, especially in areas with a high prevalence of TB-HIV coinfection. Third, there was a higher proportion of salivary sputum than mucous sputum in this study. This could affect the diagnostic performance of both the index and the reference test [33]. Both the quality and quantity of sputum specimens were associated with the positivity of smear, molecular testing methods (Xpert MTB/RIF and PCR), and TB culture [34,35]. Thus, some patients with pulmonary TB might be classified as smear-negative, LAMP-negative, or even culture-negative cases. Sensitivity and specificity would be improved if higher quality sputum is obtained [36,37].

Finally, 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]. With a higher quality reference standard, the sensitivity of the in-house LAMP should be increased when a portion of three 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 [38]. In our study, two different culture techniques, L-J and MGIT, were used to increase the diagnostic rate of TB [39]. We also applied a strict diagnostic definition in calculating specificity by considering only patients with smear-negative and culture-negative results [40].

Conclusions

In conclusion, a LAMP test is a practical and affordable nucleic amplification technique for the diagnosis of pulmonary TB, which should be implemented in resource-limited settings where Xpert MTB/RIF is unavailable. The diagnostic accuracy of the in-hose LAMP was

310 similar to previous studies for specificity. To improve the test sensitivity, a better sputum
311 processing and DNA extraction method is essential. The in-house LAMP test had lower
312 sensitivity than smear microscopy. Therefore, a parallel examination of both smear
313 microscopy and the in-house LAMP test is suggested to minimize the risk of false-negative
314 results, especially in an endemic area.
315

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455

456

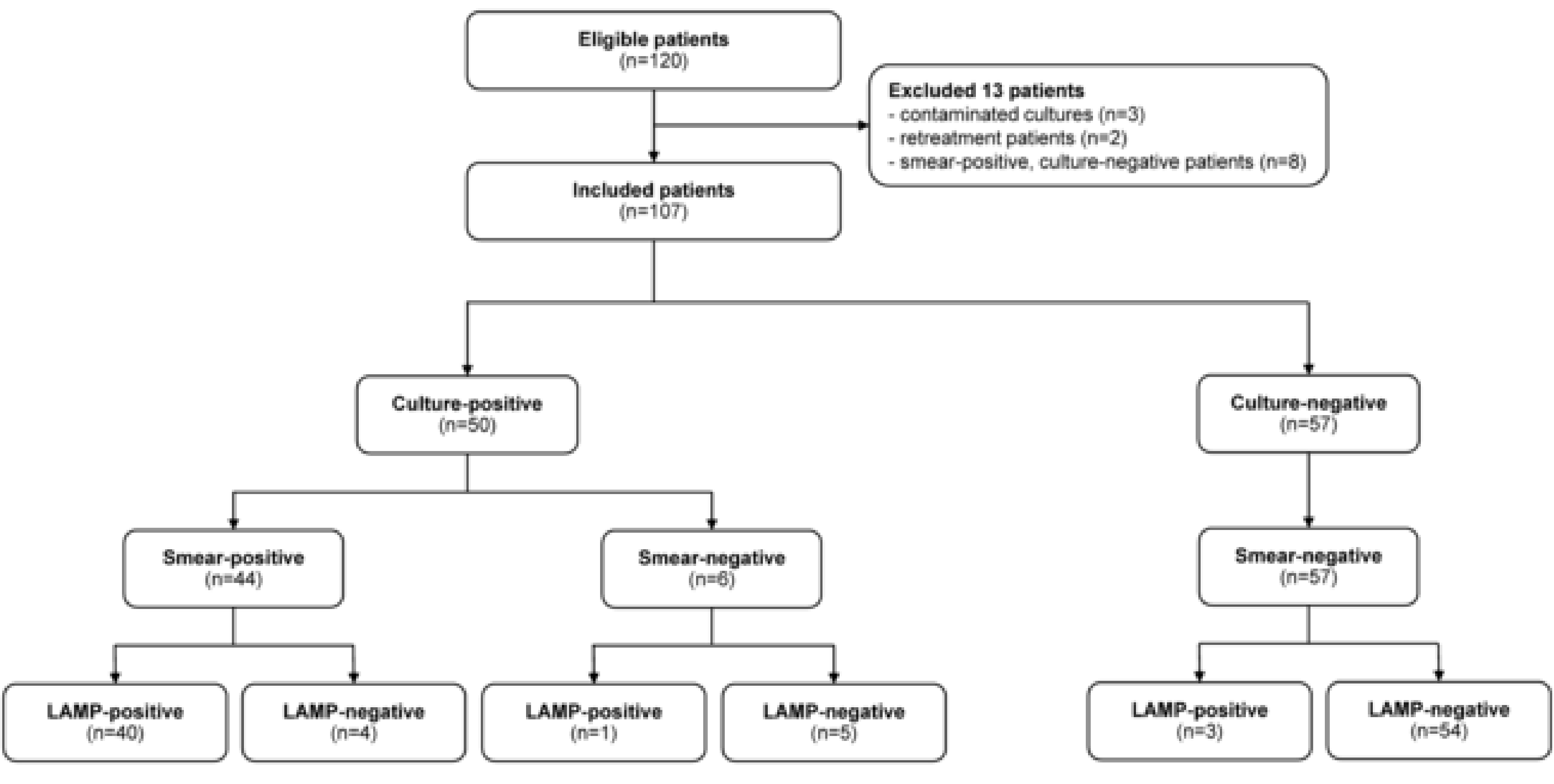
457 # Supporting information

458 **S1 Table. Review on diagnostic accuracy of in-house LAMP assays for diagnosis of**

459 **pulmonary tuberculosis** (DOCX)

460 **S2 Table. LAMP minimal dataset** (CSV)

461


Eligible patients
(n=120)
Excluded 13 patients
- contaminated cultures (n=3)
- retreatment patients (n=2)
- smear-positive, culture-negative patients (n=8)
Included patients
(n=107)
Culture-positive
(n=50)
Culture-negative
(n=57)
Smear-positive
(n=44)
Smear-negative
(n=6)
Smear-negative
(n=57)
LAMP-positive
(n=40)
LAMP-negative
(n=4)
LAMP-positive
(n=1)
LAMP-negative
(n=5)
LAMP-positive
(n=3)
LAMP-negative
(n=54)

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

S1 Review table

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Supporting Information
Table S1.docx

1 **Pragmatic accuracy of an 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 an in-house LAMP for pulmonary TB

6

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Abstract

Background: To 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-effective nucleic amplification technique. We evaluated the pragmatic accuracy of ~~the~~ an in-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 based on culture positivity was 88.8% (95/107) ~~(~~with a 95%CI of 81.2~~,~~-94.1~~)~~. The sensitivity was 90.9% (40/44) ~~(~~with a 95%CI of 78.3~~,~~-97.5~~)~~ for smear-positive, culture-positive patients, and was 16.7% (1/6) with a ~~(~~95%CI of 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% (54/57) with a ~~(~~95%CI of 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.

46 **Keywords**: Pulmonary Tuberculosis, ~~in~~In-~~house~~ House LAMP, Diagnosis, Sensitivity,

47 Specificity

48

Introduction

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

Molecular testing methods such as polymerase chain reaction (PCR) or Xpert MTB/RIF have been widely acknowledged as alternative tools to TB culture for the diagnosis of TB patients [3,5]. These nucleic amplification techniques were known for yielding rapid and accurate TB diagnosis~~,~~. ~~which~~ Thi[illegible]ld overcome the limitations of classical methods, insensitivity for smear microscopy, and lengthy incubation period for TB culture. However, several obstacles remain for the application of these molecular tests as point-of-care testing in community settings. This is because of their complexity ~~in executions~~to execute and substantial requirements for financial and personnel resources [3,6]. Loop-mediated isothermal

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73 amplification (LAMP) assay is another recently developed nucleic acid amplification
74 technique. Unlike PCR, where the amplification of DNA fragment occurs in temperature-
75 dependent steps, the reaction of LAMP assay functions in isothermal or constant temperature
76 conditions [7,8]. In 2016, WHO ~~suggested~~ endorsed the use of commercial TB-LAMP assay
77 (Eiken Chemical Co., Tokyo, Japan) as a replacement for smear microscopy for the diagnosis
78 of TB ~~in patients with symptoms suggestive of TB~~ [9]. TB-LAMP assay has a low cost per
79 test, does not required advanced technological facilities, and can be routinely practiced in
80 general hospital laboratories [6,10].
81
82 As financial resources are usually limited in countries with high TB prevalence, ~~setting up an~~
83 ~~infrastructure to support the~~ a commercial TB-LAMP could still be unattainable. ~~A~~
84 ~~more~~More affordable in-house LAMP assays ~~was~~ were later developed and applied in ~~2008~~
85 several centers [11–15]~~[11]~~. ~~The main advantage of the in-house assay was that it could be~~
86 ~~implicated on the readily-available infrastructure of any laboratory, even in the decentralized~~
87 ~~one.~~ However, it did require extra-training and skill of technicians to process the clinical
88 specimens. In the past decades, several clinical studies and meta-analyses had evaluated the
89 diagnostic accuracy of ~~the~~ in-house LAMP tests for the diagnosis of pulmonary TB
90 [14,16,17]~~[12–14]~~ (S1 Table). From the latest meta-analysis, the overall sensitivity and
91 specificity of ~~the~~ in-house LAMP ~~was~~ 93.0% (95%CI 88.9-95.7) and 91.8% (95%CI 86.4-
92 95.1), respectively [17]~~[14]~~. One recent study in Thailand reported the sensitivity and the
93 specificity of the in-house LAMP at 94.4% (95%CI 88.9-97.7) and 94.3% (95%CI 87.2-
94 98.1), respectively [15]. However, the reported accuracy could be overestimated if ~~being~~ it is
95 assessed in qualified laboratories with highly skilled technicians and sufficient resources
96 where molecular tests are usually ~~are~~ available [17]~~[14]~~. Therefore, this study aimed to

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97 evaluate the pragmatic accuracy of the in-house LAMP assay for the diagnosis of pulmonary

98 TB in a ~~peripheral~~ community hospital of a developing country with a high TB burden.

99

Materials and Methods

Ethics Statement

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

Setting

The study was ~~settled~~ performed in Maesot General Hospital, a large-sized community hospital with 365 in-patient beds. ~~The hospital~~ It ~~is~~ located in Maesot ~~district~~ District, ~~in~~ Tak Province ~~(province)~~, which shares the border with Myanmar. The hospital ~~and~~ provides standard health care to both Thai and non-Thai patients (Burmese immigrants and ethnic minorities). According to the Health Data Center, the ~~ministry~~ Ministry of ~~public~~ Public ~~health~~ Health Thailand, the incidence rate of pulmonary TB in Maesot was 351 per 100,000 in 2019. The ~~level o~~ th care system of the hospital is considered rural. Maesot hospital has its reference laboratory with biosafety cabinet infrastructure, BSC class II. There are four lab technicians and one lab assistant within each working shift. Power generator (350 kW) and UPS (2.7 kW) were available in case of power outages, which was infrequent. The m~~M~~edian LAMP test workload per day was 6 (range 4-10).

Study Design

This prospective diagnostic accuracy research was conducted from April to August 2016. 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 culture or missing cultures were excluded from the study.

Methods

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 of more than 3 ml, was used for the whole investigation procedures as routinely done.~~Only one sputum specimen with adequate sputum containing both mucoid or mucopurulent characters and a sample volume of more than 3 ml was selected ~~to be used in~~ all investigation procedures. ~~-~~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. The s~~S~~mear-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 AFB smear-negative~~ was conversely defined.

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. The ~~and were~~specimens were subsequently mixed by vortexing for 30 seconds and left at room

temperature (20-25℃) 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 (pH 6.8) was used for resuspension of the specimens.

For TB culture, the reference test, we performed both conventional culture method on L-J (Lowenstein-Jensen) medium and BBL MGIT 960 (mycobacterial growth indicator tube) culture method. The culture media were inoculated with processed sputum specimens and incubated at 35 to 37℃ and monitored weekly for growth until 8 weeks. The sputum samples were considered as "culture-positive" if growth was detected in either of L-J or MGIT 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-negative" or "non-TB patients". Patients with smear-positive and culture-negative, which were generally considered as probable TB, were excluded from the analysis. Both smear microscopy and culture methods were performed according to the standard protocols [18]~~[16]~~.

In-house LAMP test

The LAMP test consists of three steps as follows: DNA extraction, isothermal amplification, 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 since 2009.~~In this study, we followed the TB Fast AMP technique, which was developed by the National Institute of Health of Thailand and was described in our previous studies [13,15,19]. ~~-~~The procedures were described as follow. Flexi Gene® DNA Kit (Qiagen co., USA) and Protenase K Kit (Qiagen co., USA) were used for DNA extraction~~ [17,18]~~. ~~Four~~

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172 Six primers ~~(MTB primers, MAV primers, MIN primers, and Muniv primers)~~ were used for

173 the recognition of ~~six~~ eight distinct regions on the 16S ribosomal RNA gene of M.

174 *tuberculosis*. Each single LAMP reaction includes 12 µl of TB-Fast AMP mixture (FastAMP

175 master mix includes 2 µl 10Xbuffer, 4 µl 2mM dNTPs, 3.2 µl 5M betaine, 1.2 µl 100 mM

176 $MgSO_4$, 1.6 µl primer mixture), 1 µl *Bst* DNA polymerase enzyme (New England Biolabs,

177 Ipswich MA, USA), 1 µl fluorescent detection reagent (FDR; Eiken Chemical Tokyo, Japan)

178 and 6 µl of extracted DNA samples. Amplification of reaction mixture was performed in the

179 heating blocks at 65°C for 60 minutes, then examined directly by visual observation. The

180 LAMP assay was considered "positive" if the color of the reaction mixture changed from

181 orange to green, or fluorescence was directly observed with the naked eyes. The test was

182 considered "negative" if the color of the mixture remained unchanged. For quality control,

183 positive control (test tube with ~~M~~ *Mycobacterium*~~.~~ *tuberculosis* genetic materials) and

184 negative control (test tube without *M. tuberculosis* genetic materials) were included in all

185 runs.

186

187 Statistical Analysis

188 We used Fisher's exact probability test for comparison of differences in independent

189 proportions and Student's t-test for two independent means. The sensitivity, specificity,

190 positive predictive values (PPV), negative predictive values (NPV), and positive and negative

191 likelihood ratios were calculated and reported with its 95% confidence interval. The 95%

192 confidence interval were estimated using the Clopper Pearson binomial exact method. The

193 comparison of sensitivity, specificity, and overall test accuracy between the LAMP test and

194 smear microscopy methods was performed with McNemar's exact probability test. Pairwise

195 testing to compare the specificity between the LAMP test and the smear microscopy methods

196 was not performed as the specificity of the latter was affected by incorporation bias and

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197 would not be comparable to the in-house LAMP. The inter-rater reliability and the ~~agreement~~
198 correlation of the LAMP test with smear microscopy methods was analyzed with Kappa's
199 statistics and Spearman's rank correlation, respectively. P-values of less than 0.05 were
200 considered statistically significant. All statistical analyses were done using Stata version 16
201 (StataCorp, Texas).
202

Results

A total of 120 patients to be evaluated for TB were consecutively included from April to August 2016. Three patients with two contaminated cultures, two patients who subsequently were detected as previously documented TB cases, and eight patients who had smear-positive and culture-~~positive~~ negative 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~~ year~~. Fifty (46.7%) were culture-positive TB patients and 57 (53.3%) were culture-negative patients. The baseline demographic data between culture-positive and culture-negative patients were comparable (Table 1). For clinical characteristics, the presence of cavitary lesions on chest radiographs and the ~~character of collected sputum~~character of collected sputum was ~~found to be~~statistically ~~significantly~~ differen~~t~~t~~ (Table 1)~~. Culture-positive TB patients had higher proportion of cavitary lesions (14.0% vs. 1.8%, p=0.024) and mucous sputum specimen (52.0% vs 24.6%, p=0.005) than ~~patients~~ those with negative TB culture. The proportion of patients with salivary sputum was significantly lower than mucous sputum in both smear-positive and ~~,~~LAMP-positive results ~~was 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).

221

222 **Table 1. Demographic and clinical characteristics of the patients by TB culture status**

Characteristics	TB Culture Positive (S+ or S-, C+)	TB Culture Negative (S-, C-)	P-Value
	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)	

223 Abbreviations: TB, tuberculosis; C, culture (+ positive or – negative); S, smear microscopy (+ positive or – negative); SD,
224 standard deviation.

225

226 **Fig. 1. Study flow diagram of patient enrollment and results of index and reference test**
227 **based on culture result**

228

229

230 The overall sensitivity of the LAMP test was 82.0% (95%CI 68.6-91.4), whereas the
231 sensitivity in smear-positive, culture-positive patients and smear-negative, culture-positive
232 was 90.9% (95%CI 78.3-97.5) and 16.7% (95%CI 0.4-64.1), respectively. The overall
233 sensitivity of both the AFB and the fluorescence stain was slightly higher than that of the
234 LAMP test; however, the differences were non-significant (Table 2). The specificity, positive
235 predictive value, and negative predictive value of the LAMP test was 94.7% (95%CI 85.4-
236 98.9), 93.2% (95%CI 81.3-98.6), and 85.7% (95%CI 74.6-93.3), respectively. The positive
237 and negative likelihood ratios of the LAMP test was 15.6 (95%CI 4.47-82.12) and 0.19
238 (95%CI 0.08-0.44), respectively. ~~Even though the~~The accuracy measures for the diagnosis of
239 TB cases were shown to vary across different test methods (LAMP test, AFB stain, and
240 fluorescence stain), the differences were without statistical significance (Table 2).
241 LAMP test results were highly correlated with those of AFB and fluorescence stain
242 (Spearman's rho 0.85~~,~~, 95%CI 0.74-0.95, p<0.001) in the diagnosis of culture-positive TB
243 cases (Table 3). The in-house LAMP also showed substantial to an almost perfect agreement
244 with both microscopy methods in the diagnosis of culture-positive cases (Kappa 0.85, 95%CI
245 0.74~~,~~-0.95, p<0.001) (Table 3).
246

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

Method	Sensitivity% (95% CI), no. corrects			Specificity% (95%CI), no. corrects	Accuracy% (95%CI), no. corrects (n=107)	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)					
LAMP	90.9 (78.3,97.5), N=40	16.7 (0.4,64.1), n=1	82.0 (68.6,91.4), n=41	94.7 (85.4,98.9), n=54	88.8 (81.2,94.1), n=95	93.2 (81.3,98.6)	85.7 (74.6,93.3)	15.6 (4.5,82.1)	0.2 (0.1,0.4)
AFB stain	-	-	88.0 (75.7,95.5), n=44	100.0 (93.7,100.0), n=57	94.4 (88.2,97.9), n=101	100.0 (93.7,100.0)	90.5 (80.4,96.4)	-	-
Fluorescence stain	-	-	88.0 (75.7,95.5), n=44	100.0 (93.7,100.0), n=57	94.4 (88.2,97.9), n=101	100.0 (93.7,100.0)	90.5 (80.4,96.4)	-	-
LAMP test vs. AFB stain			P=0.375*	P=0.250*	P=1.000*				
LAMP test vs. Fluorescence stain			P=0.375*	P=0.250*	P=1.000*				

*P-values from McNemar's Exact probability test

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

Table 3. ~~Diagnostic agreement and correlation~~Inter-rater reliability and diagnostic agreement between ~~the~~ an in-house LAMP test and AFB stain-fluorescence stain.

LAMP Test	AFB Stain & Fluorescence stain		
	Positive	Negative	Total
Positive	40	4	44
Negative	4	59	63
Total	44	63	107
Agreement (%)	92.5%		
Kappa (95%CI, p-value)	0.85 (0.74-0.95, p<0.001)		
Spearman's rho (p-value)	0.85 (0.74-0.95, p<0.001)		

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

260 # Discussion

261 This study ~~had~~ has demonstrated the pragmatic diagnostic performance of ~~the~~ our in-house
262 LAMP assay in a remote hospital of a high TB burden country. ~~It was revealed that the~~The
263 overall sensitivity ~~of the in-house LAMP in our study~~ was lower than ~~the numbers reported in~~
264 the majority of the previous in-house LAMP studies [11,15,20–23]. Nonetheless, the
265 specificity was comparable to other figures reported in the literature [11,12,15,21,22]. -In
266 comparison to microscopy methods (~~, the~~ AFB and fluorescence stain), the in-house LAMP
267 was ~~found to be~~ inferior in terms of overall sensitivity ~~(82.0% vs. 88.0%, p=0.375) and~~
268 ~~accuracy (88.8% vs. 94.4%, p=1.000); however, the comparative statistical test revealed non-~~
269 ~~significant results~~. Based on the result of our study, we suggest that the in-house LAMP
270 should not be a substitute to conventional smear methods, but should be done in parallel,
271 which would result in a higher sensitivity with fewer false-negative TB cases.
272
273 ~~In this study, the sensitivity of the in-house LAMP test was 82.0% (95%CI 68.6-91.4) in~~
274 ~~culture-positive TB patients, respectively.~~ In the past, several studies ~~had~~ reported a higher
275 sensitivity of ~~the~~ in-house LAMP tests, ~~which ranges~~ranging from 90.0 to 100.0% [11,15,20–
276 25]~~[11,15,19–24]~~. Most of these studies were reported from either ~~University~~ university
277 hospitals, TB-specialized centers or hospitals, or national TB-specialized laboratories, which
278 were generally equipped with ~~highly-trained~~highly trained personnel and adequate
279 infrastructural supports [17]. The overall sensitivity of our in-house LAMP was consistent
280 with two previous studies from India and Zambia, which was 79.5% (95%CI 64.0-89.0) and
281 81.4% (95%CI 71.6-89.0), respectively [12,16]~~[12,25]~~. Although both studies were performed
282 in ~~University~~ university hospitals, the LAMP procedures were modified to suit local
283 conditions, and sputum processing and DNA extraction ~~was~~ were done with commercial kits.
284 The higher sensitivity of the acid-fast stain and the fluorescence stain in our study could be

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explained by the high prevalence of TB, the absence of HIV patients or less fewer number of patients with paucibacillary sputum, and the availability of skilled technicians [16,26–28][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 [16,29][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.

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The overall specificity of the LAMP test was 94.7% (95%CI 85.4-98.9) for non-TB patients, respectively. This was in concordance with a recent meta-analysis, which reported pooled specificity of the in-house LAMP tests at of 91.8% (95%CI 86.4-95.1) [17][14]. However, it was concluded that the specificity of the in-house assays was lower than that of the Loopamp 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 explained by multiple factors such as higher temperature, higher humidity, suboptimal reagents volume, and crossover contamination [17,30][14,30]. For in-house LAMP, an extensive laboratory technician training and continuous quality assessment should be conducted to lessen the risk of false-positive results. However, other potential factors might still account for the low specificity, such as temperature controls and volume of reaction used. For 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 reaction volume of 30-35 µl due to the risk of self-priming in concentrated reagents [30].

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309 Currently, the WHO only ~~supported~~ endorses the use of two rapid molecular tests for the
310 diagnosis of pulmonary TB, which were Xpert MTB/RIF and the commercialized TB-LAMP
311 assay [9]. According to previous studies, both had shown comparable performance in smear-
312 positive samples, but higher sensitivity was shown in Xpert MTB/RIF than in the LAMP test
313 [6,12]~~[6,25]~~. Xpert MTB/RIF has been endorsed for use in the diagnosis of TB in many
314 countries, including Thailand [4,31]. ~~Nonetheless, Xpert MTB/RIF might not be suitable in~~
315 ~~peripheral regions with poor infrastructure as the instrument requires a stable electricity~~
316 ~~supply and an appropriate environment. The device also requires high continuous~~
317 ~~maintenance costs leading to a relatively high cost per test compared to the LAMP test. The~~
318 ~~LAMP test is readily available and can be done in any resource-poor settings with regular~~
319 ~~infrastructure and technicians with adequate training. In Thailand,~~However, only a portion of
320 patients, ~~not including~~excluding foreigners and ethnic minorities, could reimburse the cost for
321 Xpert MTB/RIF due to the regulation stated by The National Health Security Office (NHSO).
322 To ~~effectively~~ better ~~prevent~~ control the spread of TB, ~~an~~ access to rapid diagnostic tools
323 should be provided to all patients ~~to be evaluated for TB~~with symptoms suggestive of TB
324 ~~should have equal access to high-quality diagnostic tools~~[3]. ~~Therefore~~~~For this reason~~Thus, a
325 ~~smear microscopy and the~~ LAMP ~~test~~ assay may be more applicable in terms of accessibility
326 and affordability, especially in the ~~distant~~ decentralized areas ~~and the borderlands~~[4,32].
327
328 However, there ~~may be~~were some limitations to this study. First, ~~the study size might not be~~
329 ~~powered enough to confirm the statistical insignificance of the between-test comparison~~the
330 study size may not be substantial enough to provide the power required to detect a
331 statistically significant difference between tests. Second, no patients with HIV infection were
332 included during the study period, as HIV status could be influential to the diagnostic
333 performance of both the smear microscopy and the LAMP test, especially in areas with a high

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334 prevalence of TB-HIV coinfection. Third, this study hadthere was a higher proportion of

335 salivary sputum than mucous sputum in this study. This could affect the diagnostic

336 performance of both the index and the reference test [33][32]. The percentage of culture-

337 positive TB cases was lower in salivary samples than in mucous samples (35.8% vs. 65.0%,

338 p=0.005). Both the quality and quantity of sputum specimens were associated with the

339 positivity of smear, molecular testing methods (Xpert MTB/RIF and PCR), and TB culture

340 [34,35][33,34]. Thus, it was possible that some patients with pulmonary TB might be

341 classified as smear-negative, LAMP-negative, or even culture-negative cases. Sensitivity and

342 specificity would be improved if higher quality sputum is obtained No previous study had

343 officially addressed the effect of sputum quality on the LAMP test. Moreover, the character

344 of sputum specimens was rarely reported. Interestingly, it was revealed from our data that the

345 proportion of smear-positive, LAMP-positive results was also significantly lower in salivary

346 sputum than in mucous sputum (31.3% vs. 57.5%, p=0.009 and 29.9% vs. 60.0%, p=0.003,

347 respectively). Therefore, the sensitivity and accuracy of all tests, including LAMP, might be

348 underestimated. Previous studies reported that by improving the sputum quality, TB

349 diagnostic yield increased [36,37][35,36]. Thus, high-quality sputum collection must be

350 encouraged both in practice and studies.

351

352 Finally, the use of routine TB culture as a reference standard might be inadequate, as some

353 TB patients could be classified as not having TB [6]. With a higher quality reference

354 standard, the sensitivity of the in-house LAMP should be increased when a portion of three

355 remaining false-positive cases was re-classified as true-positive cases. Different culture media

356 and techniques could be used in composite to achieve different performance characteristics

357 [38][37]. In our study, two different culture techniques, L-J and MGIT, were used to increase

358 the diagnostic rate of TB [39][38]. We also applied a strict diagnostic definition in calculating

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359 specificity by considering only patients with smear-negative and culture-negative results

360 [40][39].

361

362 Conclusions

363 In conclusion, the a LAMP test is a practical and affordable nucleic amplification technique

364 for the diagnosis of pulmonary TB, which should be implemented in resource-limiteding

365 settings where Xpert MTB/RIF is unavailable. The diagnostic accuracy of the in-house LAMP

366 was similar to previous studies for specificity. Better sputum processing and DNA extraction

367 method should be identified to improve the test sensitivity.To improve the test sensitivity, a

368 better sputum processing and DNA extraction method is essential. -TThe overall accuracy of

369 the in-house LAMP test showedhad lowerminimal inferiority in terms of sensitivity tothan

370 was comparable to that of conventional microscopy and fluorescence microscopysmear

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

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

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

374

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379 Health for their technical advice and support.

380

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Supporting information

651 **S1 Table. Review on diagnostic accuracy of in-house LAMP assays for diagnosis of**

652 **pulmonary tuberculosis** (DOCX)

653 **S2 Table. LAMP minimal dataset** (CSV)

654

Response to Reviewers

We want to thank both the editor and the reviewers for granting the opportunity to revise our manuscript for publication in the PLOS one journal. We hope that our responses and revisions would substantially improve the quality of our manuscript and would be qualified for publication in the journal. If there were any further questions or minor points to be addressed or elaborated, please let us know. We would be more than eager to make any further revision.

Editor's comments

Specific comments:

1. Abstract and Tables: Change the format 95%CI to be consistent with the rest of the manuscript, for example "95%CI 78.3,97.5" should be "95%CI 78.3-97.5".
   - Changed as suggested.

2. Line 80, implicated has negative meaning, suggest changing to implemented.
   - We modified the first two sentences as "As financial resources are usually limited in countries with high TB prevalence, a commercial TB-LAMP could still be unattainable. More affordable in-house LAMP assays were later developed and applied in several centers".

3. Line 162 change M. *tuberculosis to Mycobacterium tuberculosis* since this is the first time you mentioned the bacteria. Also, all the *"*M." need to be italicized in M. *tuberculosis* throughout the manuscript.
   - Corrected as suggested.

4. Line 192 – 193, should the "smear-positive and culture-positive results" be "smear-positive and culture-negative results"?
   - Corrected as suggested.

5. Line 243 – 246: These sentences need reference(s)
   - We inserted some references to the two sentences as suggested.

Reviewer's comments

Thank you to the authors for the revisions made. This is a much better paper to present what is important work. However, I still have a few concerns. These focus on clarification of the 'in-house assay' and the discussion. Additionally, I think a review of the paper by a medical writer or any strong English editor would boost the communication of the results enormously.

1. The paper needs to be reviewed in detail for grammar and English. Other than general tidiness, in a number of places, the intent of what the authors are saying is lost due to odd grammar choices. For the best readability and better reach for the research contained, a review of the writing is recommended. I have made a few notes and suggestions in specific places.
   a. We corrected all of your English suggestions.
   b. We also modified and re-written some of the sentences in the manuscript to improve the readability.
2. The difference in assays still needs to be clearer. An 'in-house assay' is one that is not performed from a kit. You refer to 'the in-house' LAMP assay a lot as if there is only one,

which is not the case. There are many papers out there with different ‘in-house’ LAMP assays. From the introduction, it sounds like you are presenting the findings from an in-house assay you developed following the protocol presented in Pandey et al. If so, this needs to be stated very clearly. However, from the methods section, it does not necessarily sound like you are not following that protocol and that this is a unique in-house assay. Please clarify in the paper.

    a. We made the modification and improved the clarity of our in-house LAMP method as suggested.

3. When discussing previous results and meta-analyses, it needs to be clear that these refer to ‘in-house LAMP assays’ and not ‘the in-house LAMP assay’ as they are not uniform.
    a. Corrected as suggested.
4. Inclusion of ‘Accuracy’ in Table 2 is a bit odd, but it can be kept if it is defined in the statistical methods section.
    a. It was pre-specified in the methods section.
5. The discussion has a lengthy discourse on the costs of Xpert vs LAMP. But there is no referencing of the studies that have costed these two in order to make a proper comparison. It feels quite unsupported.
    a. We removed unsupported statements from the paragraph and make the paragraph more concise.
6. In the discussion, the authors state ‘No previous study had officially addressed the effect of sputum quality on the LAMP test’. I’m not sure this is true and would caution the authors not to make such a sweeping statement.
    a. We removed the sentence out of the discussion section as suggested.
7. In the discussion, “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.” 1) Do not present new results in the discussion – these need to be included in the Results section first. 3) are these sensivity? Specificity? Accuracy? 3) This is not an interpretation that makes sense. The sensitivity/specificity is reported based on the best sputum sample available from the patients – quality samples are difficult to obtain. You can instead interpret it as ‘Sensitivity and specificity would be improved if higher quality sputum is obtained’.
    a. We modified the content as suggested.
    b. We moved the findings to the results section.
8. In general, the discussion needs to be revised to make only statements supported by the literature, the study, or a comparison of the two. Much of the discussion feels like the authors musings.
    a. We modified the whole discussion sections to be as objective as possible.
9. In the discussion, I would suggest focusing on sensitivity and specificity and not accuracy as accuracy is not a common way of discussing or assessing diagnostic tests due to its difficulty of interpretation.
    a. Corrected as suggested.
10. The references as displayed in the reference section aren’t quite right. In reference #1, instead of Lancet, the Journal is listed as Lancet Lond Engl which is not correct. This inclusion of a city occurs in reference #7 as well.
    a. Corrected as suggested.