Novel targeting of the \textit{lepB} gene using PCR with confronting two-pair primers for simultaneous detection of \textit{Mycobacterium tuberculosis} complex and \textit{Mycobacterium bovis}

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Tuberculosis (TB), caused by members of the \textit{Mycobacterium tuberculosis} complex (MTC), is the leading cause of infectious disease-related mortality worldwide. The standard method for TB diagnosis usually requires long periods of mycobacteria cultivation, leading to delayed diagnosis, inefficient treatment and widespread occurrence of the disease. Therefore, a rapid method for the detection and differentiation of MTC from other mycobacteria is essential for disease diagnosis. Here, we describe the potential of using the type I signal peptidase (\textit{lepB}) gene as a novel target for TB diagnosis, based on confronting two-pair primers PCR (PCR-CTPP) that can detect MTC and simultaneously differentiate \textit{M. bovis}. The limit of detection of the developed technique was equivalent to 12–120 bacilli. PCR-CTPP was highly specific to only MTC and \textit{M. bovis}, and no cross-reaction was detected in 27 DNA of the non-tuberculous mycobacterial and bacterial strains tested. Thirty-nine blinded clinical isolates and 72 sputum samples were used to validate the PCR-CTPP in comparison with the standard mycobacterial culture method. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of PCR-CTPP were equal to 95, 100, 100 and 95 %, respectively, when tested with clinical isolates. Furthermore, upon testing with the sputum samples, the sensitivity, specificity, PPV and NPV were observed to be 84, 76, 90 and 67 %, respectively. Hence, this highly sensitive novel technique, which is rapid, easy to conduct and cost-effective, is a potential method for TB diagnosis and epidemiological studies, especially in resource-limited countries with a high TB burden.

\textbf{Abbreviations:} AFB, acid-fast bacillus; LOD, limit of detection; MTC, \textit{Mycobacterium tuberculosis} complex; NPV, negative predictive value; NTM, non-tuberculous mycobacteria; PCR-CTPP, confronting two-pair primers PCR; PPV, positive predictive value; SNP, single-nucleotide polymorphism; TB, tuberculosis.
INTRODUCTION

Tuberculosis (TB) is one of the most serious infectious diseases in the world, and is caused by an acid-fast-positive and slow-growing bacterium, known as Mycobacterium tuberculosis complex (MTC), which is composed of M. tuberculosis, M. bovis, M. africanum, M. canetti, M. microti and the subsequently identified M. caprae and M. pinnipedi (Cousins et al., 2003). In 2014, the World Health Organization (WHO) reported that approximately one-third of the world population was infected with TB, and it was predominantly reported as widespread in countries associated with a high prevalence of human immunodeficiency virus infection (WHO, 2014). M. tuberculosis is found to be the aetiological agent of most TB, but some reports show that the non-tuberculous mycobacteria (NTM) could also cause TB-like disease in the immunocompromised host (Koh et al., 2006; Glassroth, 2008; Ryoo et al., 2008; Weiss & Glassroth, 2012).

The diagnosis of TB is regularly performed by physicians, using chest X-ray results combined with common TB common symptoms such as weight loss and prolonged coughing, with or without production of sputum. However, laboratory diagnosis, including acid-fast bacillus (AFB) staining and culture methods, is required to confirm the infection. AFB staining is widely used as a screening technique. In spite of this method’s ability to provide rapid results, skilled technicians and a high bacterial load of approximately 10⁴ cells ml⁻¹ are needed (Lebrun et al., 1997; Nghiem et al., 2015). In addition, AFB cannot differentiate between MTC and NTM. The culture method is indicated as the gold standard for the diagnosis of TB; however, it is laborious, time-consuming, carries a high risk of bacterial contamination and requires the Biosafety Level Category 3 laboratory. The long-time diagnosis affects the effectiveness of TB treatment and control. Nowadays, nucleic acid amplification tests, mainly PCR, have been developed to address these problems. Additionally, PCR-RFLP is also being used as a tool for identifying Mycobacterium spp. (Plikaytis et al., 1992); however, specific restriction enzymes are required for this. PCR/hybridization, real-time PCR and sequencing (Padilla et al., 2004; Heller et al., 2008; Hong et al., 2011) are also among the techniques used, but these require specific probes or special instruments, which are costly.

PCR with confronting two-pair primers (PCR-CTPP), established by Hamajima et al. (2000), is a powerful tool to identify single-nucleotide polymorphisms (SNPs). The principle of this technique is based on amplification of allele-specific DNA by using appropriately designed two-pair primers in ordinary PCR conditions. PCR-CTPP has been demonstrated as a useful tool for genotyping and studying polymorphism of several human genes associated with clinically important diseases, such as haemoglobin E (Intorasoot et al., 2008), carcinogen-metabolite enzyme (Kawai et al., 2001) and DNA-repairing genes (Li et al., 2012). Moreover, Hamajima et al. (2001) showed that PCR-CTPP could be used for determining the association between the infection rate of Helicobacter pylori and interleukin 1 polymorphism in 241 non-cancer outpatients. To date, the application of PCR-CTPP has not been reported for the detection and the differentiation of either MTC or other bacterial species. Recently, different mycobacterial genes/sequences that specifically distinguish between MTC and other bacteria have been reported as targets for TB diagnosis. The best-known and widely used targets include IS6110, lsp65, dnaJ and 16S rRNA (Pao et al., 1990; Jonas et al., 1993; Takewaki et al., 1993; Dziadek et al., 2001).

In this study, polymorphism within the lepB gene of the Mycobacterium species was identified. The type I signal peptidase encoded by the lepB gene is essential for protein transportation via the Sec-dependent pathway to the outer membrane, since it functions in cleaving the signal peptide from the secreted protein (Paetzel et al., 2000). Inhibition of the type I peptidase function leads to the accumulation of protein in the cell and subsequent cell death. Recently, the lepB gene was also designated a new promising drug target for M. tuberculosis (Ollinger et al., 2012). The nucleotide sequence of the lepB gene is conserved among MTC, and because of this it can be differentiated from NTM and other bacterial species. Moreover, SNPs of the lepB gene can be used as a new marker to differentiate M. bovis from MTC. To date, this is the first report applying the lepB gene for TB diagnosis. Thus, the aim of this study was to develop a rapid, easy to apply and cost-effective method based on PCR-CTPP targeting of the lepB gene for TB diagnosis and identification of M. bovis. This newly developed technique will provide information leading to accurate TB diagnosis and help control further spread of TB.

METHODS

Bacterial strains. Twenty-seven bacterial species were used in this study, including 14 species of Mycobacterium spp. and 13 of other bacteria related to pulmonary diseases (Table 1). The bacteria were cultured in appropriate culture media such as trypticase soy agar (Lab M Limited); in the case of fastidious bacteria, these were cultured on sheep blood agar and incubated at 37 °C for 18–24 h. The mycobacteria were cultured in Lowenstein–Jensen medium (Becton Dickinson) and incubated at 37 °C for 6–8 weeks. Biochemical tests were performed to identify the bacteria before extraction of the genomic DNA.

Sputum specimens. A total of 72 specimens, including 53 AFB-positive and 19 -negative sputum samples (Chiangrai Prachanukroh Hospital) were used for genomic DNA extraction prior to PCR-CTPP. The ethics approval for research was obtained from the Faculty of Associated Medical Sciences, Chiang Mai University, with permission code number 006EXP/57.

DNA extraction. The genomic DNA of a total of 27 bacterial species, 39 blinded clinical isolates of mycobacteria and 72 samples from direct sputum was extracted using a commercial genomic DNA extraction kit (NucleoSpin Tissue). The procedure was conducted according to the manufacturer’s instructions. Genomic DNA was eluted in sterile distilled water and stored at −20 °C until use.

PCR-CTPP. PCR-CTPP was conducted using designed primers that specifically amplify the lepB gene. The primer sequences and the amplicon size are given in Table 2. Initially, the optimal condition of
PCR-CTPP was determined using standard strains of *M. tuberculosis* H37Ra and *M. bovis* BCG ATCC 35740. First, annealing temperatures ranging from 64 to 72 °C were tested. Then, the optimal ratio of the *lepB* primers was determined by varying the four primers used, the *lepB* specific primer (ratio 1:4:4:1), as well as 100–200 ng DNA template. Interestingly, a SNP, G766A, was found in the DNA sequence of all *M. tuberculosis* strains indicated in Table 1 were tested and the analysis performed as described above. To determine the limit of detection (LOD), the genomic DNA extracts from *M. tuberculosis* H37Ra and *M. bovis* BCG ATCC 35740 were serially diluted 10-fold and used as a template in the PCR-CTPP.

**Specificity and limit of detection.** To determine the specificity of the developed method, the genomic DNA extracts from the bacterial strains indicated in Table 1 were tested and the analysis performed as described above. To determine the limit of detection (LOD), the genomic DNA extracts from *M. tuberculosis* H37Ra and *M. bovis* BCG ATCC 35740 were serially diluted 10-fold and used as a template in the PCR-CTPP.

**RESULTS**

**Nucleotide sequence analysis of *lepB* gene**

All *Mycobacterium lepB* gene sequences available in the NCBI database were aligned using ClustalW2 (http://www.ebi.ac.uk). This revealed a highly conserved nucleotide sequence of 99 % identity (data not shown) within the 885 bp of the *lepB* gene among the MTC members, including *M. tuberculosis*, *M. bovis*, *M. canetti*, *M. africanum* and *M. microti*. However, the *lepB* gene of other *Mycobacterium* species was observed to be different in length and sequence (Fig. 1). Interestingly, a SNP, G766A, was found in the DNA sequences of all *M. bovis* strains analysed in this study. Therefore, four primers were designed based on the PCR-CTPP principle to generate three amplicons of different length, which could be distinguished by ordinary gel electrophoresis. The first two primers, LepB complex F1N2 and LepB complex R2N2, were designed specifically to amplify a 381 bp amplicon of the MTC *lepB* gene. The third primer, designated LepB bovis2 F2N2, was the *M. bovis* specific primer. This primer and the LepB complex R2N2 were designed to specifically amplify the *lepB* gene of *M. bovis* and yield a 140 bp amplicon. The last primer, designated LepB TB R1N, was specific to the common allele in all species of MTC except *M. bovis*. A 279 bp amplicon was expected

**Table 2. Primer sequences used in PCR-CTPP**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide sequences (5’→3’)</th>
<th>Target position</th>
<th>Amplified product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LepB complex F1N2</td>
<td>CGAGAACGACCTGGTCAAGC</td>
<td>503–522</td>
<td>279</td>
</tr>
<tr>
<td>LepB TB R1N</td>
<td>CCCCCTAGGCGATGTC</td>
<td>766–781</td>
<td></td>
</tr>
<tr>
<td>LepB bovis2 F2N2</td>
<td>CACTGCGCGTGCTATGTACTA</td>
<td>744–765</td>
<td>140</td>
</tr>
<tr>
<td>LepB complex R2N2</td>
<td>TACCGACCTGCTGGGAGTT</td>
<td>864–883</td>
<td></td>
</tr>
<tr>
<td>LepB complex F1N2</td>
<td>CGAGAACGACCTGGTCAAGC</td>
<td>503–522</td>
<td>381</td>
</tr>
<tr>
<td>LepB complex R2N2</td>
<td>TACCGACCTGCTGGGAGTT</td>
<td>864–883</td>
<td></td>
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</table>
from the amplification of this primer and the LepB complex F1N2. According to the Primer BLAST results (NCBI website), these newly designed primers were unable to bind the nucleotide sequences of other bacteria including NTM.

**Specificity and LOD of PCR-CTPP targeting lepB gene**

The reaction conditions of PCR-CTPP, applying the newly designed primers, were optimized using the genomic DNA of *M. tuberculosis* H37Ra and *M. bovis* ATCC 35740. To determine the specificity of the developed technique, 27 genomic DNA samples were subjected to PCR-CTPP. The results showed that amplicons of the expected sizes at 381 and 279 bp were obtained from *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv, respectively (Fig. 2a).

The PCR results for *M. bovis* BCG Pasteur and BCG also showed the expected amplicons of 381 and 140 bp (Fig. 2a). Interestingly, none of the DNA samples of NTM and other bacteria that can cause pulmonary diseases gave any positive amplicons (Fig. 2a). In addition, to ensure that an adequate quantity and quality of the DNA extracts were used in PCR-CTPP, PCR targeting the 16S rRNA gene was performed (Fig. 2b) using primers and the PCR conditions previously described (Sansila et al., 1998). To determine the LOD of PCR-CTPP, the genomic DNA extracted from the standard strains, *M. tuberculosis* H37Ra and *M. bovis* BCG, was quantified and serially diluted in ten-fold dilutions to $10^{-8}$ and subjected to PCR-CTPP. Based on the genome length of *M. tuberculosis* and *M. bovis* (~4.4 x 10^{6} bp), DNA of 5 fg can be considered as equivalent to one bacillus. Thus, the LOD of this technique was approximately 12–120 bacilli of *M. tuberculosis* and 760–7600 bacilli of *M. bovis* (Fig. 2c, d, respectively).

**Assessment of PCR-CTPP for TB diagnosis**

To evaluate the developed method, DNA samples from 39 clinically mycobacterial isolates were subjected to PCR-CTPP. The results showed that 19 samples of clinical isolates were positive for MTC, all of which were identified as MTC by standard culture methods and biochemical testing. Twenty DNA samples of clinical isolates were found to be negative for MTC by PCR-CTPP. Nevertheless, one out of 39 DNA samples was discordant with the culture method (Table 3). Therefore, the sensitivity, specificity, PPV and NPV of PCR-CTPP in the diagnosis of TB from clinical isolates were equal to 95, 100, 100 % [95 % confidence interval (CI) = 82.20–100.00] and 95 % (95 % CI = 75.05–99.17), respectively.

In addition, DNA samples directly extracted from 72 sputum specimens were examined. Of these, 53 and 19 specimens were AFB positive and negative, respectively. All the sputum specimens were diagnosed by the standard culture method, and it was found that 51 were positive for *M. tuberculosis*. Forty-eight and 24 samples were identified as *M. tuberculosis* positive and negative, respectively, by PCR-CTPP as shown in Table 3 and Fig. 3. In comparison with the culture method, eight and five samples were found to be false positive and false negative, respectively, by PCR-CTPP. Thus, the sensitivity, specificity, PPV and NPV were calculated as 84, 76, 90 and 67, respectively.

**DISCUSSION**

Several rapid nucleic acid amplification-based methods for detection and identification of MTC have been reported, however, only *IS6110, hsp65* and *dnaJ* have been widely used as targets for TB diagnosis (Pao et al., 1990; Takewaki et al., 1993; Dziadek et al., 2001). In this study, it was found that targeting the *lepB* gene allowed simultaneous detection of MTC and *M. bovis* whereas most PCR-based methods targeting MTC often require other molecular techniques for identification of *Mycobacterium* spp. The developed PCR-CTPP method was found to be highly specific to MTC and *M. bovis*. No amplified product was detected in any of 27 bacterial DNA samples tested.

In comparison with the AFB staining technique, PCR-CTPP showed higher sensitivity and its positive result...
Fig. 2. Specificity and sensitivity of PCR-CTPP. (a, b) Genomic DNA was extracted from various known *Mycobacterium* culture, Gram-positive, Gram-negative and fungi-like bacteria using a *lepB* primer set (a) and 16S rRNA gene primers (b). Lanes: M, 50 bp DNA marker; 1–15, *M. tuberculosis* H37Ra, *M. tuberculosis* H37Rv, *M. bovis* BCG Pasteur, *M. bovis* BCG, *M. microti*, *M. africanum*, *M. scrofulaceum*, *M. avium*, *M. intracellulare*, *S. aureus* ATCC 25923, *S. pneumoniae*, *E. coli* ATCC 25922, *K. pneumoniae*, *P. aeruginosa* ATCC 27853 and *Nocardia*, respectively; 16, negative control. (c, d) The sensitivity of PCR-CTPP was determined using serial 10-fold dilution genomic DNA of *M. tuberculosis* H37Ra (c) and *M. bovis* BCG (d). Lanes: M, 50 bp DNA marker; 1, undiluted sample; 2–5, diluted sample 1 : 10–1 : 10 000 (10⁻¹–10⁻⁴); 6, diluted sample 1 : 50 000 (5 x 10⁻⁵); 7, diluted sample 1 : 100 000 (10⁻⁵); 8, diluted sample 1 : 500 000 (5 x 10⁻⁵); 9, diluted sample 1 : 1 000 000 (10⁻⁶); 10, negative control.
directly indicated MTC infection. Less time and labour are needed for PCR-CTPP as it can be completed within 24 h compared with culture methods. The LOD of PCR-CTPP is as low as 60–600 fg of M. tuberculosis DNA per 20 μl PCR, which is equivalent to only 12–120 bacilli. However, at least 100 c.f.u. ml⁻¹ of specimen is required for positive culture (Vignesh et al., 2007). Multiplex PCR targeting the 500 bp fragment specific to M. bovis and the pncA gene to differentiate between M. tuberculosis and M. bovis, as developed by Shah and colleagues, showed lower LOD at 20 pg of M. tuberculosis DNA (Shah et al., 2002). Likewise, LOD at 20 pg of M. tuberculosis and M. bovis chromosomal DNA was reported for multiplex PCR based on the uninterrupted 229 bp sequence in the M. bovis and a unique 12.7 kb insertion sequence from the M. tuberculosis genome (Bakshi et al., 2005).

Notably, the purified DNA extract from clinical isolates showed higher sensitivity and specificity than clinical sputum. This is probably due to the variation in the viscosity of each sputum sample (Khatri et al., 2003). The false-negative results of the PCR-CTPP in comparison to the standard culture method were found in AFB-negative (6/8) or 1 + (2/8) sputum samples. This implied low amounts of mycobacterial DNA in these samples. Moreover, PCR inhibitors or interference in the sputum, such as haem in bloody sputum, may affect DNA amplification (Altschuler, 2006). On the other hand, false-positive results in some sputum samples might have been affected by prior antimicrobial treatment of patients since dead tubercle bacilli can be detected by PCR. Moreover, there are many factors affecting positive culture, such as loss of mycobacterial cells during the processing of the sputum and overgrowth of the contaminant microbial flora in the sample. Therefore, targeting the lepB gene base in PCR-CTPP is probably more suitable for diagnosis than for monitoring of TB treatment. PCR-CTPP based on the lepB gene should also be useful for TB diagnosis in other types of specimen such as CSF, pleural fluid or even oral swabs, a non-invasive-derived specimen that was demonstrated to be successfully used in PCR-based TB diagnosis (Wood et al., 2015).

Although in this report no M. bovis was detected in the clinical specimen, analysis is still necessary since M. bovis is naturally resistant to pyrazinamide, which is one of the front-line drug regimens in anti-tuberculosis treatment (Konno et al., 1967). Additionally, due to the need for acidic conditions in pyrazinamide drug susceptibility testing, this is not routinely performed in the laboratory (Zhang et al., 2002). Hence, the treatment of M. bovis-infected patients with the front-line drug regimen is not only presumed to be totally inadequate but is also believed to lead to the emergence of drug-resistant mycobacterial strains. Although M. bovis-infected cases are rare, their incidences was reported to be around 2.8 and 13.8 % in Africa (de Kantor et al., 2010) and Mexico (Müller et al., 2013), respectively. The identification of M. bovis infection will be beneficial in regard to the epidemiology, source of infection, prevalence of mixed infection, and prevention of the emergence of drug-resistant strains.

### Table 3. Validation of PCR-CTPP results compared with the culture method when using DNA extracted from clinical isolates and sputum samples

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Culture</th>
<th>PCR-CTPP Positive (n)</th>
<th>Negative (n)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>% PPV (95 % CI)</th>
<th>% NPV (95 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>Positive (n)</td>
<td>19</td>
<td>1</td>
<td>95</td>
<td>100</td>
<td>100 (82.20–100.00)</td>
<td>95 (75.05–99.17)</td>
</tr>
<tr>
<td></td>
<td>Negative (n)</td>
<td>0</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical isolates</td>
<td>Positive (n)</td>
<td>43</td>
<td>8</td>
<td>84</td>
<td>76</td>
<td>90 (77.33–96.49)</td>
<td>67 (44.68–84.33)</td>
</tr>
<tr>
<td></td>
<td>Negative (n)</td>
<td>5</td>
<td>16</td>
<td></td>
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</table>

### Fig. 3. Detection of MTC in clinical sputum by PCR-CTPP.
Genomic DNA was extracted from blinded sputum samples and subjected to PCR-CTPP. Lanes: M, 50 bp DNA marker; 1–8, unknown sputum numbers 35119, 35124, 36465, 36466, 36467, 36468, 36470 and 36471, respectively; 9, positive amplicon of size 381 and 279 bp using genomic DNA from M. tuberculosis; 10, positive amplicon of size 381 and 140 bp using genomic DNA from M. bovis; 11, negative control.

http://jmm.microbiologyresearch.org
Many commercial PCR-based methods for TB diagnosis are now available. Despite the fact that these methods can differentiate between MTC members and offer drug susceptibility testing, the cost per test is high, thus making them unaffordable in many low-income countries where a high burden of TB has been reported. In addition, these tests require further analysis based on hybridization. The inconclusive results observed with some specimens may impose the requirement of experienced technicians for interpretation. Unlike DNA sequencing or real-time PCR, targeting the lepB gene based on PCR-CTPP does not require specialized instruments. Accordingly, it is an inexpensive, simple and quick method with high specificity and suitability for differentiating MTC simultaneously with M. bovis from other bacterial species.

CONCLUSION

Targeting the lepB gene using PCR-CTPP enables the classification of MTC from other bacteria, and as well as differentiation of M. bovis from MTC, this could provide information related to the source of infection. In addition, this method is highly accurate, simple, fast, reproducible and cost-effective compared with commercially available diagnostic kits. Therefore, the developed technique could be applied as a routine test in microbiology laboratories and may contribute to more effective treatment and surveillance of tuberculosis.

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