Specific Differentiation of *Mycobacterium bovis* from *Mycobacterium tuberculosis* of the *Mycobacterium tuberculosis* Complex by Molecular Methods

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**ABSTRACT**

The purpose of this study was to determine whether *Mycobacterium bovis* (*M. bovis*) causes tuberculosis (TB) in Thai patients by retrospectively identify *M. tuberculosis* complex (MTBC) isolates stored in our laboratory using different simple molecular methods. Polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) of oxyR gene, PCR of the RD9 region and the putative gene RvD1-Rv2031c, a 500 bp fragment, were used in differentiating *M. bovis* from other MTBC especially *Mycobacterium tuberculosis* (MTB). During 2004-2014, a total of 22,199 isolates from TB patients were identified to be MTB (19,378 isolates) and MTBC (2,821 isolates). Four hundred and eighty six MTBC clinical isolates were retrieved. As controls we tested 1 *M. bovis*, 2 *M. bovis* BCG, 1 H37Rv *M. tuberculosis* strain and 24 *M. bovis* isolates obtained from water buffaloes (*Bubalus bubalis*). PCR-RFLP of oxyR gene and amplification of RD9 region identified MTB (486/486, 100%). The putative gene RvD1-Rv2031c, a 500 bp fragment, was present in all *M. bovis* and *M. bovis* BCG reference strains and also in some MTB clinical isolates but not present in H37Rv *M. tuberculosis* strain. PCR-RFLP of the oxyR gene and amplification of RD9 region were 100% sensitive and specific for distinguishing *M. bovis* isolates from other complex members, but cannot differentiate *M. bovis* from *M. bovis* BCG. The GenoType MTBC assay (Hain-LifeScience, Nehren, Germany) was used as gold standard. In this study, no *M. bovis* causing tuberculosis in Thai patients was identified.

**Keywords:** *Mycobacterium bovis*, Thai tuberculosis patients, Molecular techniques

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Introduction

Tuberculosis (TB) is caused by members of the *Mycobacterium tuberculosis* complex (MTBC). In 2014, an estimate of 9.6 million new TB cases and 1.5 million TB deaths, including 0.4 million among people who were HIV-positive were reported. The number of incident TB cases relative to population size varies widely among countries. Thailand’s, incidence rate is 171 cases per 100,000 population per year (World Health Organization [WHO], 2015). The MTBC comprises a group of closely related species and subspecies, which include *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* Bacillus Calmette-Guérin (BCG), *M. microti*, *M. canetti*, *M. pinnipedii*, and *M. mungi* (Ernst et al., 2007). These species are the primary causes of TB in humans and in wild and domesticated animals.

The majority of TB cases in humans are caused by *M. tuberculosis*. However, infection with *M. bovis* which causes bovine TB (BTB) can occasionally cause TB in humans. BTB is a public health problem in developing countries. *M. bovis* is the main cause of TB in cattle, deer, and other mammals (Coleet et al., 1998). Humans can be infected by consuming contaminated and unpasteurized dairy products. Infections can also occur from direct contact during slaughter or hunting (Prevention CIIDCa, 2016).

Worldwide, *M. bovis* causes less than 1.4 percent of pulmonary TB outside Africa. Within Africa, *M. bovis* causes approximately 2.8 percent of cases of pulmonary TB, for a crude incidence of 7 cases per 100,000 population (Muller et al., 2013). The global proportion of *M. bovis* is higher among patients with extra pulmonary tuberculosis, since the pathogen is frequently acquired via oral ingestion (O'Reilly, Daborn, 1995). In developed countries where *M. bovis* in cattle is controlled and dairy products are routinely pasteurized, the proportion of *M. bovis* infection among human TB cases may be lower than global estimates. In developing countries, accurate data regarding the relative frequency of human TB due to *M. bovis* are rarely available because of limited laboratory capacity for culture and identification (Muller et al., 2013).

In particular, no report has been published regarding the prevalence of BTB among patients in Thailand. Therefore, research on bovine tuberculosis in Thai patients is expected to be beneficial for treatment and epidemiology point of view. In addition, improved understanding of *M. bovis* TB transmission dynamics would help direct the development of additional control measures. The use of selective culture and the identification of isolates using biochemical tests can establish a definitive diagnosis. However, these procedures sometimes fail in precise identification of *M. bovis* (Cosivi et al., 1998; Perez-Guerrero et al., 2008; Portillo-Gomez et al., 2008). Sreevatsan et al. (1996) described specific nucleotide polymorphism of the *oxyR* gene, which has proved to be consistent and accurate for the identification of *M. bovis* (Sreevatsan et al., 1996; Espinosa de los Monteros et al., 1998; Gutierrez et al., 1999). PCR amplification of some RD can distinguish between *M. bovis* and *M. tuberculosis* (Parsons et al., 2002). RD9 region is present in *M. tuberculosis* while this region is absent in *M. bovis* and *M. bovis* BCG strains (Parsons et al., 2002; Ahmed et al., 2004; Bikandi et al., 2004). The putative gene RvD1-Rv2031c, a 500 bp fragment has also been reported to be *M. bovis* specific. Amplification of a 500 bp fragment from the *M. bovis* genome by using the JB21-JB22 primer pair
(Rodriguez et al., 1999) can be used as a marker for comparison between *M. tuberculosis* and *M. bovis*. The putative gene RvD1-Rv2031c, a 500 bp fragment was developed using Random Amplified Polymorphic DNA (RAPD) technique and the fragment hybridized specifically to a 2,900 bp *EcoR* fragment in the *M. bovis* genome (Rodriguez et al., 1999).

Until now, there is no data of *M. bovis* infection in human among ASEAN countries. We therefore would like to retrospectively identify whether we have some *M. bovis* in *M. tuberculosis* complex isolates stored in our laboratory using different simple molecular methods.

**Objectives of the study**

The aim of this study was to determine whether *M. bovis* causes TB in Thai patients by retrospective testing with retrieved MTBC isolates from stock culture and compare different molecular methods namely PCR-RFLP analysis of allelic variation at *oxyR* nucleotide position 285, PCR of the RD9 region and the putative gene RvD1-Rv2031c, a 500 bp fragment, in differentiating *M. bovis* from other MTBC especially MTB.

**Methodology**

**Mycobacterial strains**

Four hundred and eighty six MTBC clinical isolates (obtained during 2004-2014) isolated from Drug Resistant Tuberculosis Fund, Siriraj Hospital, Mahidol University, Thailand and 1 *M. bovis*, 2 *M. bovis* BCG, 1 *M. tuberculosis* H37Rv and 24 *M. bovis* isolates obtained from water buffaloes (*Bubalus bubalis*) reference strains were used in this study. All isolates were cultured on Löwenstein-Jensen (LJ) medium and incubated at 37 °C for 3-4 weeks before DNA preparation.

**Isolation of crude genomic DNA from pure cultures of mycobacteria** (Chaiprasert et al., 2006)

Mycobacterial cells from LJ medium were suspended in 400 µl of TE buffer pH 8.0 (10 mM Tris-HCl and 1 mM EDTA) in 1.5 ml microcentrifuge tube and mixed well. The tubes were boiled at 100 °C in water bath for 20 minutes to kill viable mycobacteria and induce cell lysis. They were then centrifuged at 12,000 rpm for 5 minutes and the supernatant will be carefully removed to a new sterile microcentrifuge tube and stored at -20 °C.

**Identification of Mycobacterium tuberculosis complex** (Chaiprasert et al., 2006)

Multiplex PCR was used to detect the presence of KS4-DNA fragment (specific for MTBC) and simultaneously identify the species *M. tuberculosis* by means of the presence of a *mtp40* sequence by using the published primer set as follows: KS4-based primers TPOL, 5´-CCGGCGCTTGGGCGGAGCCACCACCGCC-3´ and TPOR, 5´-CAGGCTGCCCTGCCACCAGCCCGGTAG-3´ (Prammananan, 1994); *mtp40*-based primers PT1, 5´-CAACGCGCGTCGGTG-3´ and PT2, 5´-CCCCCAGCCACGCGGC-3´ (del Portillo et al., 1991). DNA amplification was done in 50 µl reaction volumes containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 1% gelatin, 25 µM of KS4-based primers, 10 µM of *mtp40* based primers, 0.2 mM dNTP (Thermo Fisher Scientific), 1 U of *Taq* polymerase (Thermo Fisher Scientific) and 5
μl of DNA template. Amplification was done using the Eppendorf AG 22331 Hamburg thermal cycler (Eppendorf, Hamburg, Germany) using the following profile: initial denaturation at 94 °C for 5 minutes, 25 cycles of denaturation at 94 °C for 1 minute, annealing at 70 °C for 1 minute, and extension at 72 °C for 1 minute. After 25 cycles of amplification, the second set of 35 amplification cycles was initiated. It consisted of 1 minute at 94°C for denaturation, 30 seconds at 50°C for annealing, and 1 minute at 72°C for primer extension. Amplified products were detected by agarose gel electrophoresis in 2.5% Tris-Acetate-EDTA (TAE) agarose stained with ethidium bromide.

**PCR-restriction fragment length polymorphism (RFLP) analysis of oxyR** (Sreevatsan et al., 1996)

PCR-RFLP was used to detect variation at oxyR nucleotide position 285. A 548-bp segment of oxyR was amplified by PCR using the published primer set as follows: oxyR1, 5’-GGTGATATATCACACCATA-3’ and oxyR2, 5’-CTATGCGATCAGGCGTACTTG-3’ (Sreevatsan et al., 1996). DNA amplification was done in 25 µl reaction volumes containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 1% gelatin, 25 µM of each primers (oxyR1, oxyR2), 0.2 mM dNTP (Thermo Fisher Scientific), 1 U of Taq polymerase (Thermo Fisher Scientific) and 1 µl of DNA template. Amplification was done using the Eppendorf AG 22331 Hamburg thermal cycler (Eppendorf, Hamburg, Germany) using the following profile: initial denaturation at 96 °C for 5 minutes, 35 cycles of denaturation at 96 °C for 30 seconds, annealing at 57 °C for 30 seconds, extension at 72 °C for 45 seconds, and final extension 72 °C for 6 minutes. Amplified products were detected by agarose gel electrophoresis in 2% Tris-Acetate-EDTA (TAE) agarose stained with ethidium bromide. The PCR product (10 µl) will be digested with 4 U of *AluI* (New England Biolabs, Beverly, MA, USA), a restriction enzyme that cleaves at an AGCT sequence. The reaction mix contained 12 µl water, 2.5 µl enzyme buffer, and 10 µl PCR product.

**Polymerase chain reaction (PCR) for detection of RD9 and 500 bp fragment** (Das S, Das SC, Verma, 2007)

DNA amplification was done in 30 µl reaction volumes containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 1% gelatin, 10 μM of each primers, 0.2 mM dNTP (Thermo Fisher Scientific), 1 U of Taq polymerase (Thermo Fisher Scientific) and 5 µl of DNA template. Amplification was done using the Eppendorf AG 22331 Hamburg thermal cycler (Eppendorf, Hamburg, Germany) using the following profile: initial denaturation at 95 °C for 5 minutes, 30 cycles of denaturation at 95 °C for 1 minute, annealing at 54 °C (RD9) / 58 °C (500 bp) for 1 minute, extension at 72 °C for 1 minute, and final extension at 72 °C for 10 minutes. Amplified products were detected by agarose gel electrophoresis in 2.5% Tris-Acetate-EDTA (TAE) agarose stained with ethidium bromide.

**Detection of RD9 region**

To detect the RD9 region for distinguishing between *M. tuberculosis* and *M. bovis* by using the published primer set as follow: RD9F, 5’-GTGTAGGTCAAGCCCCATCC-3’; RD9R, 5’-CAATGTTGGTGGTGC-3’ and RD9R, 5’-GCTACCCCTGACAAAGTGT-3’ (Parsonset et al., 2002).
Detection of 500 bp fragment

Five-hundred-bp fragment has also been reported to be \textit{M. bovis} specific that will be amplified from the \textit{M. bovis} genome (Rodriguez et al., 1999) by using the published primer set as follow: \textit{JBF}, 5’-TCGTCCGCTGATGCAAGTGC-3’ and \textit{JBR}, 5’-CGTCCGCTGACCCTCAAGAAG-3’ (Rodriguez et al., 1999).

GenoType MTBC assay

The GenoType MTBC assay (Hain-Lifescience GmbH, Nehren, Germany) is based on an \textit{MTBC}-specific 23S ribosomal DNA fragment, \textit{gyrB} DNA sequence polymorphisms, and the RD1 deletion of \textit{M. bovis} BCG (Kasai et al., 2000; Niemann et al., 2000; Talbot et al., 1997) for differentiation of the members of the \textit{Mycobacterium tuberculosis} complex (MTBC): \textit{M. tuberculosis}/\textit{M. canetti}, \textit{M. africanum}, \textit{M. microti}, \textit{M. bovis} subsp. \textit{bovis}, \textit{M. bovis} subsp. \textit{caprae}, and \textit{M. bovis} BCG. The assay was performed according to the manufacturer’s instructions as described previously (Richter et al., 2003).

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure1}
\caption{The specificity and targeted genes of the lines are as follows: 1) conjugate control; 2) universal control (23S rRNA); 3) MTBC specific (23S rRNA); 4 to 12) discriminative for the MTBC species (\textit{gyrB}); 13) \textit{M. bovis} BCG (RD1); M, marker line for correct orientation of the strip (Richter et al., 2003).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure2}
\caption{Interpretation Chart (Hain-Lifescience, 2016)}
\end{figure}

\footnote{Approximately 50% of the subspecies \textit{M. bovis} \textit{caprae} exhibit a banding pattern according to the right column.}
Results

Re-tested with in-house multiplex PCR for identification of *Mycobacterium tuberculosis* complex

Detect the presence of KS4-DNA fragment (specific for MTBC) and simultaneously identify the species *M. tuberculosis* by means of the presence of a *mtp40* sequence. Four hundred and sixty two isolates (95.1%) yielded an amplicon sized 768 bp (KS4-DNA fragment) and were characterized as MTBC. Twenty two isolates (4.5%) amplified both 768 bp (KS4-DNA fragment) and 396 bp (*mtp40* sequence) and were characterized as *M. tuberculosis*. Two isolates (0.4%) amplified only 396 bp (*mtp40* sequence) that show controversy results (Figure 3). These 2 isolates were determined to be *M. tuberculosis* by the GenoType MTBC assay (Figure 4).

![Gel Electrophoresis](image1)

**Figure 3** Gel electrophoresis for multiplex PCR amplicons of KS4-DNA fragment and *mtp40* sequence.

- M: Molecular weight marker (100 bp DNA ladder)
- Lane 1: Positive control (*M. tuberculosis* H37Rv)
- Lane 2: Positive control (*M. bovis*)
- Lane 3: Positive control (*M. bovis* BCG)
- Lane 4: Negative control
- Lane 5-8: Samples (MTBC)
- Lane 9, 10: Samples Positive only *mtp40* sequence
- Lane 11: Sample (MTBC)
- Lane 12, 13: Samples (*M. tuberculosis*)
- Lane 14-16: Samples (MTBC)

![GenoType MTBC Assay](image2)

**Figure 4** GenoType MTBC assay banding pattern of 2 isolates (amplified only *mtp40* sequence).

- No 1: Positive control (*M. tuberculosis* H37Rv)
- No 2,3: Samples (*M. tuberculosis*)
PCR-RFLP of oxyR gene

PCR-RFLP was used to detect variation at oxyR nucleotide position 285. A 548-bp segment of oxyR was amplified by PCR (Figure 5). All of the isolates studies (n= 486) had oxyR gene positive. The PCR product was digested with AluI (New England Biolabs, Beverly, MA, USA), a restriction enzyme that cleaves at an AGCT sequence. Four hundred and eighty six isolates (100%) present only one band at about 230 bp and were identified as M. tuberculosis after digested with AluI (Figure 5).

![PCR amplicon of oxyR gene](image1)

![PCR-RFLP amplicon of oxyR gene digested with AluI](image2)

**Figure 5** PCR amplicon of oxyR gene and PCR-RFLP amplicon of oxyR gene digested with AluI.

- M: Molecular weight marker (100 bp DNA ladder)
- Lane 1: Positive control (M. tuberculosis H37Rv)
- Lane 2: Positive control (M. bovis)
- Lane 3: Positive control (M. bovis BCG)
- Lane 4: Negative control
- Lane 5-13: Samples (M. tuberculosis complex)

**PCR detection of RD9 region**

To detect the RD9 region for distinguish between M. tuberculosis and M. bovis, four hundred and eighty six isolates (100%) yielded an amplicon sized 333 bp and were characterized as M. tuberculosis (Figure 6).

![Gel electrophoresis for PCR amplicon of RD9 region](image3)

**Figure 6** Gel electrophoresis for PCR amplicon of RD9 region.

- M: Molecular weight marker (100 bp DNA ladder)
- Lane 1: Positive control (M. tuberculosis H37Rv)
- Lane 2: Positive control (M. bovis)
- Lane 3: Positive control (M. bovis BCG)
- Lane 4: Negative control
- Lane 5-13: Samples (M. tuberculosis complex)
PCR detection of putative gene RvD1-Rv2031c (500 bp fragment)

Five-hundred-bp fragment of putative gene RvD1-Rv2031c has been reported to be *M. bovis* specific. Three hundred and ten isolates (63.8%) yielded an amplicon sized 500 bp and were positive for 500 bp fragment. One hundred seventy six isolates (36.2%) did not amplify the 500bp fragment and were negative for 500 bp fragment (Figure 7). According to our results, it is different from the publication (Das *et al.*, 2007). So we need the second method to confirm whether it’s real *M. tuberculosis* or it’s other species that can have 500 bp fragment by using the GenoType MTBC assay to prove and found that they determined to be *M. tuberculosis* (Figure 8).

![Image of gel electrophoresis](image1)

**Figure 7** Gel electrophoresis for PCR amplicons of the 500 bp fragment.

- M: Molecular weight marker (100 bp DNA ladder)
- Lane 1: Positive control (*M. tuberculosis* H37Rv)
- Lane 2: Positive control (*M. bovis*)
- Lane 3: Positive control (*M. bovis* BCG)
- Lane 4: Negative control
- Lane 5-13: Samples (*M. tuberculosis*)

![Image of GenoType MTBC assay](image2)

**Figure 8** Sampling 5 isolates that amplified the 500 bp fragment to confirm the species by GenoType MTBC assay.

- No 1-5: Samples (*M. tuberculosis*)
Discussion and Conclusions

This study summarizes the epidemiological survey of bovine TB in Thai patients during 2004-2014 by retrospectively identify whether we have some M. bovis in M. tuberculosis complex isolates stored in our laboratory using different simple molecular methods. There were 486 MTBC clinical isolates identified as MTB (486/486, 100%) by PCR-RFLP of oxyR and amplification of RD9. The putative gene RvD1-Rv2031c, a 500 bp fragment, was present in all M. bovis and M. bovis BCG reference strains and also in some MTB clinical isolates. Our data differ from the published data of Rodriguez et al. (1999) reported that, in 20 M. tuberculosis clinical isolates, the 500 bp fragment was absent. However the same group mentioned in a later study (Gori et al., 1998), without presenting data, that some M. tuberculosis isolates present the 500 bp amplification band and Metaxa-Mariatou et al. (2004) found that the 500 bp PCR fragment is not M. bovis specific, which was also verified by BLAST search where it is evident that the primer sequences are also present in the CDC1551 M. tuberculosis strain. PCR-RFLP of the oxyR gene and amplification of RD9 region were 100% sensitive and specific for distinguishing M. bovis isolates from other complex members, but cannot differentiate M. bovis from M. bovis BCG. The GenoType MTBC assay (Hain-Lifescience, Nehren, Germany) was used to distinguish between BCG and non-BCG strains. In this study, no M. bovis causing tuberculosis in Thai patients was identified.

Acknowledgements

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References


