Evaluation of real-time loop-mediated isothermal amplification (RealAmp) for rapid detection of Mycobacterium tuberculosis from sputum samples

Yiming Li a, Lei Shi a, Anqi Pan a, Weiwei Cao b, Xun Chen b, Hecheng Meng a, He Yan a, Shin-ichi Miyoshi c, Lei Ye a,⁎

a College of Light Industry and Food Sciences, South China University of Technology, 510640 Guangzhou, PR China
b Guangzhou DE AOU Biotechnology Technology Co., Ltd., Innovation Building, Science Road, Guangzhou Science City, 510663 Guangzhou, PR China
c Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama 700-8530, Japan

ARTICLE INFO

Article history:
Received 23 April 2014
Received in revised form 12 June 2014
Accepted 13 June 2014
Available online 24 June 2014

Keywords:
Mycobacterium tuberculosis
RealAmp
ESE Quant tube scanner
Sputum

ABSTRACT

Tuberculosis (TB) caused by Mycobacterium tuberculosis (MTB) leads to serious health problems as a chronic respiratory infectious disease. Here we established a real-time fluorescence loop-mediated isothermal amplification assay (RealAmp) using a portable ESE Quant tube scanner as a convenient rapid detection method for MTB. The method efficacy from sputum samples was further investigated, and the reaction time was only 20 min with the detection limit low to 10^2 CFU/ml concentration of MTB. We assessed a total of 1067 samples by the RealAmp assay, comparing the results with smear microscopy and conventional culture methods. To examine whether the failure to detect TB by culturing is due to low sensitivity or true absence, we examined the culture negative samples by commercial real time PCR MTB detection kit, and the results were compared with RealAmp. The data showed that RealAmp assay had a higher positive rate than that of sputum smear and culture methods. RealAmp had a sensitivity of 96.70% and a specificity of 91.55% when compared with culture. In addition, its sensitivity and specificity were 95.29% and 86.88% respectively compared with examination of smear samples using light microscopy. The sensitivity of RealAmp in comparison to real time PCR was 98.25% and specificity was 99.11% in validation of culture negative samples. The present study revealed the newly established RealAmp assay as a convenient, efficient, sensitive and specific method that could be an alternative for rapid detection of MTB and a tool to validate culture and smear negative samples. Furthermore, the portability of the ESE Quant tube scanner also contributed to the promising application for grassroots and field detection of MTB.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Tuberculosis caused by Mycobacterium tuberculosis remains a major life-threatening disease. Approximately one-third of the world population, mainly in developing countries, is exposed to MTB at some stage (WHO, 2010). Culturing based approaches that are considered as the gold standard of MTB diagnosis take 3–6 weeks (Osores et al., 2006), leaving the less sensitive smear microscopy as the only feasible rapid test presently (Kisa et al., 2003; Kramme et al., 2004; Telenti et al., 1993). Thus more rapid and sensitive detection methods are required for the early diagnosis of MTB.

PCR based nucleic acid amplification methods are powerful diagnostic tools for the detection of MTB. Real time PCR, especially, is widely utilized in many advanced countries with its advantages of process visibility, high-efficiency and high-sensitivity. Yet this method is still not routinely applicable worldwide, particularly in developing countries and areas, due to its expensiveness of materials and high demand of laboratory facilities as well as skilled technologists (Cho, 2007; Huggett et al., 2009; Noordhoek et al., 1996).

The loop-mediated isothermal amplification (LAMP) method is a DNA amplification method based on strand displacement reaction and stem-loop structure under isothermal conditions (60 °C–65 °C) using the Bacillus stearothermophilus (Bst) polymerase. The LAMP assay is simple, less expensive and more rapid than PCR, and can be performed by using water bath or heat block benefiting from its isothermal reaction requirement (Notomi et al., 2000). The results are directly visible by naked eyes when a nucleic acid stain (e.g., SYBR Green) is added. However, such method is sensitivity limited compared with the fluorescence-based LAMP.

The ESE Quant tube scanner (ESE GmbH, Stockach, Germany) is a recently introduced real-time fluorescence detection system which is highly efficient for the detection of LAMP generated products (Lucchi et al., 2010). This scanner is a small and portable device and can work...
without external electric power supply. This conveniently portable detection system cooperated with simple and rapid RealAmp assay provides promising applications in remote areas with low resource settings and point-of-care cases. In the present study, we have evaluated the efficacy of a newly developed RealAmp method using ESE Quant tube scanner for the rapid and sensitive detection of MTB in sputum samples.

2. Materials and methods

2.1. Strains and clinical specimens

Mycobacterial reference strains M. tuberculosis (ATCC27294), M. bovis (ATCC19210), M. africanum (ATCC25420), M. avium (ATCC29211), M. kansasi (ATCC 12478), M. intracellulare (ATCC13950), M. abscessus (ATCC23003), M. gordonae (ATCC14470), M. fortuitum (ATCC6841), M. smegmatis (ATCC19420), M. chelonea (ATCC35752), M. gilvum (ATCC43909), M. nonchromogenicum (ATCC19530), M. phlei (ATCC11758), M. terrae (ATCC15755), as well as Nocardia brasiliensis (ATCC19296), Corynebacterium pokenense (SCTCC200239), Streptococcus pneumoniae (ATCC49619), legionella pneumophila (ATCC33152), Bordetella pertussis (CCTCCMM88052) were used in the study.

Sputum samples were collected and provided by Guangdong Provincial Tuberculosis Control Center in China. A total of 1067 sputum samples were analyzed in the study. All sputum samples were collected from May to September 2011.

2.2. Smear microscopy and culture

Sputum samples were smeared directly on a slide and subjected to Ziehl–Neelsen staining and microscopy. Sputum samples were also processed with sodium hydroxide for decontamination, and subjected to cultivation on solid medium (egg-based Löwenstein–Jensen) (Kent et al., 1985; Narvaiz et al., 1998).

2.3. DNA extraction

A 3 times volume of 4% NaOH was added to the concentrated sputum sample and vortexed. After being left for 15 min, 1 ml specimen was centrifuged at 12,000 rpm for 10 min. After discarding the supernatant, 1 ml 0.9% NaCl was added, centrifuged at 12,000 rpm for 10 min, discarded the supernatant. Chloroform was added and mixed by vortexing and the aqueous phase was harvested and stored in the refrigerator until DNA extraction. The DNA was extracted by boiling for 10 min, than freezing in ice for 10 min, centrifuged at 12,000 rpm for 10 min. The extracted DNA was stored in the refrigerator for LAMP assay (Yang et al., 2011).

2.4. LAMP primer design

Using the online Primer Explorer V4 software (EikenChemical Co. Ltd., Tokyo, Japan) (http://primerexplor-er-er.jp/elamp4.0.0/index.html), IS6011-specific LAMP primers were selected according to the general criteria described by Notomi et al. (2000). All the primers were synthesized by commercial provider (Sangon Biotech, Shanghai, China).

2.5. The nucleic acid amplification assay and DNA sequencing

The RealAmp reaction was carried out in a total of 25 μl reaction mixture containing 40 pmol of the inner primers (FIP and BIP), 5 pmol of the outer primers (F3 and B3), 20 pmol of the loop primers, 1.4 mM deoxyxynucleoside triphosphates, 0.8 M betaine, 0.1% Tween 20, 10 mM (NH₄)₂SO₄, 8 mM MgSO₄, 10 mM KCl, 20 mM Tris–HCl (pH 8.8), 8 units of Bst DNA polymerase (New England Biolabs, Beijing, China), and 2 μl of DNA template. The RealAmp assay was accomplished by incubating the reaction mixture at 63 °C for 30 min in an ESE Quant tube scanner (ESE Gmbh, Stockach, Germany). The tests were performed in duplicate.

Real-time PCR reaction was performed by using commercial MTB DNA detection kit which was approved by SFDA (Mycobacterium Tuberculosis (TB) PCR Kit, DAAN Gene Co., Ltd. Of Sun Yat-sen University, China) according to the manufacturer’s instructions.

LAMP or real time PCR products with expected sizes were purified using the QiAQuicks kit (Qiagen, Valencia, CA) following the manufacturer’s instruction. DNA sequencing was performed by Sangon (Shanghai, China).

2.6. Statistical analysis

The sensitivity and specificity values were calculated by using spss program (ver. 13.0) (Chicago, IL, USA).

3. Results and discussion

There is no requirement for expensive reagents and equipment; the ESE-Quant tube scanner provides a major advancement toward “electricity-free” (an alternate power source such as battery can be used to operate the tube scanner) and real time technology for LAMP technology and offers a single-step amplification and product detection step; a portable fluorescent reader equipped with a battery pack (ESE-Quant Tube Scanner) is sufficient to run a RealAmp assay (Lucchi et al., 2010). Therefore, the ESE Quant tube scanner system can be a very useful tool for the research laboratories and in primary health care settings for pre-treatment diagnosis in developing areas. Results in the present study suggested that the RealAmp is an alternative assay for rapid detection of MTB.

To evaluate specificity of the newly developed RealAmp assay, we tested 15 mycobacterial reference strains, as well Nocardia brasiliensis, Corynebacterium pkenense, Streptococcus pneumoniae, legionella pneumophila, and Bordetella pertussis. Amplification curves were observed in the tubes containing genomic DNAs from MTB. No amplification was found with all other strains tested after 30 min of incubation. Aryan et al. utilized the IS6110 repetitive element as a MTB complex-specific target to establish a highly efficient LAMP method for the detection of MTB; the sensitivity of developed LAMP method was 20 times higher than that of PCR method, as well as 50 and 20 times higher than that of LAMP methods based on target gene gyrB and RRS (Aryan et al., 2010). Results in the present study showed that the IS6110-based RealAmp assay specifically detects only MTB. In addition, the RealAmp assay was accelerated by addition of the loop primers; the amplification curves for the detection of MTB strains were produced within 10 min (~10⁶ CFU per ml). However, without the loop primers, the amplification curves for detection of the same concentration of target strains were produced at about 25 min.

To evaluate the sensitivity of the RealAmp assay, we tested serial 10-fold diluted genomic DNA extracted from culture of MTB (ATCC27294). Positive LAMP reactions were detected by the ESE tube scanner. The results indicated that RealAmp assay was able to efficiently detect a very low number of MTB with 30 minute-incubation in the ESE tube scanner. The detection limit of RealAmp assay was up to 10² CFU/ml of MTB, and the amplification curves of the detection limit were produced with in 20 min (Fig. 1). Detection of MTB by LAMP required 60–90 min in previous studies (Geojith et al., 2011; George et al., 2011; Poudel et al., 2009; Yang et al., 2011). Data in our study suggest that the primers designed in this study are highly efficient and minimized the total detection time to less than 30 min.

Out of 1067 sputum samples, 297 cases (27.83%) were positive by smear microscopy. The culture was positive in 333 cases (31.20%) and RealAmp assay was positive in 384 cases (35.99%). The positive rate of RealAmp assay was higher than that of sputum smear and culture methods. The higher positivity rate detected by RealAmp may be due to the possibility that LAMP can detect very low number and even
can be detected by RealAmp assay. The sensitivity of RealAmp assay in comparison to culture was 96.70% and specificity was 91.55%, overall coincidence rate was 93.16%, and Kappa value was 0.8471 (Table 1), which means that the RealAmp results has a consistency with gold standard. The data in the present study validated with larger sample sets is comparable with a previous study with a small number of samples, which shows that in a total of 202 samples, there is a sensitivity of 97.00% and specificity of 94.12% in comparing with culture; and a sensitivity of 91.09% and specificity of 89.11% in comparing with microscopy (Poudel et al., 2009). The overall sensitivity (96.70%) of RealAmp was higher than that of the smear (84.38%), but the overall specificity (91.55%) is slightly lower than that of smear (97.82%) when compared to culture (n = 1067) (Table 1).

Of 718 smear and culture negative samples, 55 of them showed RealAmp positive; combining the results of smear/culture negative but RealAmp positive results, RealAmp appears to pick up additional positives compared with smear and/or culture negative. Of 52 culture positive but smear negative samples, there were 46 RealAmp positives while 6 RealAmp negatives. Of 16 culture negative but smear positive samples, there were 7 RealAmp positives while 9 RealAmp negatives. The RealAmp showed good sensitivity (98.22%) and specificity (92.34%) for those samples with analogous culture and smear results (n = 999), as shown in Table 1. RealAmp showed good sensitivity (88.46%) but poor specificity (56.25%) for the samples with discordant culture and smear results (n = 68, Table 1). The data above indicated that RealAmp can be used as a tool to validate culture and smear negative sample, and minimize the rate of false negatives in culture and smear diagnosis. However the utility of RealAmp as a tool to resolve differences between the culture and smear results is questionable, and large scale evaluation still needs to be performed.

The existence of uncultured or dead bacteria may lead to false negative results in the culture method. In the present study, the negative sample determined by the culture method was also detected by the commercial real time PCR MTB detection kit and the results were compared to RealAmp (Table 2). Different results detected by real time PCR and RealAmp were subsequently sequenced. There were six samples negative by real time PCR but positive by RealAmp and the sequencing results of a sample that was both positive by real time PCR and by RealAmp showed this sample was also positive. The discordant results in some samples may have been due to DNA concentrations from these samples close to the lowest detectable limit.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Culture +</th>
<th>Culture −</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Overall coincidence (%)</th>
<th>K-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RealAmp +</td>
<td>322</td>
<td>62</td>
<td>96.70</td>
<td>91.55</td>
<td>93.06</td>
<td>0.8449</td>
</tr>
<tr>
<td>RealAmp −</td>
<td>11</td>
<td>672</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 1067</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RealAmp +</td>
<td>283</td>
<td>Smear −</td>
<td>95.29</td>
<td>86.88</td>
<td>89.22</td>
<td>0.7539</td>
</tr>
<tr>
<td>RealAmp −</td>
<td>14</td>
<td>669</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 1067</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smear +</td>
<td>281</td>
<td>Culture −</td>
<td>84.38</td>
<td>97.82</td>
<td>94.16</td>
<td>0.8471</td>
</tr>
<tr>
<td>Smear −</td>
<td>52</td>
<td>718</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 1067</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RealAmp +</td>
<td>276</td>
<td>Culture −</td>
<td>98.22</td>
<td>92.34</td>
<td>93.99</td>
<td>0.8591</td>
</tr>
<tr>
<td>RealAmp −</td>
<td>5</td>
<td>663</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 999</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RealAmp +</td>
<td>46</td>
<td>Culture −</td>
<td>88.46</td>
<td>56.25</td>
<td>80.88</td>
<td>0.4570</td>
</tr>
<tr>
<td>RealAmp −</td>
<td>6</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Positive results marked as ‘+’ and negative results as ‘−’.
of these two assays causing inconsistent results. As nucleic acid amplification technology, RealAmp and real time PCR possessed a high consistency with each other; the sensitivity of RealAmp assay in comparison to real time PCR was 98.25% and specificity was 99.11%, the overall coincidence rate was 99.05 %, the overall predictive value was 95%, and Kappa value was 0.8471. The RealAmp positive but culture negative results highlighted the higher sensitivity and specificity of RealAmp than culture method.

The ESE Quant tube scanner is comparable to the real-time PCR machine in the sense that both are capable of detecting samples in real-time as well as analysis of the melting curves using a computer with the corresponding software (Lucchi et al., 2010; Njiru et al., 2012). Therefore, the ESE Quant tube scanner system can be a very useful tool for the research laboratories and in primary health care settings for pre-treatment diagnosis in developing areas. The culture method that is considered as the 'gold standard' of TB diagnosis takes 3–8 weeks, leaving the less sensitive smear microscopy as the only feasible rapid test presently. The results of RealAmp compared to culture and microscopy showed that RealAmp has good sensitivity as well as highly specificity in the rapid detection of MTB; it is able to cover the shortage of microscopy and culture method in clinical diagnosis. The present study, to our knowledge, is the first report demonstrating the efficacy of RealAmp assay for rapid detection of MTB. The RealAmp assay developed in this study has the important advantage in that it significantly reduces the total detection time (less than 30 min). Combined with less than 2 h for pretreatment of sputum samples and RealAmp reactions, the complete LAMP detection system was markedly faster than the conventional methods. Therefore, this assay may provide a sensitive and rapid detection method to improve MTB detection.

Acknowledgments

This work was supported by the Important National Science & Technology Major Projects of China, no. 2013ZX10003001; and the Project of Guangdong Province Training Outstanding Young University Teachers (Yq2013117).

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Real time PCR</th>
<th>+</th>
<th>-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>RealAmp +</td>
<td>56</td>
<td>6</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>RealAmp −</td>
<td>1</td>
<td>671</td>
<td>672</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>57</td>
<td>677</td>
<td>734</td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>98.25%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>99.11%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coincidence rate</td>
<td>99.05 %</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References


