

Comparison of the Efficacies of Loop-Mediated Isothermal Amplification, Fluorescence Smear Microscopy and Culture for the Diagnosis of Tuberculosis

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Abstract

Background: Despite the advent of novel diagnostic techniques, smear microscopy remains as the most practical test available in resource-limited settings for tuberculosis (TB) diagnosis. Due to the low sensitivity of microscopy and the long time required for culture, feasible and accessible rapid diagnostic methods are urgently needed. Loop-mediated Isothermal Amplification (LAMP) is a promising nucleic-acid amplification assay, which could be accessible, cost-effective and more suited for use with unpurified samples.

Methodology/Principal Findings: In the current study, the objective was to assess the efficacy of a LAMP assay for tuberculosis compared with fluorescence smear microscopy as well as Löwenstein-Jensen (LJ) and Mycobacteria Growth Indicator Tube (MGIT) cultures for the diagnosis of pulmonary tuberculosis using sputum samples. Smear microscopy and culture were performed for decontaminated and concentrated sputum from TB suspects and the LAMP was also performed on these specimens. The LAMP and smear microscopy were compared, in series and in parallel, to culture. LAMP and smear microscopy showed sensitivities of 79.5% and 82.1% respectively and specificities of 93.8% and 96.9% respectively, compared to culture. LAMP and smear in series had sensitivity and specificity of 79.5% and 100.0% respectively. LAMP and smear in parallel had sensitivity and specificity of 82.1% and 90.6% respectively.

Conclusions/Significance: The overall efficacies of LAMP and fluorescence smear microscopy in the current study were high and broadly similar. LAMP and smear in series had high specificity (100.0%) and can be used as a rule-in test combination. However, the performance of LAMP in smear negative samples was found to be insufficient.

Citation: George G, Mony P, Kenneth J (2011) Comparison of the Efficacies of Loop-Mediated Isothermal Amplification, Fluorescence Smear Microscopy and Culture for the Diagnosis of Tuberculosis. PLoS ONE 6(6): e21007. doi:10.1371/journal.pone.0021007

Editor: Shuang-yong Xu, New England Biolabs, Inc., United States of America

Received: March 12, 2011; **Accepted:** May 16, 2011; **Published:** June 17, 2011

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Funding: The authors acknowledge the salary support for Geojith G., which was provided by a research grant from the Research Council of Norway (179342: TB Trials). The initial funding for this project was also obtained from Aeras Global TB Vaccine Foundation. The funders were involved in this project with regard to sample collection, preliminary processing for smear examination/culture and storage (which has been used in this study to compare the LAMP technique). This was primarily for quality control purposes to determine laboratory functionality and efficacy. The funders had no other role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The study received no other external funding.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Tuberculosis (TB) is one of the oldest diseases that still afflict mankind. The dual specters of TB and AIDS have drawn recent attention to the lack of a suitable diagnostics for TB [1,2,3,4]. TB case detection is the first hurdle towards tackling the TB epidemic [5]. However, the culture which is considered as the 'gold standard' of TB diagnosis takes 3–6 weeks, leaving the less sensitive smear microscopy as the only feasible rapid test presently. Even the automated liquid culture systems like BACTEC or Mycobacteria Growth Indicator Tube (MGIT) take 1–6 weeks for growth detection. The utility of microscopy decreases radically in paucibacillary and HIV positive TB suspects. Smear negative carriers, even if considered less infectious, can still spread TB [6]. The long delays in diagnosis result in patients dropping out or continuing to spread TB till they are correctly diagnosed, found and treated [7,8]. While treating all suspected cases adds significantly to the cost of TB control programs, it also exposes subjects to unnecessary

drugs and worsens the emergence of drug resistance. A highly sensitive rule-in test can significantly improve the case detection whereas a highly specific rule-out test can reduce the turnaround time and the duration of respiratory isolation as well as avoid unnecessary administration of potentially toxic drugs [9,10,11].

Despite the recent advances in TB diagnosis [12], cost and accessibility continue to be the major limiting factors in the effort to eradicate tuberculosis [13]. Notwithstanding the advent of novel diagnostic techniques, smear microscopy remains the most practical test available in resource-limited settings, where majority of the TB is present. Considerable effort and resources have been invested in developing novel diagnostics and improving existing ones [5,14,15]. However, the improvements in sensitivity and specificity achieved thus far have not been extensively demonstrated or rigorously evaluated with actual patient samples in field conditions. Nucleic-acid amplification based tests (NAATs) are of particular interest, since they may be eminently suited for use with respiratory specimens [16] and due to their rapidity and

specificity, especially compared to serological tests [17,18,19]. Nucleic acid assays are also more amenable to miniaturization and microfabrication, opening new vistas for cost reduction and automation [20]. Loop-mediated Isothermal Amplification (LAMP) was shown to be a promising nucleic-acid amplification assay, which could be accessible and cost-effective [21]. It could also be more robust than other nucleic acid amplification tests, retaining the specificity across wider pH and temperature gradients and showing lesser inhibition in unpurified samples [22]. Suitability of LAMP as a point of care test for the diagnosis of pulmonary tuberculosis is beginning to be evaluated with clinical samples [23,24].

The LAMP assay was found to be suitable for the laboratory identification of *M. tuberculosis* (MTB) in culture isolates by the authors previously [25]. In the current study, the objective was to assess the efficacy of a LAMP assay for tuberculosis, alone and in combination with fluorescence smear microscopy as well as Löwenstein-Jensen (LJ) and Mycobacteria Growth Indicator Tube (MGIT) cultures for the detection of *M. tuberculosis* from archived sputum samples.

Materials and Methods

Ethics Statement

This study was reviewed and cleared by the St. John's Medical College Hospital ethics review board. Written informed consent was obtained from all the participants. The data were analyzed anonymously.

Participants

To compare 'LAMP' with the fluorescence smear microscopy and LJ culture, we used 78 sputum samples obtained from as many TB suspects from the Palamaner region in the state of Andhra Pradesh in southern India.

Procedures

Samples were collected over a period of six months starting from January 2007. These were decontaminated using NALC-NaOH method and stored at -20°C . Sputum sample digestion-decontamination, LJ culture [26] and auramine O fluorescence microscopy were performed as per standard literature [27]. MGIT cultures were performed as per the manufacturer's instructions. Decontaminated sputum was processed (January to February 2010) using the 'AMPLICOR Respiratory Specimen Preparation Kit' (Roche Diagnostics GmbH, Mannheim), according to manufacturer's instructions and the resulting lysate was stored at -20°C . LJ and MGIT cultures were used as the 'gold standard' against which other tests could be assessed. The tests were executed and read by experienced personnel, who were blinded to the results of the other tests.

LAMP reaction

The *M. tuberculosis* specific LAMP reaction was carried out as published previously [28], but was modified to suit local conditions [25]. Briefly, the primers, along with 5 μl of the sample lysate were heat denatured for 3 minutes and then annealed prior to adding the enzyme-dNTP mix. The LAMP reaction was carried out in a final volume of 25 μl in a 120 minute format and was terminated by heating at 80°C for 2 minutes. This assay is specific for the *rimM* sequence of *M. tuberculosis* and *M. bovis*. DNA extracted from *M. tuberculosis* ATCC strain H37Rv was used as the positive control and PCR grade water as the negative control. The *Bst* polymerase (Large fragment) was purchased from New England Biolabs Inc. The primers and SYBR Green I were acquired from Sigma-Aldrich Corporation, India. The final reaction volume was 25 μl

(including sample volume 5 μl). The results were visualized by adding 2 μl of 10-fold diluted original SYBR Green I after amplification, and confirmed by agarose gel electrophoresis [29].

Statistical methods

Data was analyzed using SPSS software - version 17 (© IBM corporation 2010). Sensitivity, specificity [30] as well as positive and negative predictive values (PPV & NPV) [31] were calculated for the LAMP and smear microscopy in comparison with culture as the gold standard. LAMP and smear microscopy testing in series and parallel were also compared against the culture [32]. For this purpose, LAMP and smear microscopy were compared in series (LAMP performed only if smear positive and considered TB positive if both tests are positive or TB negative if either test is negative) and in parallel (LAMP performed for all samples and considered TB positive if either smear or LAMP is positive and TB negative if both are negative). Serial testing improves the specificity but lowers the sensitivity. It also reduces the cost of the second test as it is performed for only those samples positive by the first test. Parallel testing improves the sensitivity, while decreasing the specificity. Cohen's kappa was calculated as a measure of agreement between the tests [33].

Results and Discussion

The results of the study are outlined in table 1. Samples were considered to be culture negative if no growth was detected in both LJ and MGIT cultures. Samples were considered to be 'culture positive' if growth was detected in either LJ or MGIT cultures. Of the 78 samples tested, 7 showed contamination for both LJ and MGIT cultures and were omitted from analysis. 34 samples were LAMP positive, of which one was detected to be contaminated on both LJ and MGIT cultures. Of the 44 LAMP negative samples, 6 were detected to be contaminated on both LJ and MGIT cultures. 33 samples were smear positive. Of the 45 smear negative samples, 7 were detected to be contaminated on both LJ and MGIT cultures. Non-contaminated culture results were available for 33 positive and 38 negative samples each for the LAMP and smear. The performance of LAMP and smear as compared to the LJ or MGIT culture is given in table 2. Efficacy of the LAMP and smear microscopy in series and in parallel is depicted in table 3.

LAMP detects the presence/absence of the genetic material of *M. tuberculosis*. Smear microscopy detects the morphology and culture methods differentiate based on the physiology of the viable organism. These different approaches could be complementary. In this study, the LAMP was observed to have high sensitivity and specificity for samples with concordant culture and smear results. The overall efficacy of LAMP and fluorescence smear microscopy in the current study was high and broadly similar. However, the performance of LAMP in smear negative samples was found to be suboptimal.

The LAMP showed excellent sensitivity (96.7%) and specificity (100.0%) for those samples with analogous culture and smear results ($n = 59$), as shown in table 2. These values are similar to those reported earlier [24]. LAMP showed poor sensitivity (22.2%) and specificity (33.3%) for the samples with discordant culture and smear results ($n = 12$, Table 2). The overall sensitivity (79.5%) and specificity (93.8%) of LAMP was slightly lower than that of the smear microscopy (82.1% and 96.9% respectively) for the entire samples ($n = 71$) when compared to culture. LAMP showed a sensitivity and specificity of 96.9% and 100.0% respectively for smear positive samples ($n = 33$). The specificity of LAMP for smear negative samples ($n = 38$) was 93.6%. The sensitivity of LAMP for smear negatives could not be calculated due to the limited number of samples. When both the LAMP and smear microscopy were

Table 1. Results of LAMP, smear and culture.

LAMP	Smear	Number of cultured samples	LJ or MGIT culture			Number of total samples
			Either +	Both -	Both Contaminated	
+	+	31	31	0	0	31
+	-	2	0	2	1	3
-	+	2	1	1	0	2
-	-	36	7	29	6	42
Total		71	39	32	7	78

*Positive results marked as '+' and Negative results as '-'.
doi:10.1371/journal.pone.0021007.t001

performed serially, the overall sensitivity and specificity observed were 79.5% and 100.0% respectively (n = 71). When both the LAMP and smear microscopy were performed in parallel (n = 71), the sensitivity and specificity observed were 82.1% and 90.6% respectively (Table 3). Commercial NAATs had shown pooled sensitivity and specificity of 96.0% and 85.0% respectively among smear positive samples and 66.0% and 98.0% respectively among smear negative samples[34]. In-house PCR assays were reported to show pooled sensitivity and specificity of 96.0% and 81.0% respectively for smear positive samples [35], indicating that a standardized LAMP can perform potentially better than or equivalent to PCR-based methods.

LAMP does not appear to pick up any additional true positives or pick up any that the smear missed, in smear negative samples. Hence the LAMP assay in the current format may be useful only in diagnosis of smear positive samples or as a rule-in test for smear

negative samples (specificity of 93.6%). The utility of LAMP as a tool to resolve differences between the culture and smear results is questionable, especially when smear negative samples are involved. However, this has to be validated with larger sample sets.

Discordant results between the LAMP and smear or culture can arise if non-tuberculous Mycobacteria (NTM) are present [36,37] or if cultures are contaminated [38]. NTM have been reported at high frequencies from southern India [39,40]. The development of a LAMP assay capable of identifying both MTBC and NTM can improve the sensitivity and specificity significantly. In this study, the one sample that was LAMP negative but smear and LJ positive was later identified as MTB by the 'GenoType MTBC' assay (HAIN lifesciences GmbH, Germany) as well as by LAMP for culture lysates. These rule out NTM or sequence variation as reasons for amplification failure with this sample and indicate the lower concentration of nucleic acid present or the presence of

Table 2. Three-way comparison of LAMP, smear and culture.

	Culture +	Culture -	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Positive likelihood ratio	Negative likelihood ratio	k-value
LAMP +	31	2	79.5	93.8	93.9	79.0	12.7	0.2	0.7
LAMP -	8	30							
n = 71	95% CI =		64.5-89.2	79.9-98.3	80.4-98.3	63.7-88.9	3.3-49.1	0.1-0.4	0.6-0.9
	Culture +	Culture -							
Smear +	32	1	82.1	96.9	97.0	81.6	26.3	0.2	0.8
Smear -	7	31							
n = 71	95% CI =		67.3-91.0	84.3-99.5	84.7-99.5	66.6-90.8	3.8-181.7	0.1-0.4	0.6-0.9
	Smear +	Smear -							
LAMP +	31	2	93.9	94.7	93.9	94.7	17.9	0.1	0.9
LAMP -	2	36							
n = 71	95% CI =		80.4-98.3	82.7-98.5	80.4-98.3	82.7-98.5	4.6-69.0	0.0-0.2	0.8-1.0
	Culture +	Culture -							
	Smear +	Smear -							
LAMP +	29	0	96.7	100.0	100.0	96.7	NA	0.0	1.0
LAMP -	1	29							
n = 59	95% CI =		83.3-99.4	88.3-100.0	88.3-100.0	83.3-99.4	NA	0.0-0.2	0.9-1.0
	Culture +	Culture -							
	Smear -	Smear +							
LAMP +	2	2	22.2	33.3	50.0	12.5	0.3	2.3	NA
LAMP -	7	1							
n = 12	95% CI =		6.3-54.7	6.2-79.2	15.0-85.0	2.2-47.1	0.1-1.4	0.5-12.0	NA

doi:10.1371/journal.pone.0021007.t002

Table 3. Comparison of LAMP and smear in series and parallel.

		Culture +	Culture –	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Positive likelihood ratio	Negative likelihood ratio	k-value
LAMP on smear positives	Positive	31	0	96.9	100.0					
	Negative	1	1							
	(n = 33)	95% CI =		84.3–99.5	20.7–100.0					
2 tests – serial	Positive	31	0	79.5	100.0	100.0	80.0	NA	0.2	0.8
	Negative	8	32							
	(n = 71)	95% CI =		64.5–89.2	89.3–100.0	89.0–100.0	65.2–89.5	NA	0.1–0.4	0.6–0.9
LAMP on smear negatives	Positive	0	2	NA	93.6					
	Negative	7	29							
	(n = 38)	95% CI =		NA	79.3–98.2					
2 tests – parallel	Positive	32	3	82.1	90.6	91.4	80.6	8.8	0.2	0.7
	Negative	7	29							
	(n = 71)	95% CI =		67.3–91.0	75.8–96.8	77.6–97.0	65.0–90.2	3.0–26.0	0.1–0.4	0.6–0.9

doi:10.1371/journal.pone.0021007.t003

inhibitors. All samples negative by LAMP were spiked with *M. tuberculosis* DNA and re-amplified. As all these samples amplified, presence of inhibitors can be ruled out.

The high sensitivity observed for smear microscopy in this study may be due to the nature of sampling, better lab practices or the high prevalence of TB as observed elsewhere [41,42]. One of the factors reducing the sensitivity of the LAMP may be the lysis of specimens upon storage [43]. Subsequent supernatant removal and NaOH treatment of lysed specimens may reduce the amount of amplifiable DNA available. Washing and NaOH-free methods for sputum processing will be of considerable value to NAATs.

Conclusions

LAMP appears to be suitable for smear positive specimens with concordant culture results, but was ineffective in smear negative samples. LAMP and smear in series had high specificity (100.0%) and can be used as a rule-in test combination. An improved LAMP alone or together with smear microscopy can offer same-day diagnosis and has the potential to reduce the drop-out rate substantially. However, these findings need to be substantiated further with larger sample sizes and in different geographical settings. Inter-reader reproducibility and the performance with smear negative samples also need further evaluation. Suitably modified, LAMP can potentially improve the diagnosis of TB in resource limited settings.

References

- Perkins MD, Cunningham J (2007) Facing the crisis: improving the diagnosis of tuberculosis in the HIV era. *Journal of Infectious Diseases* 196: 15.
- WHO (2010) Global tuberculosis control: WHO report 2010.: World Health Organization. WHO/HTM/TB/2010.7 WHO/HTM/TB/ 2010.7.
- Pai M, Minion J, Steingart K, Ramsay A (2010) New and improved tuberculosis diagnostics: evidence, policy, practice, and impact. *Current opinion in pulmonary medicine* 16: 271.
- Davies P, Pai M (2008) The diagnosis and misdiagnosis of tuberculosis [State of the art series. Tuberculosis. Edited by ID Rusen. Number 1 in the series]. *The International Journal of Tuberculosis and Lung Disease* 12: 1226–1234.
- Keeler E, Perkins M, Small P, Hanson C, Reed S, et al. (2006) Reducing the global burden of tuberculosis: the contribution of improved diagnostics. *Nature* 444: 49–57.
- Behr M, Warren S, Salamon H, Hopewell P, de Leon AP, et al. (1999) Transmission of *Mycobacterium tuberculosis* from patients smear-negative for acid-fast bacilli. *The Lancet* 353: 444–449.
- Millen S, Uys P, Hargrove J, Van Helden P, Williams B (2008) The effect of diagnostic delays on the drop-out rate and the total delay to diagnosis of tuberculosis. *PLoS One* 3: 1933.
- Sreeramareddy CT, Panduru KV, Menten J, Van den Ende J (2009) Time delays in diagnosis of pulmonary tuberculosis: a systematic review of literature. *BMC Infectious Diseases* 9: 91.
- Moore DF, Guzman JA, Mikhail LT (2005) Reduction in turnaround time for laboratory diagnosis of pulmonary tuberculosis by routine use of a nucleic acid amplification test. *Diagnostic microbiology and infectious disease* 52: 247–254.
- Campos M, Quartín A, Mendes E, Abreu A, Gurevich S, et al. (2008) Feasibility of shortening respiratory isolation with a single sputum nucleic acid amplification test. *American journal of respiratory and critical care medicine* 178: 300.
- Bailey S, Roper M, Huayta M, Trejos N, Alarcon L, et al. (2011) Missed opportunities for tuberculosis diagnosis. *The International Journal of Tuberculosis and Lung Disease* 15: 205–210.

Limitations

In the current study, sample size under certain subtypes was too limited to arrive at statistically significant estimates of efficacy. The sampling was not continuous due to limited availability of stored samples. Speciation of all the cultured samples were not performed and as such it is not possible to determine how many of the culture or smear positives were NTM, which the LAMP will not detect using current set of primers. The HIV status was not ascertained for the study samples. However, it is unlikely that HIV had a significant impact on the findings, as other studies in the same region indicate <1% prevalence for HIV.

Acknowledgments

We acknowledge the TB Trials Group and part funding by the Research council of Norway (TB Trials 179342). We would also like to thank Bijo Thomas and the SETRI lab, Palamaner, Andhra Pradesh.

Author Contributions

Conceived and designed the experiments: JK. Performed the experiments: GG. Analyzed the data: GG JK PM. Contributed reagents/materials/analysis tools: JK PM. Wrote the paper: GG JK.

12. Nyendak M, Lewinsohn D, Lewinsohn D (2009) New diagnostic methods for tuberculosis. *Current Opinion in Infectious Diseases* 22: 174.
13. Lemaire J, Casenghi M (2010) New diagnostics for tuberculosis: fulfilling patient needs first. *Journal of the International AIDS Society* 13: 40.
14. Lange C, Mori T (2010) Advances in the diagnosis of tuberculosis. *Respirology* 15: 220–240.
15. Pai M, Minion J, Sohn H, Zwerling A, Perkins MD (2009) Novel and improved technologies for tuberculosis diagnosis: progress and challenges. *Clinics in Chest Medicine* 30: 701–716, viii.
16. Dinnes J, Deeks J, Kunst H, Gibson A, Cummins E, et al. (2007) A systematic review of rapid diagnostic tests for the detection of tuberculosis infection. *Health Technology Assessment* 11: 1–196.
17. Morris K (2011) WHO recommends against inaccurate tuberculosis tests. *The Lancet* 377: 113–114.
18. CDC (2009) Updated Guidelines for the Use of Nucleic Acid Amplification Tests in the Diagnosis of Tuberculosis. *Morbidity and Mortality Weekly Report (MMWR)* 58: 7–10.
19. Neonakis IK, Gitti Z, Krambovitis E, Spandidos DA (2008) Molecular diagnostic tools in mycobacteriology. *Journal of Microbiological Methods* 75: 1–11.
20. Kiechle FL, Holland CA (2009) Point-of-care testing and molecular diagnostics: miniaturization required. *Clinics in laboratory medicine* 29: 555–560.
21. Dowdy D, OBrien M, Bishai D (2008) Cost-effectiveness of novel diagnostic tools for the diagnosis of tuberculosis. *The International Journal of Tuberculosis and Lung Disease* 12: 1021–1029.
22. Francois P, Bento M, Hibbs J, Bonetti E, Boehme C, et al. (2011) Robustness of loop-mediated isothermal amplification reaction for diagnostic applications. *FEMS Immunology & Medical Microbiology*.
23. Neonakis I, Spandidos D, Petinaki E (2011) Use of loop-mediated isothermal amplification of DNA for the rapid detection of *Mycobacterium tuberculosis* in clinical specimens. *European Journal of Clinical Microbiology & Infectious Diseases*; DOI 10.1007/s10096-10011-11195-10090.
24. Boehme C, Nabeta P, Henostroza G, Raqib R, Rahim Z, et al. (2007) Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. *Journal of clinical microbiology* 45: 1936.
25. Geojith G, Dhanasekaran S, Chandran S, Kenneth J (2011) Efficacy of Loop Mediated Isothermal Amplification (LAMP) assay for the laboratory identification of *Mycobacterium tuberculosis* isolates in a resource limited setting. *Journal of Microbiological Methods* 84: 71–73.
26. Levin W, Brandon GR, Mc MS (1950) The culture method of laboratory diagnosis of tuberculosis. *American Journal of Public Health and The Nation's Health* 40: 1305–1310.
27. Kent P, Kubica G, (US) CfDC (1985) Public health mycobacteriology: a guide for the level III laboratory: US Dept. of Health and Human Services, Public Health Service, Centers for Disease Control .
28. Zhu R-Y, Zhang K-X, Zhao M-Q, Liu Y-H, Xu Y-Y, et al. (2009) Use of visual loop-mediated isothermal amplification of rimM sequence for rapid detection of *Mycobacterium tuberculosis* and *Mycobacterium bovis*. *Journal of Microbiological Methods* 78: 339–343.
29. Iwamoto T, Sonobe T, Hayashi K (2003) Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. *Journal of clinical microbiology* 41: 2616–2622.
30. Altman DG, Bland JM (1994) Statistics Notes: Diagnostic tests 1: sensitivity and specificity. *BMJ* 308: 1552.
31. Altman DG, Bland JM (1994) Statistics Notes: Diagnostic tests 2: predictive values. *BMJ* 309: 102.
32. Fletcher RH, Fletcher SW (2005) *Clinical epidemiology: the essentials*: Lippincott Williams & Wilkins.
33. Cohen J (1960) A coefficient of agreement for nominal scales. *Educational and psychological measurement* 20: 37–46.
34. Greco S, Girardi E, Navarra A, Saltini C (2006) Current evidence on diagnostic accuracy of commercially based nucleic acid amplification tests for the diagnosis of pulmonary tuberculosis. *Thorax* 61: 783.
35. Greco S, Rulli M, Girardi E, Piersimoni C, Saltini C (2009) Diagnostic accuracy of in-house PCR for pulmonary tuberculosis in smear-positive patients: meta-analysis and metaregression. *Journal of clinical microbiology* 47: 569.
36. Lee JS, Kim EC, Joo SI, Lee SM, Yoo CG, et al. (2008) The incidence and clinical implication of sputum with positive acid-fast bacilli smear but negative in mycobacterial culture in a tertiary referral hospital in South Korea. *Journal of Korean medical science* 23: 767.
37. Vidal R, Juan A, Miravittles M, Martín-Casabona N, Falgueras T (1996) Incidence and significance of acid-fast bacilli in sputum smears at the end of antituberculous treatment. *Chest* 109: 1562.
38. Lai CC, Tan CK, Lin SH, Liao CH, Chou CH, et al. (2010) Molecular evidence of false-positive cultures for *Mycobacterium tuberculosis* in a Taiwanese hospital with a high incidence of TB. *Chest* 137: 1065–1070.
39. Chauhan M (1993) Non-tuberculous mycobacteria isolated from an epidemiological survey in rural population on Bangalore district. *Indian Journal of Tuberculosis* 40: 195–197.
40. Paramasivan C, Govindan D, Prabhakar R, Somasundaram P, Subbammal S, et al. (1985) Species level identification of non-tuberculous mycobacteria from South Indian BCG trial area during 1981. *Tubercle* 66: 9–15.
41. Shea Y, Davis J, Huang L, Kovacs J, Masur H, et al. (2009) High sensitivity and specificity of acid-fast microscopy for diagnosis of pulmonary tuberculosis in an African population with a high prevalence of human immunodeficiency virus. *Journal of clinical microbiology* 47: 1553.
42. Pandey BD, Poudel A, Yoda T, Tamaru A, Oda N, et al. (2008) Development of an in-house loop-mediated isothermal amplification (LAMP) assay for detection of *Mycobacterium tuberculosis* and evaluation in sputum samples of Nepalese patients. *Journal of Medical Microbiology* 57: 439–443.
43. Pathak D, Chakravorty S, Hanif M, Tyagi J (2007) Lysis of tubercle bacilli in fresh and stored sputum specimens: implications for diagnosing tuberculosis in stored and paucibacillary specimens by PCR. *BMC microbiology* 7: 83.