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.

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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. canettii*, *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 (Cole*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 CfDCa, 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*I 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 werethen 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´-CCGGCGCTTGCGGGCGGACCCACCGCC-3´ and TPOR, 5´-CAGGCTGCCCT GCCCCACGCCCCGGTAG-3´ (Prammananan, 1994); *mtp40*-based primersPT1, 5´-CAACGCGCCGTCGGTGG-3´ and PT2, 5´-CCCCCCACGGCACCGC-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 MgCl$_2$, 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 ºC for 5 minutes, 25 cycles of denaturation at 94 ºC for 1 minute, annealing at 70 ºC for 1 minute, and extension at 72 ºC for 1 minute. After 25 cycles of amplification, the second set of 35 amplification cycles was initiated. It consisted of 1 minute at 94ºC for denaturation, 30 seconds at 50ºC for annealing, and 1 minute at 72ºC 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 $MgCl_2$, 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 ºC for 5 minutes, 35 cycles of denaturation at 96 ºC for 30 seconds, annealing at 57 ºC for 30 seconds, extension at 72 ºC for 45 seconds, and final extension 72 ºC 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 $MgCl_2$, 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 ºC for 5 minutes, 30 cycles of denaturation at 95 ºC for 1 minute, annealing at 54 ºC (RD9) / 58 ºC (500 bp) for 1 minute, extension at 72 ºC for 1 minute, and final extension at 72 ºC 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´-GTGTAGGTCAGCCCCATCC-3´; RD9I, 5´-CAATGTTTGTTGCGCTGC-3´ and RD9R, 5´-GCTACCCTCGACCAAGTGTT-3´ (Parsons*et al*., 2002).

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Detection of 500 bp fragment

Five-hundred-bp fragment has also been reported to be *M. bovis* specific that will be amplifiedfrom the *M. bovis* genome (Rodriguez *et al.*, 1999) by using the published primer set as follow: JBF, 5′-TCGTCCGCTGATGCAAGTGC-3′ and JBR, 5′-CGTCCGCTGACCTCAAGAAG-3′ (Rodriguez *et al.*, 1999).

GenoType MTBC assay

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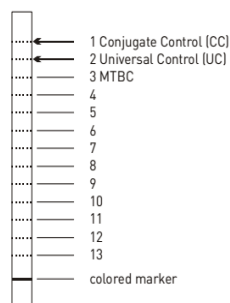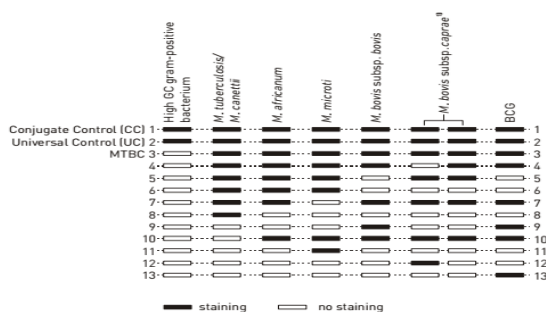

Figure 1 The specificity and targeted genes of the lines are as follows: 1) conjugate control; 2) universal control (23S rRNA); 3) MTBC specific (23S rRNA); 4 to 12) discriminative for the MTBC species (*gyrB*); 13)*M. bovis* BCG (RD1); M, marker line for correct orientation of the strip (Richter *et al.*, 2003).

Figure 2 Interpretation Chart (Hain-Lifescience, 2016)

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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).
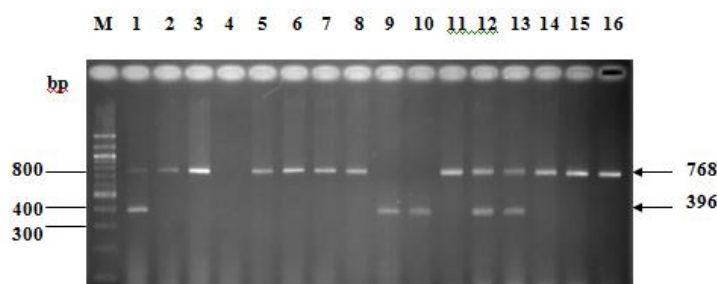

Figure 3 Gel electrophoresis for multiplex PCR amplicons of KS4-DNA fragment and *mtp40* sequence.

M: Molecular weight marker (100 bp DNA ladder)   Lane 5-8: Samples (MTBC)

Lane 1: Positive control (*M. tuberculosis* H37Rv)   Lane 9, 10: Samples Positive only *mtp40* sequence

Lane 2: Positive control (*M. bovis*)   Lane 11: Sample (MTBC)

Lane 3: Positive control (*M. bovis* BCG)   Lane 12, 13: Samples (*M. tuberculosis*)

Lane 4: Negative control   Lane 14-16: Samples (MTBC)
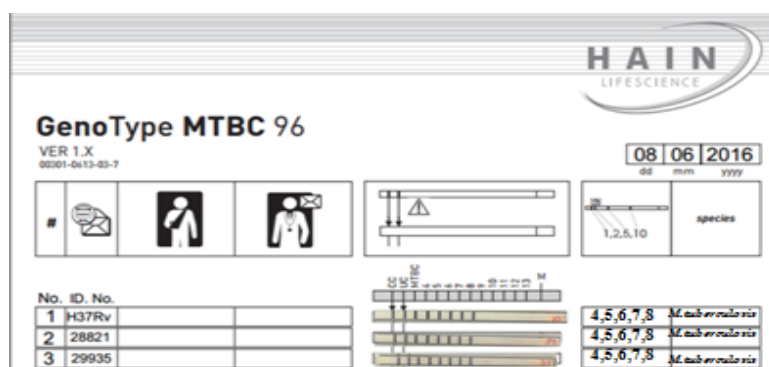

Figure 4 GenoType MTBC assay banding pattern of 2 isolates (amplified only *mtp40* sequence).

No 1: Positive control (*M. tuberculosis* H37Rv)

No 2,3: Samples (*M. tuberculosis*)

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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 AG□CT 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).
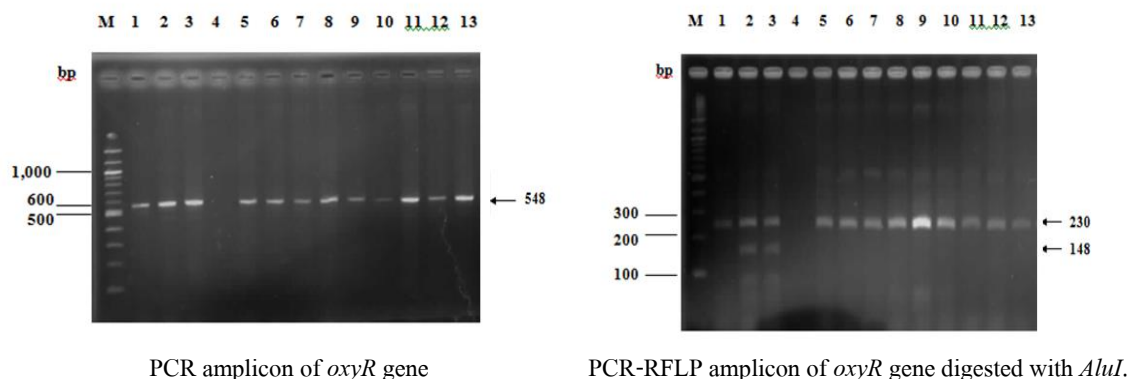

PCR amplicon of *oxyR* gene

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

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 3: Positive control (*M. bovis* BCG)

Lane 1: Positive control (*M. tuberculosis* H37Rv)   Lane 4: Negative control

Lane 2: Positive control (*M. bovis*)   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).
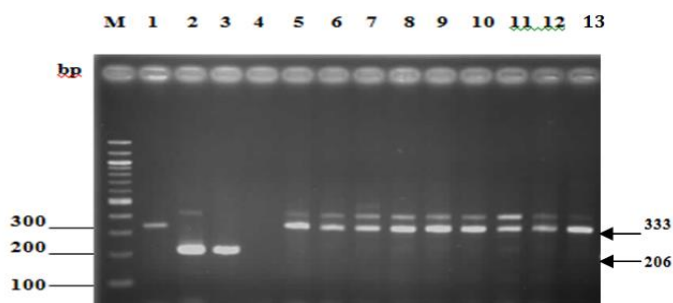

Figure 6 Gel electrophoresis for PCR amplicon of RD9 region.

M: Molecular weight marker (100 bp DNA ladder)   Lane 3: Positive control (*M. bovis* BCG)

Lane 1: Positive control (*M. tuberculosis* H37Rv)   Lane 4: Negative control

Lane 2: Positive control (*M. bovis*)   Lane 5-13: Samples (*M. tuberculosis* complex)

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PCR detection of putative gene RvD1-Rv2031c (500 bp fragment)

Five-hundred-bp fragment of putative gene RvD1-Rv2031chas 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 difference 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).
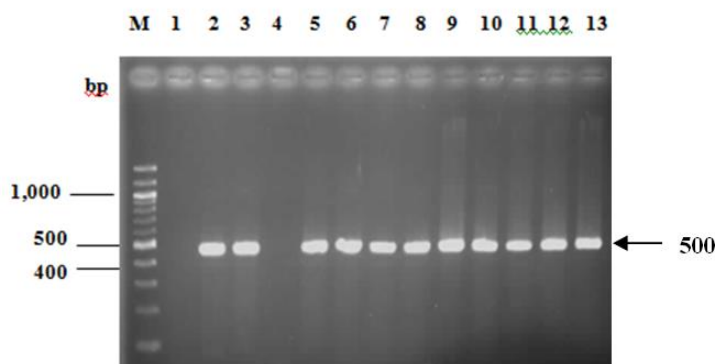

Figure 7 Gel electrophoresis for PCR amplicons of the 500 bp fragment.

M: Molecular weight marker (100 bp DNA ladder)   Lane 3: Positive control (*M. bovis* BCG)

Lane 1: Positive control (*M. tuberculosis* H37Rv)   Lane 4: Negative control

Lane 2: Positive control (*M. bovis*)   Lane 5-13: Samples (*M. tuberculosis*)

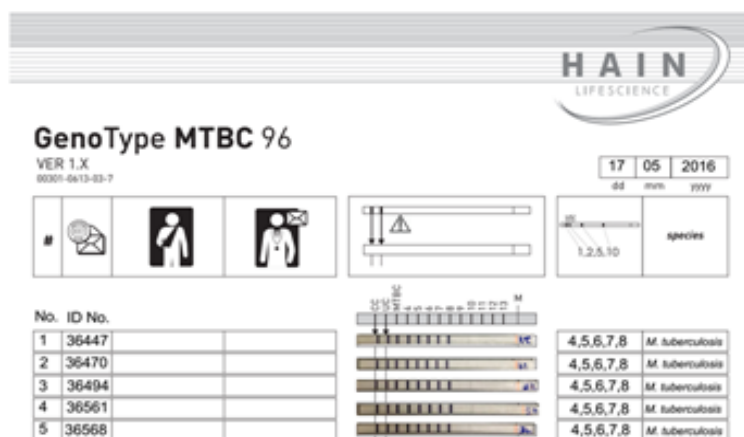**Figure 8** Sampling 5 isolates that amplified the 500 bp fragment to confirm the species by GenoType MTBC assay.

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

Discussion andConclusions

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 were486 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 mentionedin 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 byBLAST search where it is evident that the primer sequencesare 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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