การตรวจพิสูจน์และจำแนกเชื้อ *Mycobacterium tuberculosis* Complex จากตัวอย่างชิ้นเนื้อโคป่วยด้วยวิธีปฏิกิริยาลูกโซ่โพลิเมอเรส

นายศุภสวัสดิ์ บุรณเวช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาสัตวแพทยศาสตรมหาบัณฑิต

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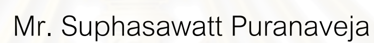
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

IDENTIFICATION AND DIFFERENTIATION OF *MYCOBACTERIUM TUBERCULOSIS*

COMPLEX ORGANISMS IN BOVINE CLINICAL SAMPLES

BY POLYMERASE CHAIN REACTION



Mr. Suphasawatt Puranaveja

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Veterinary Public Health

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ศุภสวัสดิ์ บุรณเวช : การตรวจพิสูจน์และจำแนกเชื้อ *Mycobacterium tuberculosis* Complex จากตัวอย่างชิ้นเนื้อโคป่วยด้วยวิธีปฏิกิริยาลูกโซ่โพลิเมอเรส (IDENTIFICATION AND DIFFERENTIATION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX ORGANISMS IN BOVINE CLINICAL SAMPLES BY POLYMERASE CHAIN REACTION) อ.ที่ปรึกษา : ผศ.น.สพ.ดร.อลงกร อมรศิลป์, อ.ที่ปรึกษาร่วม : ผศ.สพ.ญ.ดร.รุ่งทิพย์ ชวนชื่น, 69 หน้า. ISBN 974-14-3495-2

เชื้อ *Mycobacterium bovis* และ *Mycobacterium tuberculosis* เป็นเชื้อที่มีความสำคัญในด้านสาธารณสุข ทั้งนี้เนื่องจากเชื้อทั้งสองชนิดเป็นสาเหตุสำคัญที่ทำให้เกิดวัณโรคในคนและสัตว์ ซึ่งสามารถติดต่อระหว่างกันได้ โดยเฉพาะอย่างยิ่งในโคนมและเกษตรกรที่ทำงานในฟาร์ม การศึกษาครั้งนี้ได้เก็บตัวอย่างชิ้นเนื้อ 36 ตัวอย่างจากโคนมที่ให้ผลบวกต่อการทดสอบด้วย tuberculin ในจังหวัดนครปฐม ซึ่งการติดเชื้อ *Mycobacterium* นั้น ได้รับการตรวจยืนยันด้วยวิธีทางจุลพยาธิวิทยาและการย้อมสีด้วยวิธี acid-fast ส่วนการตรวจพิสูจน์ชนิดของเชื้อนั้น ใช้วิธีปฏิกิริยาลูกโซ่โพลิเมอเรสโดยอาศัย primers ที่จำเพาะต่อยีน 3 ตำแหน่ง (16SrRNA, Rv1970 และ JB fragment) และการหาลำดับเบสจากยีน *oxyR* พบว่า 30 จาก 36 ตัวอย่าง (83.33%) สามารถตรวจพบเชื้อ *Mycobacterium* ซึ่งใน 30 ตัวอย่างนั้น 12 ตัวอย่าง (40%) พิสูจน์ได้ว่าเป็นเชื้อ *M. tuberculosis* และ 18 ตัวอย่าง (60%) จาก 30 ตัวอย่าง ตรวจพิสูจน์ได้ว่าเป็นเชื้อ *M. bovis* สำหรับการทดสอบทาง DNA fingerprinting โดยวิธีการหาความผันแปรของจำนวนเบสซ้ำต่อเนื่อง (Variable Number Tandem Repeat: VNTR) นั้น พบว่าสามารถจำแนกเชื้อ *M. bovis* ออกได้เป็น 6 รูปแบบ แต่ไม่สามารถจำแนกเชื้อ *M. tuberculosis* ได้ จากผลการทดลองครั้งนี้พบว่า การใช้วิธีปฏิกิริยาลูกโซ่โพลิเมอเรสรวมกับการหาลำดับเบสให้ผลดีสำหรับการตรวจพิสูจน์เชื้อ *M. bovis* และ *M. tuberculosis* โดยตรงจากตัวอย่างชิ้นเนื้อโคป่วย นอกจากนี้วิธี VNTR ยังสามารถใช้ในการจำแนกสายพันธุ์และบอกถึงความหลากหลายของพันธุกรรมเชื้อได้เป็นอย่างดี โดยเฉพาะจากเชื้อ *M. bovis*

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต..

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่ออาจารย์ที่ปรึกษา..

ปีการศึกษา 2549 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม..

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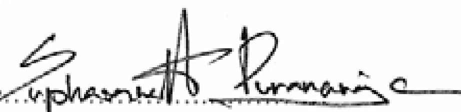
KEY WORD: *MYCOBACTERIUM TUBERCULOSIS* COMPLEX / IDENTIFICATION / DIFFERENTIATION / PCR / VNTR

SUPHASAWATT PURANAVEJA : **IDENTIFICATION AND DIFFERENTIATION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX ORGANISMS IN BOVINE CLINICAL SAMPLES BY POLYMERASE CHAIN REACTION** THESIS ADVISOR: ASST. PROF. ALONGKORN AMONSIN, THESIS CO-ADVISOR: ASST. PROF. RUNGTIP CHUANCHEN, 69 PAGES ISBN: 974-14-3495-2

Mycobacterium bovis and *Mycobacterium tuberculosis* are the public health important bacteria that cause diseases in both animals and humans, especially in cattle and dairy farm workers. In this study, 36 tissue samples were collected from 24 dairy cows previously diagnosed as tuberculin positive in Nakhon Pathom province, Thailand. Infection with *Mycobacterium* organisms was confirmed by histopathologic examination and AFB staining. Species identification was made by using a panel of PCR based on 3 specific genes (*16SrRNA*, Rv*1970*, JB fragment) and direct sequencing using *oxyR* gene. Thirty of 36 (83.33%) clinical tissue samples were identified as *Mycobacterium* spp., 12 from 30 (40%) were positive for *M. tuberculosis* while 18 from 30 (60%) were positive for *M. bovis* strains. DNA fingerprints using variable number tandem repeat (VNTR) was used to differentiate among *M. bovis* and *M. tuberculosis* isolates. Eighteen of *M. bovis* isolates can be grouped into 6 VNTR patterns. However, VNTR analysis indicated that 12 of *M. tuberculosis* isolates infected in cattle were identified in only one VNTR pattern. Our result showed that the combination of PCR and gene sequencing are very useful for identification of *M. bovis* and *M. tuberculosis* from clinical tissue samples. Moreover, VNTR is a simple useful tool for strain differentiation and for the study of genetic diversity, especially for *M. bovis*.

Department Veterinary Public Health Student's Signature 

Field of Study Veterinary Public Health Advisor's Signature

Academic Year 2006 Co-advisor's Signature

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LIST OF ABBREVIATIONS

A	adenosine
AFB	acid fast bacilli
AIDS	Acquired Immunodeficiency Syndrome
bp	base pair
^{o}C	degree celsius
DNA	deoxynucleic acid
DW	distilled water
e.g.	exempli gratia, for example
EDTA	ethylenediamine tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
et al.	et alii, and others
ETR	exact tandem repeat
g	gram (s)
G	guanosine
h	hour (s)
H & E	hematoxylin & eosin
HCSM	heat-concentrated synthetic medium
HPLC	High Performance Liquid Chromatography
i.e.	id est, such as
kDa	kilodalton
M	molar
M.	*Mycobacterium*
mg	milligram (s)
$MgCl_2$	magnesium chloride
min	minute (s)
µl	microliter
µM	micromolar
ml	millilitre

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mM	millimolar
MTBC	*Mycobacterium tuberculosis* complex
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
pg	picogram
pH	the negative logarithm of hydrogen ion concentration
PPD	purified protein derivative
RFLP	Restriction Fragment Length Polymorphism
RNA	ribonucleic acid
rep-PCR	repetitive-sequence-based PCR
rpm	round per minutes
rRNA	ribosomal ribonucleic acid
SDS	sodium polyanetholesulfonate
sec	second (s)
TB	tuberculosis
TBE	Tris-borate –EDTA
TE	Tris-EDTA
U	unit
UV	ultraviolet
V	voltage
VNTR	Variable Number Tandem Repeat

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CHAPTER I

INTRODUCTION

Tuberculosis is one of the most important zoonotic diseases, which causes serious problems affecting both public health and economy worldwide, particularly in developing and tropical countries (Pollock et al., 2006; Prasad et al., 2005). About 2 million humans die from tuberculosis each year, and it was estimated that about one-third of the world's population is infected with mycobacteria. Infections with *Mycobacterium bovis* and *Mycobacterium tuberculosis* have been known to cause bovine and human tuberculosis, respectively. In fact, infections with *M. tuberculosis* and *M. bovis* have shown to occur across a wide spectrum of species, and are not limited to the susceptible hosts (Abalos and Retamal, 2004; Oh et al., 2002).

Tuberculosis, caused by *M. bovis*, is a zoonosis that can spread to humans through inhalation of infectious droplet nuclei and by ingestion of raw milk (Thoen et al., 2006). It is predominant as an opportunistic pathogen in patients with Acquired Immunodeficiency Syndrome (AIDS). However, many reports show that human has become a more dangerous source of infection for cattle than vice versa. Several cases of *M. bovis* transmission from infected farm workers to animals in the herds have been documented (Kubica et al., 2006; Pollock et al., 2006). Furthermore, *M. tuberculosis* that was primarily considered as a human pathogen has been reported in a wide range of domestic or wildlife animal species, most frequently living in close and prolong contact with humans (Alfonso et al., 2004; Erwin et al., 2004; Parra et al., 2003). For example, in the Los Angeles Zoo, *M. tuberculosis* was detected in two Asian elephants (*Elepha maximus*), three Rocky Mountain goats (*Oreamnos americanus*), and one black rhinoceros (*Diceros bicornis*) during 1997 to 2000 (Oh, et al., 2002). Among domestic animals, infection with *M. tuberculosis* was most frequently reported in cattle (Ocepek et al., 2005). It raised an interesting question that how cattle are infected with *M. bovis* or *M. tuberculosis*.

Both of *M. bovis* and *M. tuberculosis* belong to the "*M. tuberculosis* complex" (MTBC). The list of its members consists of *M. bovis*, *M. tuberculosis, M. africanum* and *M. microti*. In addition, *M. canetti* (Brosch et al., 2002), *M. pinnipedii* (Counsins et al., 2003) and *M. caprae* (Aranaz et al., 1999) now recognized as separated species also belong to this complex.

Incidence of *M. tuberculosis* and *M. bovis* are now increasing in both domesticated animals and humans (Prasad et al., 2005). Identification and differentiation of these closely related mycobacterial species would help to determine the sources, reservoirs of infections, and disease burden due to diverse mycobacterial pathogens. In addition, identification and differentiation of the mycobacteria responsible for tuberculosis has important ramifications for infection control and the selection of antimicrobial therapy (Liebana et al., 1995; Shrestha et al., 2003). In general, identification of the mycobacterium is based on the conventional methods including the Ziehl-Neelsen acid-fast staining, a determination of bacterial morphologies (Peterson et al., 1999; Sulieman and Hamid, 2002). Biochemical tests e.g. tests for niacin, catalase, nitrate reduction, and urease are used to identify species (Valente et al., 2002; Vitale et al., 1998). The Ziehl-Neelsen staining is a simple and rapid method, but it yields low specificity and cannot be used to distinguish between *M. bovis* and *M. tuberculosis*. In addition, the bacterial culture and biochemical tests are cumbersome procedures that usually require 4 to 8 weeks to obtain the sufficient growth.

Recently, molecular approaches have been developed for the diagnosis of tuberculosis. Polymerase chain reaction (PCR) and nucleic acid hybridization greatly reduces time consumption (Sansila et al., 1998; Zink and Nerlich, 2004) and provides the sensitivity of detection in clinical specimen (Liebana et al., 1995). Several genes and insertion sequences have been targeted in attempts to develop PCR assays to identify and differentiate *Mycobacterium* spp. Many studies have also shown that PCR is a powerful technique for identification and differentiation of mycobacterial infection in cattle (Aagaard et al., 2003; Vitale et al., 1998).

High performance liquid chromatography (HPLC), which is used to identify species, is a rapid method but it needs standardized growth condition (Luquin, 1991). Probe hybridization e.g. AccuProbe (Gen-Probe®, CA) is an ideal choice for the rapid detection of MTBC, but requires a well-grown culture and testing with several probes and detects only a narrow range of mycobacterial species (Gillman et al., 2001). PCR-based method using PCR primer pairs specific to several members such as the Rv*0577*, Rv*1510*, Rv*3877/8* or Rv*3120* loci was developed and used for identification of mycobacteria (Huard et al., 2003).

For example, the *mtp40* gene, which was considered specific for *M. tuberculosis* (Herrera and Segovia, 1996), was used to distinguish between *M. tuberculosis* and *M. bovis*. However, the use of this gene for species identification has recently been invalidated because the gene is neither presents in all strains of *M. tuberculosis* nor absent in all *M. bovis* strains (Espinosa de los Monteros et al., 1998; Weil et al., 1996).

The 16SrRNA gene is the most widely accepted gene used for mycobacterial identification (Broshch et al. 2002; Frothingham 1995; Khan et al., 2005). Primers specific to the Rv1970 (*lprM*) gene encoding for lipoprotein which belongs to *M. tuberculosis*, was designed and used for differentiation of *M. tuberculosis* from the other *Mycobacterium* complex subspecies (Huard et al., 2003). Meanwhile, Rodriquez et al. (1999) reported a PCR assay, which amplifies a 500-bp fragment from the *M. bovis* genome by using the JB21-22 primer pair. The fragment is species-specific detection of *M. bovis*, and it is also well conserved and used to confirm the results obtained with the 16SrRNA primers (Kidane et al., 2002; Shah et al., 2002).

Sequence-based identification, such as with the *recA* (Blackwood et al., 2000), *rpoB* (Kim et al., 1999), *gyrA, katG, hsp65* and *gryB* (Goh et al., 2006) genes used to specify certain MTBC groupings through sequence analysis and/or digestion of PCR products followed by restriction fragment length polymorphism analysis (Huard et al., 2003). Single nucleotide changes in the *pncA* gene (Espinosa de los Monteros et al., 1998) and in the *oxyR* gene has been exploited in the differentiation between these two

species (Kurabachew et al., 2004; Sreevatsan et al., 1996). Assays targeting nucleotide difference at position 285 in the *oxyR* gene have been used successfully to differentiate between *M. bovis* and *M. tuberculosis,* regardless of their host origins (Kurabachew et al., 2004). Despite this diagnostically improvement, there are few reports on molecular techniques for the identification of different mycobacterial species in archival tissue samples.

Over the last decade, most of the molecular methods have been developed with the aim of revealing epidemiological features of tuberculosis, surveying its spread, and identifying risk factors associated with the dissemination of mycobacterial strains (Brudey et al., 2004). Although the gold standard method for studying the epidemiology of tuberculosis is *IS6110*-based restriction fragment length polymorphism analysis, it is still a cumbersome method. It may be successfully replaced by easier PCR-based methods, such as spoligotyping and variable number of tandem DNA repeats (VNTR) analysis, which have high discriminatory indices and reproducibilities when they are used in combination (Filliol et al., 2003; Smittipat et al., 2005).

The purposes of this study are i) to apply a DNA amplification system that could be used for the direct detection, identification and differentiation of *M. bovis* and *M. tuberculosis* in animal tissue samples collected from slaughterhouses ii) to provide a rapid confirmation of bovine tuberculosis by using PCR panel (with specific primers: 1*6SrRNA*, Rv*1970*, and JB fragment) and direct sequencing using *oxyR* and iii) to differentiate set of *M. bovis* and *M. tuberculosis* samples by using Variable Number Tandem Repeat (VNTR) analysis.

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CHAPTER II

REVIEW LITERATURES

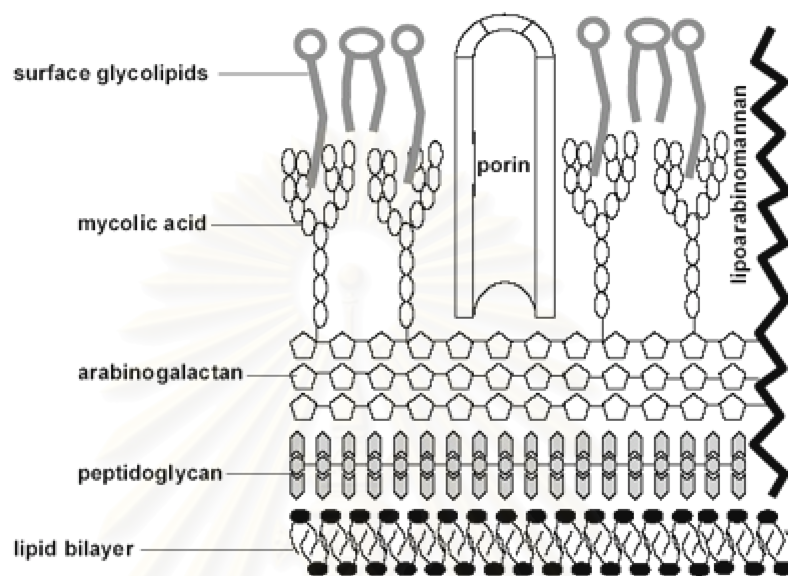
1. General characteristics and classification

The genus *Mycobacterium* is the only member of the family Mycobactericeae, which belongs to the order Actinomycetales. Members of genus *Mycobacterium* share some characteristics, including parts of their antigenic determinants with other members of the same order e.g. *Nocardia, Corynebacterium, Rhodococcus* and *Actinomyces*. Members of the genus *Mycobacterium* are gram-positive, non-motile, non-endospore forming and catalase positive. They are slightly curved or straight rod-shaped organisms of 0.2-0.6 x 1-10 μm in size and occasionally filamentous morphology.

The cell wall of mycobacteria (Figure 1) contains a high amount of long chain peptidoglycolipid (up to 24% of 60-90 carbon lipids) connected to arabinogalactan that is covalently linked to mycolic acids. The mycolyl-arabinogalactan-peptidoglycan complex strongly bind to carbol-fuchsin and can not be removed during decolorization with 3% acetic-alcohol solution, and some also resists decolorization with 95% ethanol. These characteristics, referred to as acid fastness and acid-alcohol fastness, respectively, are used to distinguish mycobacteria from other genera and species (Valente et al., 2002). Based on their cell wall structure, mycobacteria are very tolerant to both chemical and physical influences. Mycobacteria can survive in refrigerator condition (4-10°C), in general, more than 6 months without any loss of the ability to multiply. Heat inactivation of mycobacteria can be done at 70 and 65°C for 5 and 15 minutes, respectively (Valente et al., 1997).

The optimal-growing temperatures vary from 25°C to over 50°C under aerobic to microaerophilic condition (5-10% CO_2). Mycobacteria are strictly aerobic and growth is slow or very slow, therefore colonies can be visible in 2-60 days. Colonies are often pink, orange or yellow, especially when exposed to light, pigment is not diffusing, and

surfaces are commonly dull or rough. The most rapidly growing species generally grow on simple media in 2 to 3 days and most Mycobacteria associated with disease require 2-6 weeks of incubation on complex media.



(Source: modified from Murray et al., 1999)

Figure 1. Schematic representation of the mycobacterial cell wall structure.

Based on etiological data, mycobacteria can be characterized into four groups.

1. The *M. tuberculosis* complex that consists of *M. bovis*, *M. tuberculosis, M. africanum* and *M. microti*. In addition *M. canetti* (Brosch et al., 2002), *M. pinnipedii* (Counsins et al., 2003) and *M. caprae* (Aranaz et al., 1999) are now recognized as separated species belonging to this complex.

2. The *M. avium-intracellulare* complex that was first determined as pathogenic in birds.

3. Mycobacteria that are not members of *M. tuberculosis* complex or *M. avium-intracellulare* complex. They may be isolated from animals and environmental sources.

4. *M. avium* spp. *paratuberculosis* that is the causative agent of paratuberculosis or Johne's disease in cattle.

2. Occurrence and epidemiology of bovine tuberculosis

As being one of the most important zoonotic diseases, bovine tuberculosis caused by *M. bovis* is well known all over the world (Romero et al., 1999). In developing countries, tuberculosis in animals usually occurs as severe outbreaks in herds. All species, including humans, are susceptible to *M. bovis*. Although *M. bovis* can survive for several months in the environment, transmission is mainly through aerosols generated by infected cattle. Dairy cattle are particularly at risk because husbandry methods allow close contact between animals during milking. Calves can become infected by ingesting contaminated milk and ingestion is the probable route of transmission to humans. The higher prevalence in dairy than beef cattle may reflect closer confinement, longer life spans, and greater productivity stress among dairy cows. Exemption from pregnancy and lactation may explain the lower disease prevalence in bulls than cows. The incidence of pulmonary tuberculosis in humans caused by mycobacterium is higher in farm and slaughterhouse workers than in urban inhabitants (Pullock and Neill, 2002).

Although cattle are considered to be the true hosts of *M. bovis*, the disease has been reported in many domesticated and non-domesticated animals. Isolations can be made from ruminants (e.g. buffaloes, bison, sheep, goats), wild animals (e.g. otters, deer, antelopes, foxes, raccoons, hares, tapirs, elephants), domestic animals (e.g. equines, pigs, dogs, cats), primates and several predatory felines including lions, tigers, leopards and lynx (de Lisle et al., 2001)

3. Pathogenesis and pathogenicity

Tubercle bacilli are transmitted via the respiratory and alimentary routes through contaminated airborne droplet nuclei, feces, urine, genital discharges, milk from infected mammary glands, or contaminated feed and water. Aerosol exposure of cattle to *M. bovis* and *M. tuberculosis* is considered the most frequent route of infection. Intrauterine infection of claves occurred when bovine tuberculosis was common. Cattle

often develop primary foci in lymph tissues associated with the intestinal tract. As gross lesions usually involve lungs and thoracic lymph nodes, however, *M. tuberculosis* does not produce progressive pulmonary diseases in cattle (Murray et al., 1999).

The virulence of mycobacterium relates to its ability to survive and multiply in host macrophages (Pollock and Neill, 2002). The macrophage accumulation at the primary site of infection is initially a response to the foreign body effect of waxes and lipids in the mycobacterial cell wall. Survival within the cytoplasm of macrophages is promoted by interference with phagosome-lysosome fusion and failure of lysosomal digestion. Bacilli released from dead macrophages are engulfed by surrounding viable phagocytes. Migration of macrophages containing viable mycobacteria can disseminate infection. The gradual accumulation of macrophages in the lesion and the formation of granulomatous response lead to the development of a tubercle, the typical host response in the delayed-type hypersensitivity to mycobacterial infections (Neill et al., 1994; Pollock et al., 2006).

4. Clinical signs and pathology

Clinical signs of mycobacterial infection are low fever and coughing with mucous secretion that could be detected months or years after infection in most host species. In human, the disease is insidious in onset. Patients typically have nonspecific complaints of malaise, weight loss, cough and night sweats. Sputum may be scant or bloody with purulent. In generalized form, various clinical signs may be observed depending on the affected organs, for example, weight loss for intestinal tuberculosis or arrhythmia for pericardial tuberculosis (Scorpio et al., 1997).

In cattle, clinical signs are evident only in advanced disease, however cattle with extensive lesions may appear to be in good health. In advanced pulmonary tuberculosis, animals may develop a cough and intermittent pyrexia. Lymph nodes are often greatly enlarged and obstruct air passages, the alimentary tract, or blood vessels. Extreme emaciation and acute respiratory distress may occur during the last stages.

Involvement of mammary tissue may result in tuberculous mastitis, often accompany by supramammary lymph node enlargement (Thoen, et al., 2006). In the early stages of the disease, lesions may be difficult to detect at postmortem examination. The small lesions are composed of aggregates of epitheloid cells. Multinucleate Langhans' giant cells, formed from the fusion of macrophages, may also be present. The typical pathoanatomic lesions caused by *Mycobacterium* are caseous masses with or without calcification in lung, lung lymph nodes and tissues in the thoracic cavity. In generalized cases, lesions in liver, spleen, kidneys, brain, bone marrow and mammary glands could be observed (Thoen et al., 2006). The presence of the disease is usually signaled by detection in carcasses at slaughterhouse. A presumptive diagnosis of tuberculosis in humans and cattle is often made on history, clinical findings, necropsy findings, results of tuberculin skin tests and/or interferon-gamma assay.

5. Diagnostic techniques

5.1 Microscopic examination

The classical diagnostic technique used to detect mycobacterial cells is a histopathological examination and microscopic detection by Ziehl-Neelsen stain or acid fast bacilli (AFB) (Mahaisavariya, et al., 2005). Due to high content of mycolic acid in the cell wall, the positive result of Ziehl-Neelsen stain shows bright red bacilli in size of 0.2-0.6 x 1.0-10 um (Haddad et al., 2004), but mycobacteria are not the only microorganism that is acid-fast in a smear. Some species of Nocardia, Corynebacteria, fungi and tissue debris can also be Ziehl-Neelsen positive (Sulieman and Hamid 2002). Another method for the detection of mycobacterial cells in tissues is the use of a fluorescent microscope to observe specimen stained with fluorochrome e.g. auramine O (Tenover et al., 1993).

Disadvantage of microscopic examination is the presence of mycobacteria that must be approximately more than 10^4 mycobacterial cells. Ziehl-Neelsen stain can not distinguish viable and dead bacteria, and do not permit the species identification (Peterson et al., 1999).

The most efficient microscopic examination to identify lesions of mycobacterial infections is the examination of sections stained by hematoxylin and eosin (H&E). Typical lesions in cattle are described as having a center of caseous necrosis, usually with some calcification, a boundary of epithelioid cells and some of which form multinucleated giant cells (Pollock et al., 2006). An outer border of fibrous connective tissue is usually present, giving the lesion of a focal appearance and providing encapsulation.

5.2 Delayed hypersensitivity test – the tuberculin test

The current method for detection of bovine tuberculosis in living cattle is the tuberculin test, which involves the intradermal injection of bovine tuberculin purified protein derivative (PPD) and the subsequent detection of swelling (delayed hypersensitivity) at the site of injection 3 days later. In the past, heat-concentrated synthetic medium (HCSM) tuberculin was used, but in most countries, HCSM tuberculin is replaced by PPD tuberculin. The HCSM tuberculins can yield a good potency if they are correctly standardized for biological activity. However, their specificity is inferior to PPD tuberculins (Gonzalez Llamazares et al., 1999).

The tuberculin test can be performed on the mid-neck, but in Thailand the test usually performed in the caudal fold of the tail. It is not recommended to use this test when the epidemiological data suggests that the herd or animals be suspected to contact with infected animals. This is because it may result in false-negative responses and the decreased-sensitivity of the test (Gormley et al., 2004). False negative responses may occur in the early stage of the disease and in severely infected animals. Therefore, complete eradication is difficult if only a single tuberculin test is used. Because of the low diagnostic accuracy, displaying both false-positive and false-

negative results, the immune status of cattle has subjected to repeated testing (Gonzalez Llamazares et al., 1999; Mostrom et al., 2002; Ryan et al., 2000).

5.3 Gamma-interferon assay

Besides the classical intradermal tuberculin test, a number of new diagnostic blood tests have become available (Gonzalez Llamazares et al., 1999). Due to the cost and the more complex nature. They are usually used as ancillary tests to confirm or negate the results of an intradermal skin test. The lymphocyte proliferation assay and the gamma-interferon assay correspond to cellular immunity, while the enzyme-linked immunosorbent assay (ELISA) corresponds to humoral immunity.

In this test, the release of a lymphokine (interferon gamma) in a whole-blood culture system is measured. The assay is based on the release of gamma-interferon from sensitized lymphocytes during a 16-24 hour incubation period with specific antigen (PPD-tuberculin). The quantitative detection of bovine gamma-interferon is carried out with a sandwich ELISA that uses two monoclonal antibodies. The test yields higher sensitivity in comparison to the skin test, but it gives less specificity (Buddle et al., 2001).

5.4 Molecular biological identification

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In recent years, molecular biological techniques have been developed and widely used as routine identification procedures because of a more rapid turnover and an improved specificity of identification (Broccolo et al., 1993). Many of molecular biological methods are based on distinct patterns or sizes of DNA fragments. Various primers are used, including primers that targets 16S-23SrRNA, the insertion sequences IS6110 and IS1081, and genes coding for MTBC-specific protein, such as MPB70 and the 38 kDa antigen (Araujo, et al., 2005).

All the members of MTBC have high genetic relatedness and completely conserved DNA sequences in several gene regions such as 16SrRNA and the 16S-to-23SrRNA internal transcribed spacer (Broshch et al. 2002; Frothingham, 1995; Khan et al., 2005). Despite this close relationship, they show a large variability in their phenotypic properties, epidemiology, and incidence of tuberculosis (Amadio et al., 2005).

The 16SrRNA sequence is considered as the molecular gold standard for mycobacterial species identification due to its high degree of conservation (Broshch et al., 2002; Khan et al., 2005; Sansila et al., 1998). Moreover, the hypervariable region of 16SrRNA serves as target for detection of variation in the same species. Hughes et al. (1993) and Han et al. (2002) described the method for the identification of genus, and species of mycobacteria simultaneously using conserved and variable sequences of the 16SrRNA.

PCR-based method using PCR primer pairs specific to the loci Rv*0577*, Rv*1510*, Rv*3877/8* or Rv*3120* was developed and used for the identification of mycobacteria (Huard et al., 2003). Design of the Rv1970 primers are based on Rv*1970* fragment (*IprM* gene) to identify *M. tuberculosis* from the other *Mycobacterium* complex subspecies (Huard et al., 2003; Romero et al., 1999). Meanwhile, Rodriguez et al. (1995) reported a PCR assay that amplified a 500-bp fragment from the *M. bovis* genome by using the JB21-22 primers. Detection of the JB fragment is species-specific for *M. bovis*, and it is also well conserved and used to confirm the results obtained by the 16SrRNA primer (Kidane et al., 2002; Shah et al., 2002).

Partial sequences of many other genes were analyzed e.g. *recA* (Blackwood et al., 2000), *rpoB* (Kim et al., 1999), *mtp40*, *katG*, *gyrA, hsp65* and *gryB* (Goh et al., 2006; Zink and Nerlich, 2004). It was show that *pncA* and *oxyR* testing for the presence or absence of variable gene regions are useful for the identification of MTBC. The *mtp40* sequence was shown to be specific for *M. tuberculosis* but absent in *M. bovis* (Huard et al., 2003). The sequence polymorphisms of the *katG* and *gryA* gene are not sufficiently reliable to distinguish both species since there are some strains of *M. tuberculosis* containing the cited change (Espinosa de los Monteros et al., 1998). Single point

mutations of the *pncA* and *oxyR* locus have been used to differentiate between *M. bovis* and *M. tuberculosis* isolates.

One single nucleotide changes in the *oxyR* gene, functioning as a sensor and transcriptional regulator of proteins involved in the oxidative stress response, are exploited. (Sreevatsan et al., 1997; Kurabachew et al., 2004). Assays targeting nucleotide difference at position 285 in the *oxyR* gene have been used successfully to identify between *M. bovis* and *M. tuberculosis,* regardless of their host origin (Kurabachew et al., 2004).

5.5 Molecular biological differentiation

During the last decade, many differentiation techniques have been developed for subtyping mycobacterial isolates. High-performance liquid chromatography (HPLC), analyzing mycobacterial mycolic acid patterns, was described as a fast and efficient method (Butler et al., 1992). However, the HPLC equipment is costly and needs substantial numbers of samples to maintain the system. Genetic fingerprinting allows laboratories to distinguish between different strains of mycobacteria and reveals patterns of origin and transmission. The most widely used method is spoligotyping (from spacer and oligotyping) that allows the differentiation of strains inside each species belonging to the MTBC (Zink and Nerlich, 2004). Other new techniques are currently under development in order to differentiate the strains that have the same spoligotype. The insertion sequence *IS6110* is considered as specific to the members of the MTBC, and the differences in the location and number of copies of the insertion sequences indicate polymorphisms between isolates.

Nowadays, a number of molecular biological techniques have been applied to study the epidemiology of mycobacterial strains. Restriction fragment length polymorphism (RFLP) using insertion element IS6110 as a probe are the most widely used method for differentiating strains of *M. tuberculosis* isolates (Haddad et al., 2004). In RFLP techniques, bacterial DNA will be digested by restriction enzymes, which cleave DNA molecules at 4-6 specific base pair recognition sites depending on the

particular enzyme used. After digestion, DNA fragments will be separated by electrophoresis, and transferred from agarose gel to special membrane by blotting. The membrane bound DNA fragments can be hybridized and later detected either by enzyme-colorimetric or chemilunescent substrate. Genetic relationship among the samples can be analyzed by computer program according to the similarity of band patterns. RFLP typing is restricted to mycobacterial culture. Twenty to forty days incubation is required before sufficient mycobacterial cells are available to obtain adequate DNA needed. This time restriction limits the usefulness of RFLP typing (Sansila et al., 1998).

Pulsed-field gel eletrophoresis (PFGE) has been used as the gold standard for determining genetic relationship among the mycobacterium strains (Hughes et al., 1993). It allows separation of DNA chains ranging in size from <20 kilobases up to 10 megabases. In comparison to PFGE, conventional electrophoresis can only be used for DNA separation in size up to 50 kilobases. PFGE is a fastidious technique that requires an actively growing culture and several days for completion. Standardization may be not achieved by cell clumping and difficulties in controlling cell lysis resulting in different DNA yields from strain to strain. Moreover, some strains or species are untypeable by PFGE because of DNA degradation (Haddad et al., 2004).

Although strains of the *M. tuberculosis* complex vary in host range, virulence and other phenotypes, they have highly conserved DNA sequences (Frothingham and Meeker-O'Connell, 1998). A repetitive-sequence-based PCR (rep-PCR) system was recently adapted for use on mycobacteria. The method takes advantage of repetitive element interspreadsed throughout bacterial genomes. These elements generate highly discriminative genomic fingerprints when amplified by PCR (Gutierrez et al., 1995).

Differentiation of repetitive DNA, such as the variable number tandem repeats (VNTR) were first described in eucaryotic genomes a few years ago. VNTR typing is based upon repeat number polymorphism within these tandemly arranged repetitive DNA sequences. Many of these tandem repeat loci display hypervariability, enabling their exploitation for strain typing in numerous bacterial species (Skuce et al., 2002). The

novel VNTR markers described the discrimination possible in strain typing of *M. bovis* and *M. tuberculosis* with the allele copy number of the individual VNTRs providing a profile. VNTR typing was shown to be a valuable technique with great potential for further development and application to epidemiological tracing of tuberculosis transmissions (Roring et al., 2002; Sola et al., 2003). Mycobacterial isolates can be differentiated due to variations in a tandem repeat copy number at several loci. This is a convenient, highly discriminatory, highly reproducible and high-throughput strain typing technique for *M. bovis* and *M. tuberculosis* (Roring et al., 2004; Skuce et al., 2002).

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CHAPTER III

MATERIALS AND METHODS

In this study, the experiment is divided into 3 phases, including Phase I Collection and preparation of samples, Phase II Identification of *Mycobacterium tuberculosis* Complex and Phase III Differentiation of *M. bovis* and *M. tuberculosis*. The conceptual framework is shown in Figure 2.

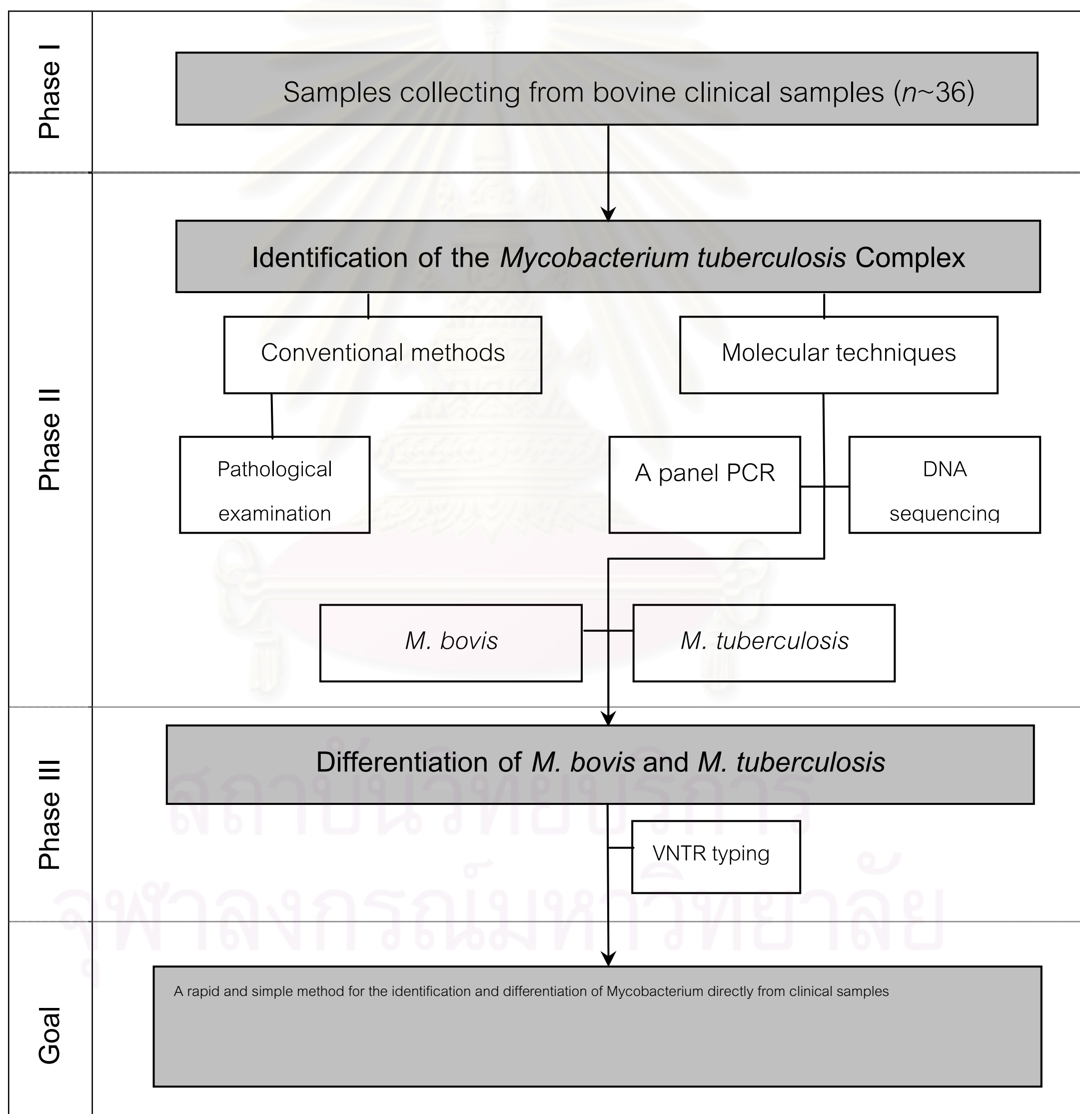

Figure 2. The conceptual framework in this study.

Phase I Collection and preparation of samples

A total of 36 clinical samples were obtained from 24 cattle, which were slaughtered after showing positive reactions to the single intradermal comparative tuberculin test or gamma interferon test. The samples, organ with or without tuberculous-like lesions in lymph nodes, lungs or liver, were collected from the slaughterhouses located in Nakhon Pathom province during 2004-2005. The history clinical records of these cattle were reviewed. These samples were placed in sterile containers and kept at 4°C during transportation. Then, they were stored at -80°C until used.

Phase II Identification of *Mycobacterium tuberculosis* Complex

1. Conventional approach

All 36 specimens were excised at the affected areas in two pieces (1.5x2cm) for histopathological examination and acid-fast bacilli (AFB) staining. They were fixed in 10% formalin and embedded in paraffin wax as standard procedure. The histological sections were 0.6 μm thickness.

The classical histopathological lesions include a caseous necrosis, epithelioid cells, multinucleated Langhans' giant cells and macrophages. The presence of well-formed granuloma, poorly formed granuloma, caseous necrosis and Langhans' giant cells was separately assessed and recorded. We identified cases having well-formed granuloma or poorly formed granuloma as "chronic granulomatous inflammation", regardless of the presence of caseous necrosis and Langhans-type giant cells. We identified other cases showing only chronic inflammation without the features of granulomatous inflammation as "chronic inflammation".

The sections were stained and microscopically examined by 2 independent observers. In cases with discordant findings, the two observers reexamined the sections for the final decision.

The result of AFB was reported in numbers based on the criteria of Miller et al. (2002) at a magnification of 400X: no AFB seen = negative; one to three per slide = 1+ (rare); one to nine per 10 fields = 2+ (few); one to nine per field = 3+ (moderate); and more than nine per field = 4+ (many).

2. Molecular approaches

2.1 Preparation of DNA from tissues

In pilot study, four mycobacterium negative tissue samples spiked with *M. tuberculosis* H37Rv were processed with two DNA extraction methods to optimize the method for preparation of mycobacterial DNA from bovine tissue samples. In addition, part of each sample was spiked with positive mycobacterium DNA to detect inhibitors in the sample as a susceptible control of the extractions.

The methods for preparation of DNA were performed in a class II laminar flow exhausting hood (Astec® SC1800; Astec Environmental Systems, England). Two DNA extraction procedures were preformed in this study.

2.2 Extraction of DNA

2.2.1 Method A: NucleoSpin® silica column purification

DNA was extracted by using the NucleoSpin® Tissue kit (Macherey-Nagel Inc., Germany), according to the manufacturer 's instructions. In this method, DNA absorbs to silica in the presence of a chaotrope, was washed with buffer, and finally eluted from the column in 50 µl of 10 mM Tris with 0.1 mM EDTA buffer at pH 8.5.

2.2.2 Method B: Proteinase K and phenol-chloroform method

Ten microlitres of proteinase K solution (1mg of proteinase K per ml, 5% Triton X-100, and 200 mM Tris-HCL [pH 8.3}; 10X) were added to the tissue sections and the mixture was incubated at 60°C overnight. The enzyme was inactivated by boiling for 15 min. The protein was extracted by adding 1 of total volume of phenol-chloroform-iso amyl alcohol (25:24:1) into the mixture and mixed extensively on the vortex mixer. The tube was centrifuged at 12,000 rpm for 15 sec. The aqueous phase was transferred to a

new microcentrifuge tube, followed by adding 0.1 of total volume of 3M sodium acetate and 2 of total volume of cold absolute ethanol. The solution was mixed and DNA was precipitated at –20°C overnight. DNA was centrifuged at 12,000 rpm for 10 min and supernatant was discarded. The final DNA was dissolved in TE buffer and stored at –20°C.

2.3 Identification of mycobacteria by using PCR assay

2.3.1 DNA amplification

The target gene loci and their primer names, primer sequences, amplification product sizes and the programs used to amplify are listed in Table 1. Each PCR mixture was prepared with 10 µl of MasterMix (Eppendorf®, Germany), 11 µl of water, 1.5 µl of each primer at 10 µM and 1 µl of DNA (equaling 100 – 200 ng). The sole difference was with the Rv1970 primer pair, which required 1 µl of Mg^{2+} for good amplification.

Table 1. Sequence of the primers and size of the amplification products

Target locus	Primer name	Nucleotide sequence	Size(bp)	Program	References
16SrRNA	16SrRNAF	5'-ACG GTG GGT ACT AGG TGT GGG TTT C-3'	543	1	(Hughes et al., 1993; Khan et al., 2005)
	16SrRNAR	5'-TCT GCG ATT ACT AGC GAC TCC GAC TT-3'			
Rv*1970 (lprM gene)*	Rv1970F	5'-GCG CAG CTG CCG GAT GTC AAC-3'	1116	2	(Huard et al., 2003; Romero et al., 1999)
	Rv1970R	5'-CGC CAG CTG CCG GAT GTC AAC-3'			
JB fragment	JB21	5'-TCG TCCGCT GAT GCA AGT GC-3'	500	3	(Rodriguez et al., 1995; Shah et al., 2002)
	JB22	5'-CGT CCG CTG ACC TCA AGA AAG-3'			
oxyR gene	oxyRF	5'-GGT GAT ATA TCA CAC ACC ATA-3'	548	3	(Sreevatson et al., 1996; Huard et al., 2003)
	oxyRR	5'-CTA TGC GAT CAG GCG TAC TTG-3'			

The PCR reactions were performed in 25 µl mixture on a PCR Sprint Thermocycler® (Thermo Electron Corporation®, Cambridge, UK), using either program 1 (with an initial denaturation step of 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 60°C and 1.30 min at 72°C and ending with a final elongation step for 10 min at 72°C while program 2 (program 1 but with an annealing temperture of 55°C) or program

3 (program 1 but with an annealing temperture of 53°C). When DNA samples were limited or of low concentration, the number of PCR cycles was increased to 40. All PCR products were identified by gel electrophoresis in a 2% agarose gel and were visualized by ethidium bromide staining under ultraviolet light. The positive result of PCR showed a single band of fragment compared with the 100-bp DNA ladder (GeneRuler®, Fermentas). *M. tuberculosis* H37Rv (KK11-20) and *M. bovis* (ATCC 19210) were included as positive and negative controls in every batches of PCR reaction. Furthermore, all negative and unexpected positive PCR results were repeated and confirm at least once again.

2.3.2 Sequencing of the *oxyR* locus

A 548-bp segment of oxyR was amplified with the oligonucleotide primers as previously described in Table 1. A PCR Sprint Thermocycler® (Thermo electron corporation®, Cambridge, UK) was used with the following parameters: denaturation temperature of 94°C for 1 min, annealing temperature at 53°C for 1 min and extension step at 72°C for 1 min 30 sec. Each reaction was preceded by an initial denaturation step at 94°C for 5 min and was terminated with a final extension step at 72°C for 10 min.

PCR products were purified using QIAquick® PCR Purification Kit (Eppendorf®, Hamburg, Germany). The purified PCR fragments were further sequenced by the Macrogen (Soul, South Korea), using a 3730xl DNA analyzer. The PCR amplification primers were also used as sequencing primers, and a minimal single overlap from two directions for each was usually achieved. The *oxyR* gene sequencing outputs were assembled and edited by using computer software (Chromas version 1.45 and Bioedit version 7.0.0). DNA sequences were aligned to identify polymorphism at nucleotide position 285. The *M. bovis* poses nucleotide at position 285 as adenine (A), whereas *M. tuberculosis* has a guanine (G) (Espinosa de los Monteros et al., 1998; Sreevatsan et al., 1996). All sequence data were submitted to GenBank and compared with a published sequence for the *oxyR* region.

Phase III Differentiation of *M. bovis* and *M. tuberculosis*

Differentiation of Mycobacteria by analysis of VNTR loci

The Variable Number Tandem Repeat (VNTR) analysis was used as a molecular typing assay for the differentiation of *M. bovis* and *M. tuberculosis* isolates. The VNTR technique was carried out as described by Frothingham and Meeker-O'Connell (1998). In this study, VNTR was amplified of each of 5 loci (ETR-A to ETR-E) containing exact tandem repeats (ETRs). These loci vary in the length of internal repeat units, giving alleles that vary in size. Strains can then be called on the basis on the number of repeats at each allele, e.g., 7-5-5-4-3 would have 7 copies of allele A, 5 of allele B and C, etc. Primers used were as described previously by Roring et al. (2002) for the *M. tuberculosis* H37Rv and *M. bovis* ATCC 19210 exact tandem repeat A to E. Primer product sizes were predicted for each locus (Table 2).

PCRs were set up using the Eppendorf® MasterMix with the following mix: 11 µl of MasterMix, 2 µl of each primer (at 10 µmol/ml), 9 µl of water and 1 µl of the DNA. The thermal cycle used for amplification was an initial denaturation at 95°C for 12 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min and extension at 72°C for 2 min; final extension at 72°C for 10 min was followed by a holding step at 4°C. Positive and negative control reactions were performed with each set of reactions.

The presence and size of each PCR product were electrophoretically separated on 2% agarose gels in 1x Tris-EDTA buffer, followed by staining with ethidium bromide. The copy number of the amplified products was inferred from the difference between the molecular weights of the amplified products of the samples and of the positive strain. A 100-bp DNA ladder (GeneRuler®, Fermentas) was used as size markers. Allele naming tables for each tandem repeated locus were calculated, and allelic variants were named using the copy number of the repeat unit present. If there were doubts regarding the number of copies of VNTR, the amplified DNA was sequenced for validation (Roring et al., 2004).

Table 2. VNTR-PCR primer sequences used in this study (Roring et al., 2002)

Locus	Forward sequence	Reverse sequence	Repeat x copy no.*	
			M. tuberculosis H37Rv	*M. bovis* (ATCC 19210)
ETR-A	5'-AAA TCG GTC CCA TCA CCT TCT TAT-3'	5'-CGA AGC CTG GGG TGC CCG CGA T-3'	(75 x 3) + 23	(75 x 8) + 23
ETR-B	5'-GCG AAC ACCAGG ACA GCA TCA TG-3'	5'-GGC ATG CCG GTG ATC GAG TGG-3'	(57 x 3) + 8	(57 x 5) + 8
ETR-C	5'-GTG AGT CGC TGC AGA ACC TGC AG-3'	5'-GGC GTC TTG ACC TCC ACG AGT G-3'	(58 x 3) +37	(58 x 4) +37
ETR-D	5'-CAG GTC ACA ACG AGA GGA AGA GC-3'	5'-GCG GAT CGG CCA GCG ACT CCT C-3'	(77 x 3) + 7	(77 x 3) + 41
ETR-E	5'-GCG GAT CGG CCA GCG ACT CCT C-3'	5'-CGG AAC GCT GGT CAC CAC CTA AG-3'	(53 x 2) + 49	(53 x 3)

*Each ETR locus had several complete repeats and one partial repeat in the type strain. For example, the ETR-A locus contained three complete 75-bp repeats followed by an additional 23 bp of repetitive sequence.

Instruments and chemical substances

1. DNA preparation

   1.1 Lysozyme (Amersco®, Ohio, USA)

   1.2 Proteinase K (Amersco®, Ohio, USA)

   1.3 Phenol (Molecular grade)

   1.4 Iso amyl alcohol (Molecular grade)

   1.5 Choroform (Molecular grade)

   1.6 Tris-EDTA (Merck®, New Jersey, USA)

   1.7 NucleoSpin® Tissue kit (Macherey-Nagel®, Düren, Germany)

2. PCR assay

   2.1 Master Mix (Eppendorf®, Hamburg, Germany)

   2.2 DNA marker (Gibco®, Paisley, UK)

   2.3 Loading dye (Amersco®, Ohio, USA)

   2.4 Agarose gel (Molecular grade)

   2.5 Gel electrophoresis buffer

2.6 Ethidium Bromide

2.7 Perfectprep Gel Cleanup kit (Eppendorf®, Hamburg, Germany)

3. PCR cabinet
4. Thermocycler (Thermo electron corporation®, Cambridge, UK)
5. Gel electrophoresis set
6. Gel document system (Vilber Lourmat®, Marne La Valle, France)
7. PCR tube and Microcentrifuge tube 1 .5 ml
8. Centrifuge and Microcentrifuge
9. Micropipete and Micropipete tip
10. The -20°C Refrigerator
11. Experimental glassware

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CHAPTER IV

RESULTS

1. Collection and preparation of samples

Thirty-six clinical samples were obtained from twenty-four cattle, which had been slaughtered after showing positive reactions to the single intradermal tuberculin test or gamma interferon test. More than one sample was collected from six animals. The samples, organs with or without tuberculous-like lesions in lymph nodes, lungs or liver, were collected at the slaughterhouses located in Nakhon Pathom province during 2004 – 2005 (Figures 3 to 5). The total of thirty-six samples consisted of thirty-one lymph nodes, three lungs and two livers. In this study, we found that twenty-four samples showed tuberculous-like lesions. The predominant lesions were the multifocal caseous masses and the tubercles (granulomas) in size of 0.5 to 5 cm. None of them showed any signs of calcification. Various degrees of hyperemia to hemorrhages were also found in some samples (Table 3).

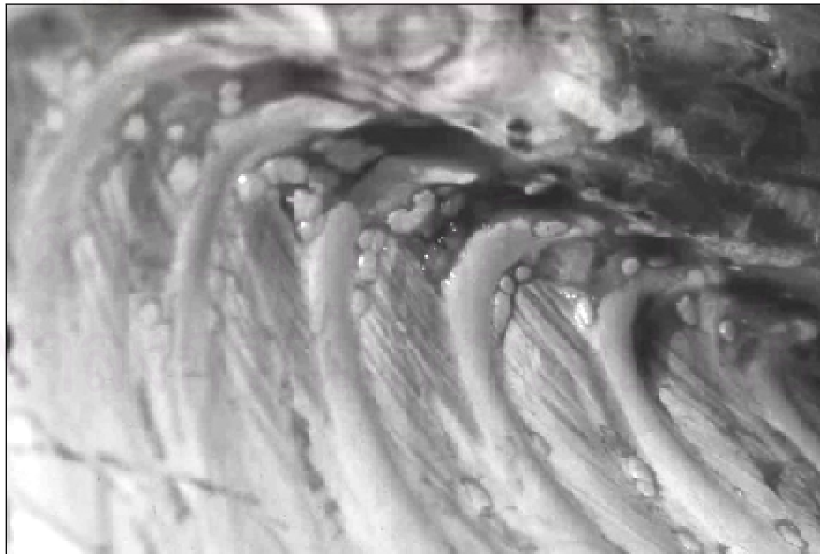

Figure 3. Advanced bovine tuberculosis with granulomatous lesions on the interior of rib cage in the thoracic cavity of *Mycobacterium* infected cow in this study.

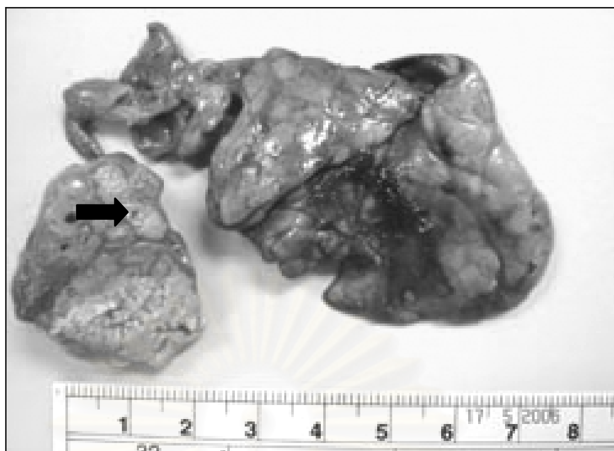

Figure 4. Gross features of granulomatous pneumonia. A caseated tubercle (arrow) in lung tissue and larger areas of caseous necrosis separated by fibrous connective tissue. Lung (No.D1), cut surface; cow.

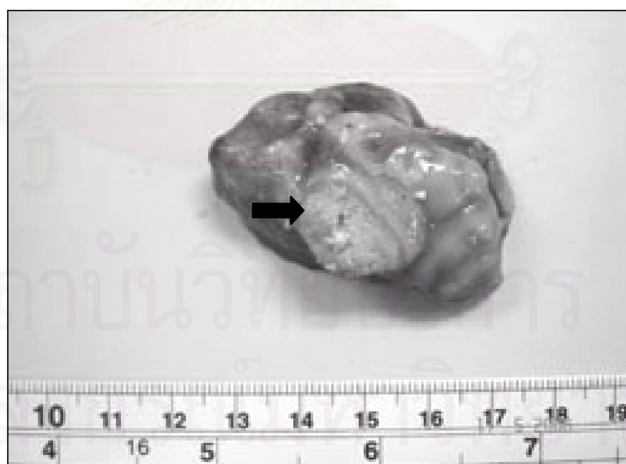

Figure 5. Gross features of granulomatous lymphadenitis. A clearly separated granulomatous tubercle (arrow) in lymph node. Mesenteric lymph node (No. G1), cut surface; cow.

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Table 3. List of 36 samples collected from 24 tuberculin positive bovines.

Isolated ID	Animals	Organs	Years	Tuberculin test	Gamma IFN test	Tuberculous-like lesion
A1	Cow1/1	Mediastinum lymph node	2004	+	N/A	Yes
A2	Cow1/2	Mesenteric lymph node	2004	+	N/A	Yes
A3	Cow1/3	Supramammary lymph node	2004	+	N/A	Yes
A4	Cow1/4	Trachiobrocheal lymph node	2004	+	N/A	Yes
B1	Cow2/1	Mammary lymph node	2005	+	N/A	No
B2	Cow2/2	Mediastinum lymph node	2005	+	N/A	Yes
B3	Cow2/3	Mesenteric lymph node	2005	+	N/A	No
B4	Cow2/4	TB lymph node	2005	+	N/A	Yes
C1	Cow3/1	Lung	2005	+	+	Yes
C2	Cow3/2	Mediastinum lymph node	2005	+	+	Yes
C3	Cow3/3	TB lymph node	2005	+	+	Yes
D1	Cow4/1	Lung	2004	+	+	Yes
D2	Cow4/2	Prefermoral lymph node	2004	+	+	No
D3	Cow4/3	Subscapular lymph node	2004	+	+	No
E1	Cow5/1	Mammary lymph node	2004	+	+	No
E2	Cow5/2	Mammary lymph node	2004	+	+	No
F1	Cow6/1	Mediastinum lymph node	2005	+	N/A	Yes
F2	Cow6/2	Supramammary lymph node	2005	+	N/A	No
G1	Cow7	Mesenteric lymph node	2005	+	N/A	Yes
H1	Cow8	Mediastinum lymph node	2005	+	N/A	Yes
I1	Cow9	TB lymph node	2005	+	N/A	Yes
J1	Cow10	TB lymph node	2004	+	N/A	Yes
K1	Cow11	Mammary lymph node	2005	+	N/A	No
L1	Cow12	Mammary lymph node	2004	+	+	Yes
M1	Cow13	TB lymph node	2004	+	+	Yes
N1	Cow14	TB lymph node	2004	+	+	Yes
O1	Cow15	TB lymph node	2004	+	+	Yes
P1	Cow16	TB lymph node	2004	+	+	Yes
Q1	Cow17	Mediastinum lymph node	2005	+	N/A	No
R1	Cow18	Trachiobrocheal lymph node	2005	+	N/A	Yes
S1	Cow19	Trachiobrocheal lymph node	2005	+	N/A	Yes
T1	Cow20	Liver	2005	+	N/A	No
U1	Cow21	Mediastinum lymph node	2005	+	N/A	Yes
V1	Cow22	TB lymph node	2005	+	N/A	Yes
W1	Cow23	Lung	2005	+	N/A	No
X1	Cow24	Liver	2005	+	N/A	No

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2. Identification of *Mycobacterium* by conventional methods

2.1 Histopathological examination

The histopathologic lesion most commonly associated with bovine tuberculosis is a granuloma, a collection of inflammatory cells within the suspect tissue. The accumulation of the living and dead macrophages or neutrophils, bacteria and tissue cells in a focal area comprises the tubercle. In chronic cases, a thick capsule may form around the tubercle, surrounding it off from other tissues and forming the granuloma (Figure 6).

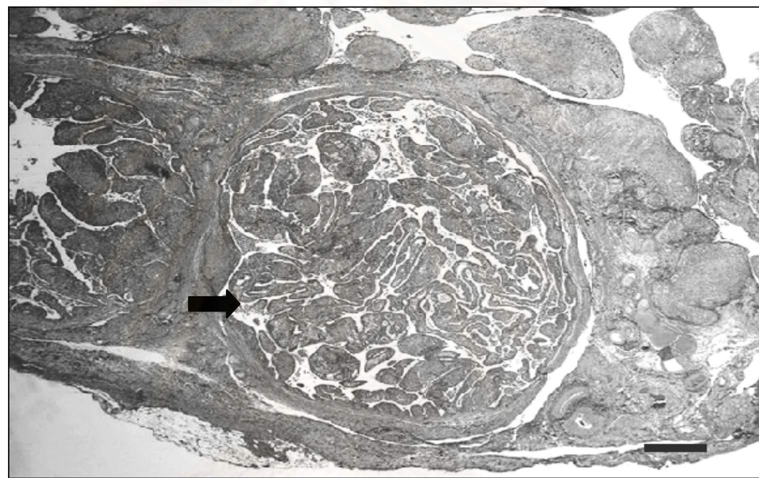


Figure 6. Typical histopathologic features of chronic tuberculous lymphadenitis from 10-year-old cow in this study. A central area of caseous necrosis (arrow) is shown with a boundary of unusually thick fibrous connective tissue encapsulation. The appearance of fibrinogen indicates a lesion of chronic disease. (Bar = 200 μm)

The classical histopathological lesions include a caseous necrosis, epithelioid cells, multinucleated Langhans' giant cells and macrophages. We categorized cases having well-formed granuloma or poorly formed granuloma as "typical granulomatous inflammation", regardless of the presence of caseous necrosis and Langhans-type giant cells (Figure 7). We categorized other cases showing only inflammation without the features of granulomatous inflammation as "atypical granulomatous inflammation" (Figure 8).

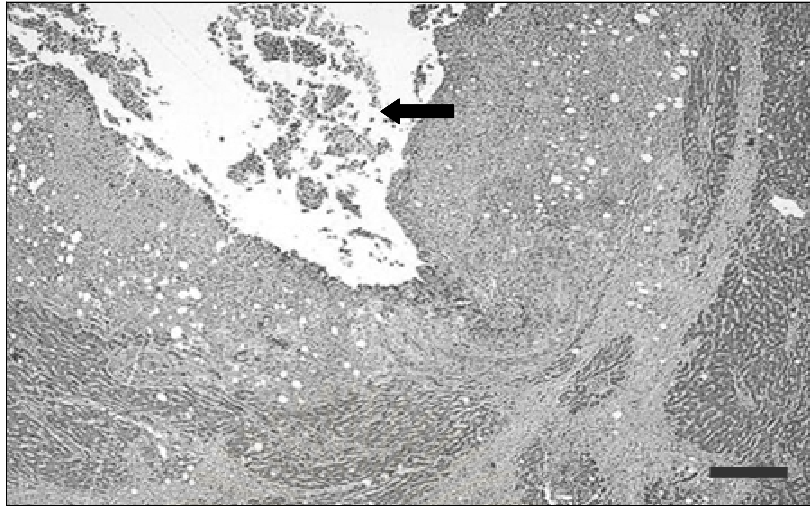


Figure 7. Histopathologic features of typical chronic granulomatous inflammation of the mesenteric lymph node (No. G1), which were a well-formed granuloma with caseous necrosis (black arrow) (Bar = 200 μm).

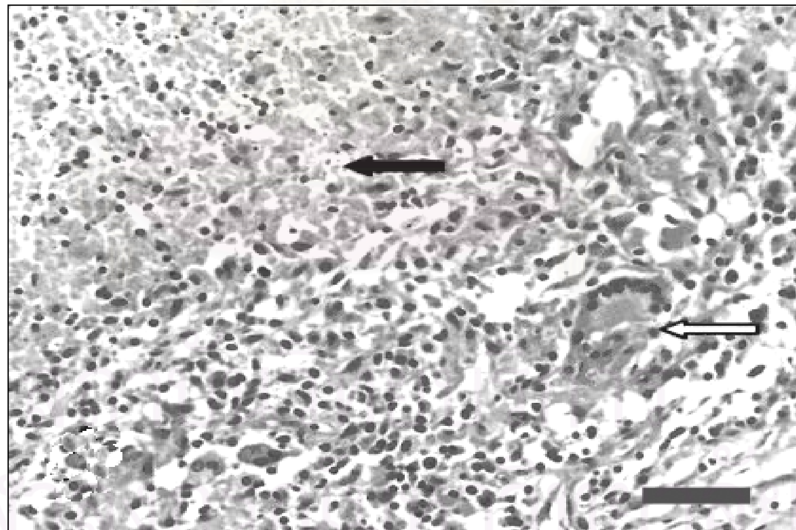
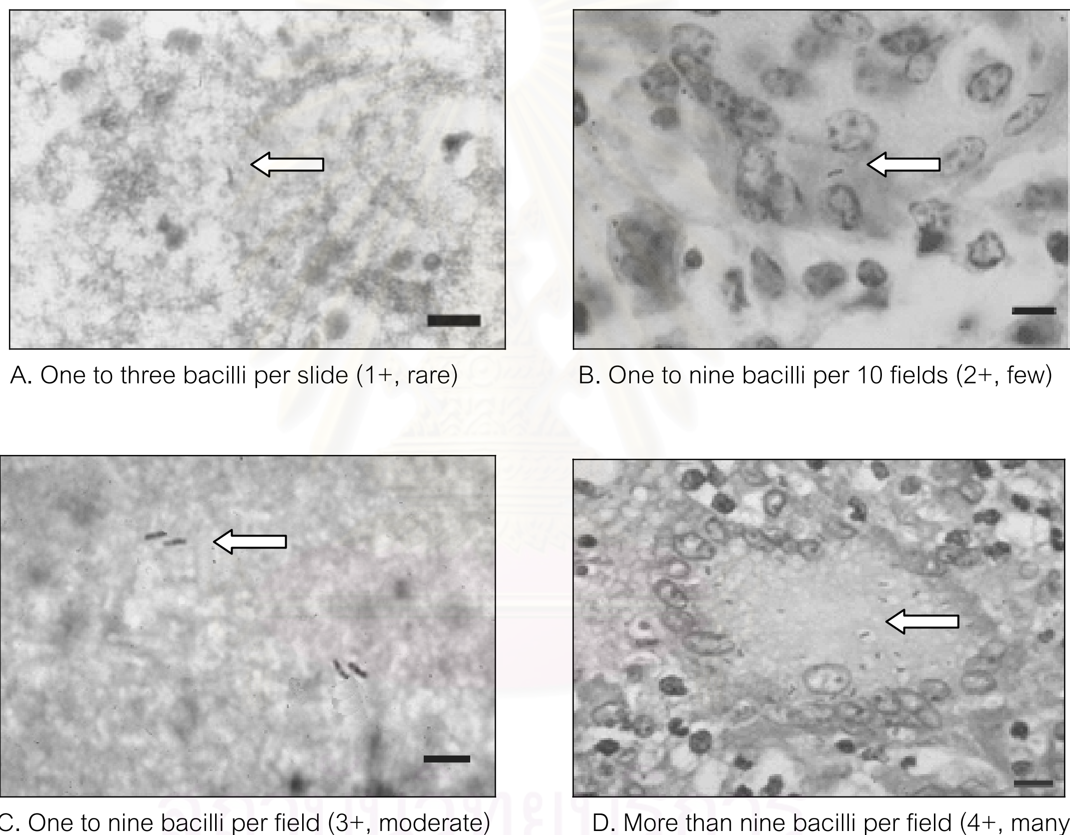


Figure 8. Histopathologic features of atypical granulomatous inflammation of the lung (No. D1) with Langhans' giant cells (white arrow) and pus cells (black arrow) (Bar = 100 μm).

2.2 Acid fast bacilli (AFB) staining

Following acid-fast staining, bacteria were appear as short red or pink rods when examined under a microscope. The result of AFB was reported in numbers based on the criteria of Miller et al. (2002) at a magnification of 400X (Table 4 and Figure 9). Criteria for categorization of AFB is as followed: no AFB seen = negative; one to three per slide = 1+ (rare) (Figure 10); one to nine per 10 fields = 2+ (few); one to nine per field = 3+ (moderate); and more than nine per field = 4+ (many) (Figure 11).



A. One to three bacilli per slide (1+, rare)

B. One to nine bacilli per 10 fields (2+, few)

C. One to nine bacilli per field (3+, moderate)

D. More than nine bacilli per field (4+, many)

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Figure 9. Criteria for categorization of acid fast stain for positive mycobacteria bacilli (arrow) in the clinical tissue samples (Bar = 100 μm), categorized to criteria of Miller et al. (2002). A: 1+ (rare), one to three per slide, B: 2+ (few), one to nine per 10 fields, C: 3+ (moderate), one to nine per field and D: 4+ (many), more than nine per field.

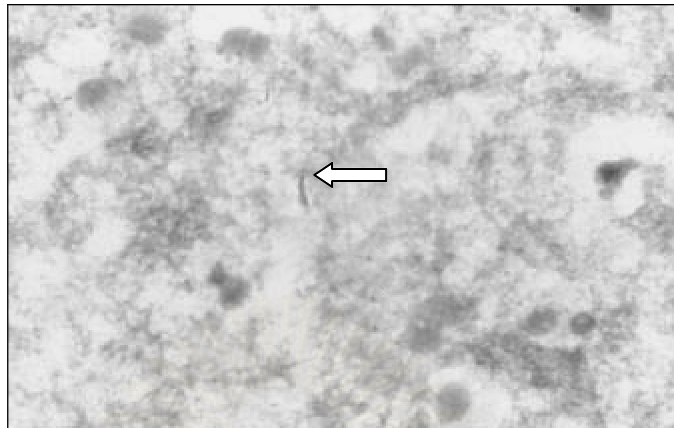

Figure 10. Ziehl-Neelsen stain for acid-fast bacilli (arrow) showing positive bacilli (1+) in the mesenteric lymph node (No. G1) (Bar = 100 μm).

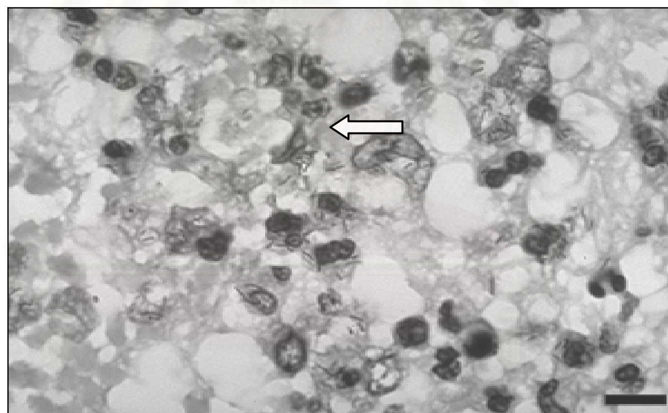

Figure 11. Ziehl-Neelsen stain for acid-fast bacilli (arrow) showing many positive bacilli (4+) in the lung tissue (No. C1) (Bar = 100 μm).

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Table 4. Comparison of results for histopathologic examination, AFB staining and PCR.

Isolated ID	Organs	Epithelioid granuloma	Caseous necrosis	Giant cells (LH)	AFB staining	PCR (16SsRNA)
A1	Mediastinum In.	+	+	+	2+	+
A2	Mesenteric In.	+	+	-	1+	Negative
A3	Supramammary In.	+	+	+	3+	+
A4	Trachiobrocheal In.	+	+	+	2+	+
B1	Mammary In.	-	+	-	Negative	+
B2	Mediastinum In.	+	-	-	1+	+
B3	Mesenteric In.	-	+	-	Negative	Negative
B4	TB In.	+	+	+	2+	+
C1	Lung	+	-	+	4+	+
C2	Mediastinum In.	+	+	+	2+	+
C3	TB In.	+	+	-	1+	Negative
D1	Lung	+	-	+	2+	+
D2	Prefermoral In.	-	+	-	Negative	+
D3	Subscapular In.	-	+	-	Negative	+
E1	Mammary In.	-	-	+	Negative	+
E2	Mammary In.	-	+	-	1+	+
F1	Mediastinum In.	+	+	+	2+	+
F2	Supramammary In.	-	+	+	Negative	+
G1	Mesenteric In.	+	-	-	1+	+
H1	Mediastinum In.	+	+	+	Negative	+
I1	TB In.	+	+	+	2+	+
J1	TB In.	+	+	-	Negative	+
K1	Mammary In.	-	+	-	Negative	Negative
L1	Mammary In.	+	+	-	Negative	+
M1	TB In.	+	-	-	1+	+
N1	TB In.	+	+	-	1+	+
O1	TB In.	+	+	-	2+	+
P1	TB In.	+	+	-	Negative	+
Q1	Mediastinum In.	-	+	+	1+	+
R1	Trachiobrocheal In.	+	+	+	3+	+
S1	Trachiobrocheal In.	+	-	-	Negative	+
T1	Liver	-	+	+	Negative	+
U1	Mediastinum In.	+	+	+	1+	+
V1	TB In.	+	+	-	1+	Negative
W1	Lung	-	+	-	Negative	+
X1	Liver	-	-	-	Negative	Negative

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3. Identification of *Mycobacterium* by molecular techniques

3.1 A panel of PCR amplification

In this study, it was necessary to have a means of confirming the presence of mycobacterial DNA. Detection of *16S rRNA* gene has been used to determine *Mycobacterium* species identity. These primers were successful in amplifying a DNA fragment. Thirty isolates out of 36 mycobacterial isolates in the collection showed a specific positive amplification product in the 16S rRNA PCR reaction (Figure 12) (Table 4). To validate species identity, the PCR amplification products from some MTBC strains were sequenced and confirmed to have the *16S rRNA* nucleotide sequence reported for that species in GenBank (data not shown). Amplification for the *16S rRNA* was chosen to provide the positive control when evaluating mycobacteria by PCR.

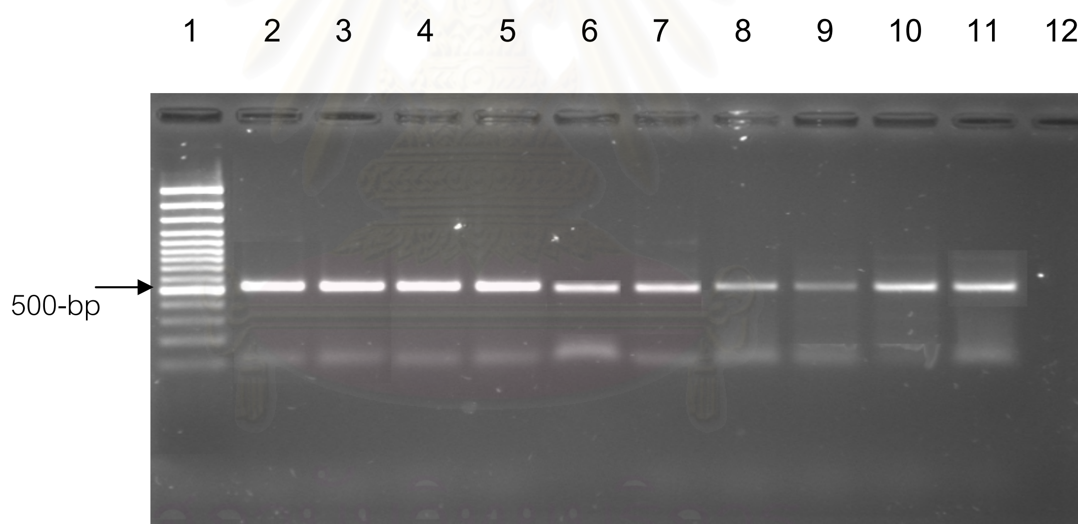

Figure 12. Agarose gel electrophoresis of PCR products from *Mycobacterium* spp. isolates using 16S rRNA primer. Amplification of mycobacterial DNA, 543-bp fragment, from 10 bovine clinical tissue samples. Lane 1, 100-bp marker; Lane 2, positive control of *M. tuberculosis* H37Rv; Lane 3, *M. bovis* (ATCC 19210); Lane 4-11, positive amplification product of samples isolated ID: D1, D2, D3, E1, E2, F1, F2 and G1, respectively; Lane 12, negative.

The Rv*1970* gene (*lprM*) is specific for *M. tuberculosis* identification from the other MTBC subspecies (Romero et al., 1999). Primers Rv1970 were used to amplify 30 mycobacterial isolates in this study, and the PCR results were positive for *M. tuberculosis* (*n* = 12) while the others (*n* = 18) failed to amplify (Figure 13) (Table 5).

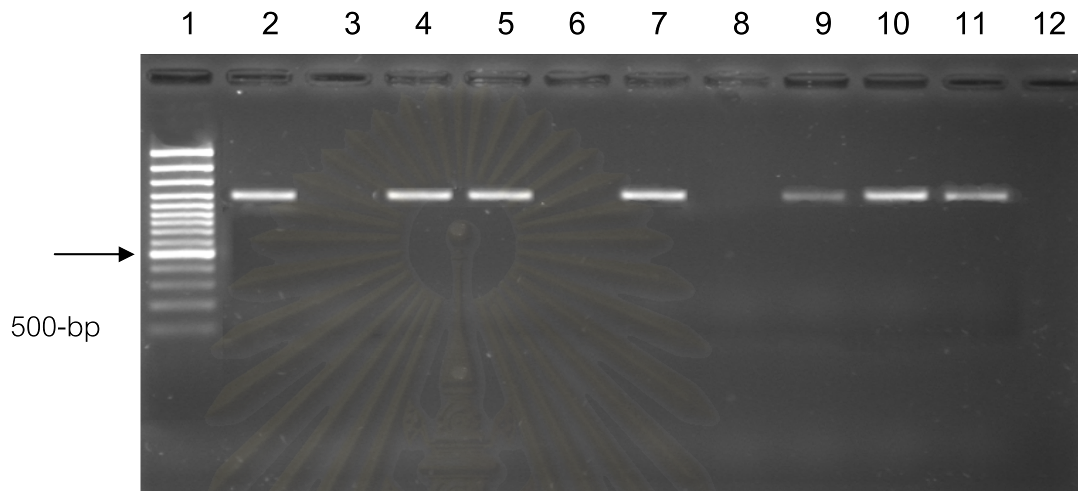

Figure 13. Agarose gel electrophoresis of PCR products from *M. tuberculosis* isolates using Rv1970 primer. Amplification of mycobacterial DNA, 1116-bp fragment, from 10 bovine clinical tissue samples. Lane 1, 100-bp marker; Lane 2, positive control of *M. tuberculosis* H37Rv; Lane 3, *M. bovis* (ATCC 19210); Lane 4-11, amplification products of isolated ID: B1, C1, D1, F1, G1, H1, J1 and M1, respectively; Lane 12, negative.

JB fragment is specific to identify *M. bovis* from the other MTBC subspecies (Shah et al., 2002). Previous studies indicated that JB21/JB22 primers could distinguish *M. bovis* from the rest of the MTBC. In our study, amplification experiments confirmed that JB fragment was present in all the evaluated *M. bovis* strains (*n* = 18) but was absent in the *M. tuberculosis* isolates (*n* = 12) (Table 5). It is noted that primer pair JB21/JB22 often gave a weak signal (Figure 14).

Three chromosomal loci (*16S rRNA,* Rv*1970* and JB fragment) were evaluated to as certain their usefulness as part of an MTBC PCR panel. Each primer pair either specifically amplified a DNA fragment of a unique size or failed, depending upon the source mycobacterial DNA. The pattern of amplification products from all of the reactions, visualized by agarose gel electrophoresis, allowed immediate identification of *M. tuberculosis and M. bovis* (Figure 15).

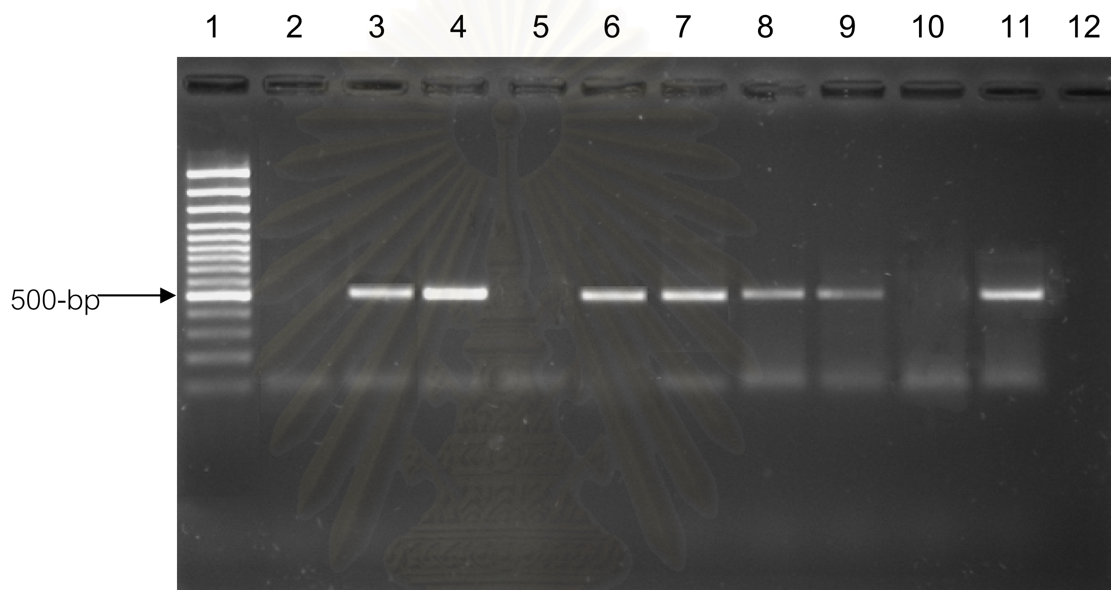

Figure 14. Agarose gel electrophoresis of PCR products from *M. bovis* isolates with JB21/JB22 primer. Amplification of mycobacterial DNA, 500-bp fragment, from 8 bovine clinical tissue samples. Lane 1, 100-bp marker; Lane 2, positive control of *M. tuberculosis* H37Rv; Lane 3, *M. bovis* (ATCC 19210), respectively. Lane 4-11, amplification products of isolated ID: E1, E2, F1, G1, I1,L1,P1, O1 and R1, respectively ; Lane 12, negative.

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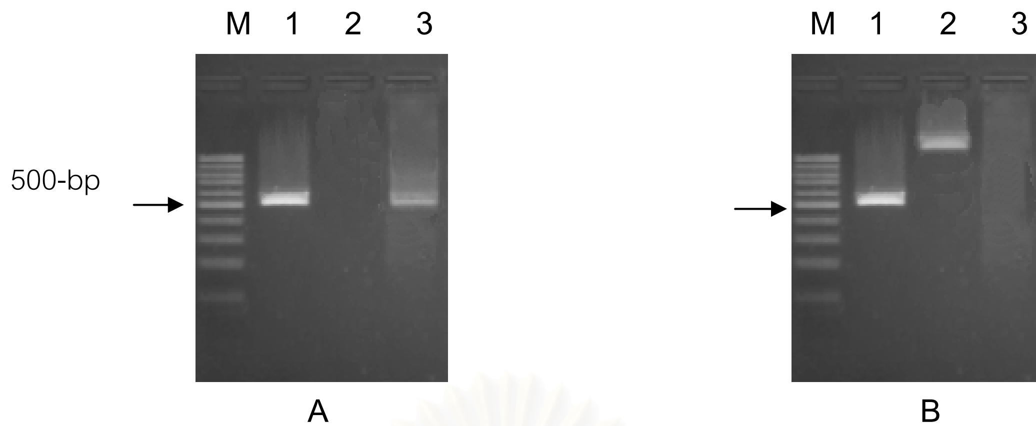

Figure 15. Patterns of the panel PCR assay result for a single representative of *M. bovis* (A) (Isolated ID: A1, 16S rRNA and JB fragment positive) and *M. tuberculosis* (B) (Isolated ID: B1, 16S rRNA and Rv1970 positive). Lane: 1, 16S rRNA; Lane 2, Rv1970; Lane 3, JB fragment. M; 100-bp marker.

3.2 *oxyR* sequencing

We used *oxyR* sequencing to distinguish *M. bovis* from *M. tuberculosis*. DNA sequence analysis has shown that the *oxyR* genes of *M. bovis* strains differ from *M. tuberculosis* at sequence position 285, where, in *M. bovis*, adenine (A) is replaced by guanine (G) (Kurabachew et al., 2004). Our results with the *oxyR* primers confirm that assumption and are in agreement with the reports by Sreevatsan et al. (1996) and Espinosa de los Monteros et al. (1998). The DNA sequence of a 548-bp segment of *oxyR* was analyzed in 30 isolates, including *M. bovis* (*n* = 18) and *M. tuberculosis* (*n* = 12). The oxyR analysis showed that all 18 *M. bovis* isolates had an adenine at position 285. In contrast, all 12 *M. tuberculosis* strains sequenced had a guanine (Figure 16 and 17) (Table 5). With the exception of an occasional rare nucleotide substitution, no additional sequence variation was found in the region studied.

Table 5. A panel PCR analysis used for mycobacterium identification in this study

Isolated ID	PCR panel				*oxyR*	Results
	16SrRNA	Rv*1970*	JB fragment	*oxyR*	sequence	
A1	+	-	+	+	A	*M. bovis*
A3	+	-	+	+	A	*M. bovis*
A4	+	-	+	+	A	*M. bovis*
B2	+	-	+	+	A	*M. bovis*
B4	+	-	+	+	A	*M. bovis*
C2	+	-	+	+	A	*M. bovis*
D1	+	-	+	+	A	*M. bovis*
D2	+	-	+	+	A	*M. bovis*
D3	+	-	+	+	A	*M. bovis*
E1	+	-	+	+	A	*M. bovis*
E2	+	-	+	+	A	*M. bovis*
G1	+	-	+	+	A	*M. bovis*
I1	+	-	+	+	A	*M. bovis*
L1	+	-	+	+	A	*M. bovis*
P1	+	-	+	+	A	*M. bovis*
Q1	+	-	+	+	A	*M. bovis*
R1	+	-	+	+	A	*M. bovis*
S1	+	-	+	+	A	*M. bovis*
B1	+	+	-	+	G	*M. tuberculosis*
C1	+	+	-	+	G	*M. tuberculosis*
F1	+	+	-	+	G	*M. tuberculosis*
F2	+	+	-	+	G	*M. tuberculosis*
H1	+	+	-	+	G	*M. tuberculosis*
J1	+	+	-	+	G	*M. tuberculosis*
M1	+	+	-	+	G	*M. tuberculosis*
N1	+	+	-	+	G	*M. tuberculosis*
O1	+	+	-	+	G	*M. tuberculosis*
T1	+	+	-	+	G	*M. tuberculosis*
U1	+	+	-	+	G	*M. tuberculosis*
W1	+	+	-	+	G	*M. tuberculosis*
A2	-	-	-	-	-	Negative
B3	-	-	-	-	-	Negative
C3	-	-	-	-	-	Negative
K1	-	-	-	-	-	Negative
V1	-	-	-	-	-	Negative
X1	-	-	-	-	-	Negative

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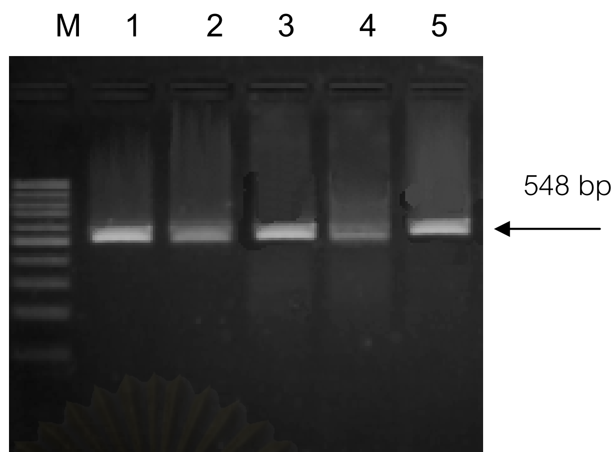

Figure 16. Agarose gel electrophoresis of PCR strategy to amplify 548-bp segment of *oxyR* in *M. bovis* and *M. tuberculosis.* M; 100-bp marker. Lane 1, positive control of *M. tuberculosis* H37Rv; Lane 2, *M. tuberculosis* DNA (Isolated ID: B1); Lane 3, positive control of *M. bovis*; Lane 4, *M. bovis* DNA (Isolated ID: A1); Lane 5, *M. bovis* DNA (Isolated ID: D1)

In conclusion, a panel molecular approach of Polymerase Chain Reaction (PCR) based on 3 specific genes, 16SrRNA, RV1970, JB fragments and *oxyR* gene sequencing can be used to confirm the identity of the *Mycobacterium* species in tuberculosis cattle (Table 5). In this study, 18 (60%) *M. bovis* and 12 (40%) *M. tuberculosis* were identified. Moreover, this PCR technique will be also directly applied on DNA extracted from the clinical sample without prior long-time culturing

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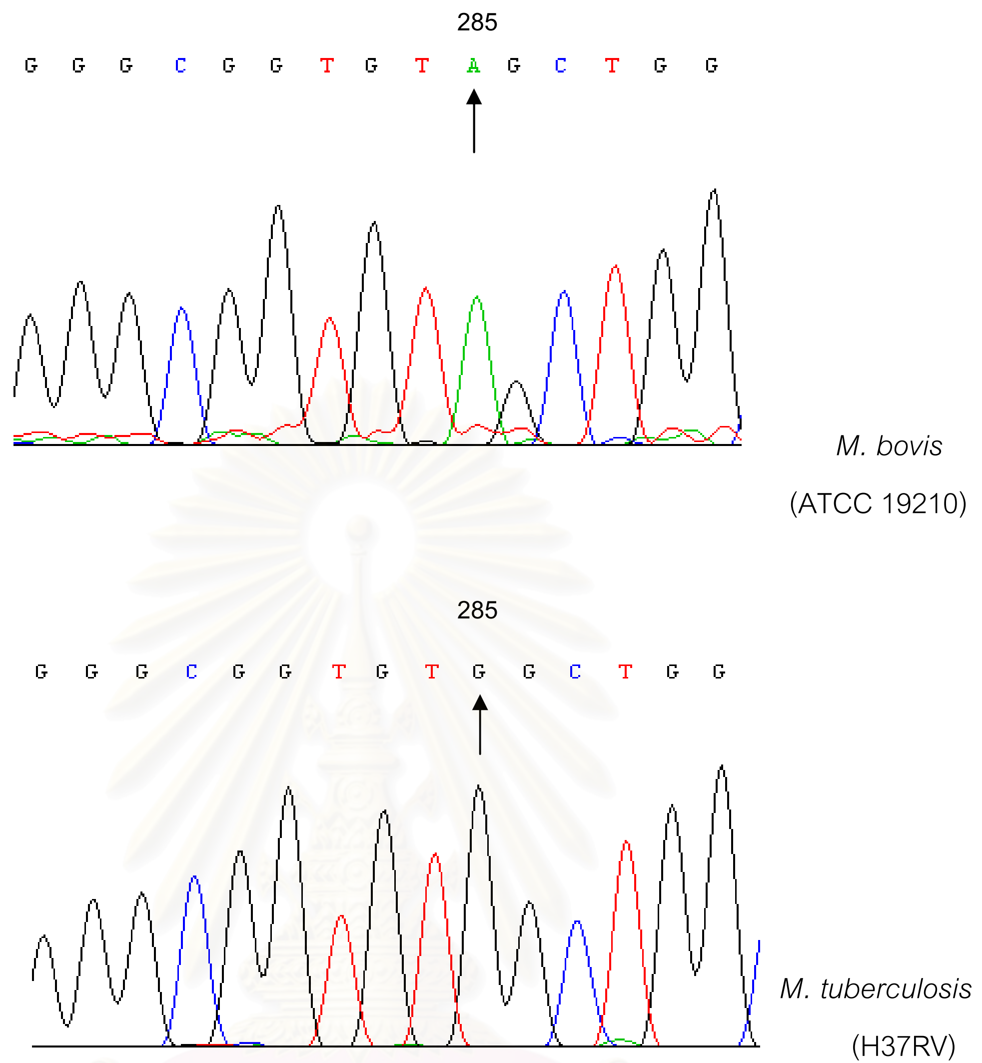

Figure 17. Identification of polymorphism at *oxyR* nucleotide 285. DNA sequencing chromatogram of the region with a polymorphism located at nucleotide 285 in *oxyR*. The *M. bovis* isolates studies had an adenine at nucleotide 285, whereas *M. tuberculosis* studied had a guanine.

4. Comparison of histopathologic examination, AFB staining and PCR identification

The results obtained from all clinical tissue samples are shown in Table 4. In all cattle studied, the corresponding tissues were not sent for TB culture due to time consuming and viability of mycobacteria. Therefore, we could not compare the PCR result with a TB culture result. Of the 36 samples studied, 28 had typical chronic granulomatous inflammation, and 8 had atypical granulomatous inflammation. Of the 28 cases having typical granulomatous inflammation, 16 cases (57.14%) were AFB positive, and 23 cases (82.14%) were PCR positive. Of the 12 cases of atypical granulomatous inflammation, 5 cases (62.50%) were AFB positive, and 7 cases (87.50%) were PCR positive (Table 6).

The results of AFB staining and PCR were not different considerably between the cases with typical granulomatous inflammation and the cases with atypical inflammation. That is, positive AFB and PCR results were common in both of the cases with typical chronic granulomatous inflammation and atypical inflammation.

A comparison of the results of PCR and the results of AFB staining is shown in Table 7. Of the 30 cases that were PCR positive, only 18 cases (60%) were AFB positive, and the 21 AFB-positive cases, 18 cases (85.71%) were PCR positive. Three cases were PCR negative and AFB positive.

Table 6. Comparison of cases by histopathologic examination, AFB staining results and PCR results

Histopathologic examination	AFB staining, No. of cases		PCR, No. of cases	
	Positive	Negative	Positive	Negative
Typical chronic granulomatous inflammation	16	12	23	5
Atypical granulomatous inflammation	5	3	7	1

Table 7. Comparison between the results of AFB staining and the results of PCR

AFB staining	PCR, No. of cases	
	Positive	Negative
Positive	18	3
Negative	12	3

5. Differentiation of *Mycobacterium* by VNTR typing

All mycobacterial isolates were subjected to analyze with five exact tandem repeat (ETR) loci which were previously described (Skuce et al., 2002; Sola et al., 2003). Five such loci (ETR-A to ETR-E), containing perfect, or near-perfect, repeats followed by partial repeats of varying lengths (Table 8), were selected for this study. Each of the five ETR loci contained large tandem repeats with identical DNA sequences in adjacent repeats (Figure 18). Each locus had a unique repeat sequence; repeat units range from 53 to 77 bp. Frothingham and Meeker-O'Connell (1998) have described the location of the ETRs previously. In this study, we determined the variability of each locus by amplifying DNA from all 30 *Mycobacterium* isolates.

PCR products from all five ETR loci demonstrated substantial length polymorphism in a panel of 30 strains. Each allele of corresponds to a different number of repeat units as determined by PCR. Length polymorphisms were easily identified on agarose gels (Figure 19). Based on the size of the PCR products, we determined the exact number of tandem repeats at each locus in each strain. PCR products were also sequenced from some loci to confirm that our PCR products correspond to the expected regions (data not shown).

Table 8. Location and arrangement of VNTR loci within *Mycobacterium* genome sequences.

Locus	Location	Repeat x copy no.	
		M. tuberculosis H37Rv	*M. bovis* ATCC 19210
ETR-A	2165204 – 2165611	(75 x 3) + 23	(75 x 8) + 23
ETR-B	2461280 – 2461550	(57 x 3) + 8	(57 x 5) + 8
ETR-C	577244 – 577500	(58 x 3) +37	(58 x 4) +37
ETR-D	580545 – 580834	(77 x 3) + 7	(77 x 3) + 41
ETR-E	3192168 – 3192370	(53 x 2) + 49	(53 x 3)

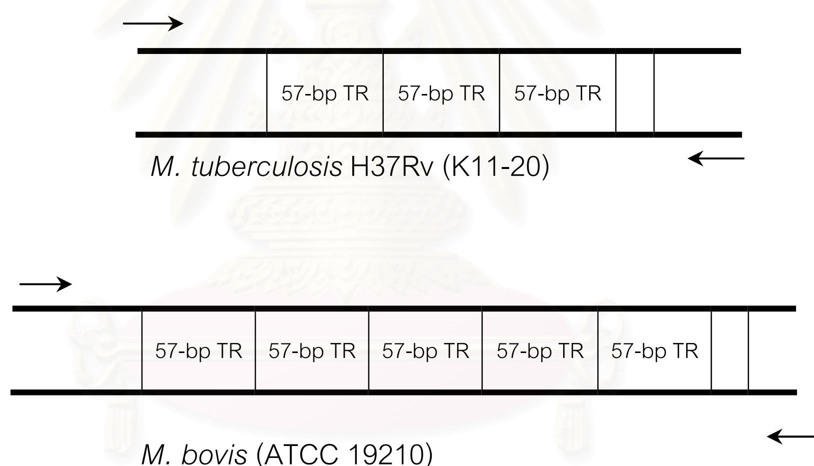

Figure 18. Example of a VNTR locus. The figure shows genomic DNA at the ETR-B locus in *M. tuberculosis* H37Rv and *M. bovis* (ATCC 19210). We amplified this locus using PCR primers complementary to flanking DNA (arrow) and sequenced the respective ~180 and ~300-bp PCR products. *M. tuberculosis* H37Rv DNA contains three complete copies of the 57-bp tandem repeat (TR), plus eight additional bases corresponding to the beginning of another tandem repeat. While *M. bovis* (ATCC 19210) DNA has five complete copies plus the same eight additional bases.

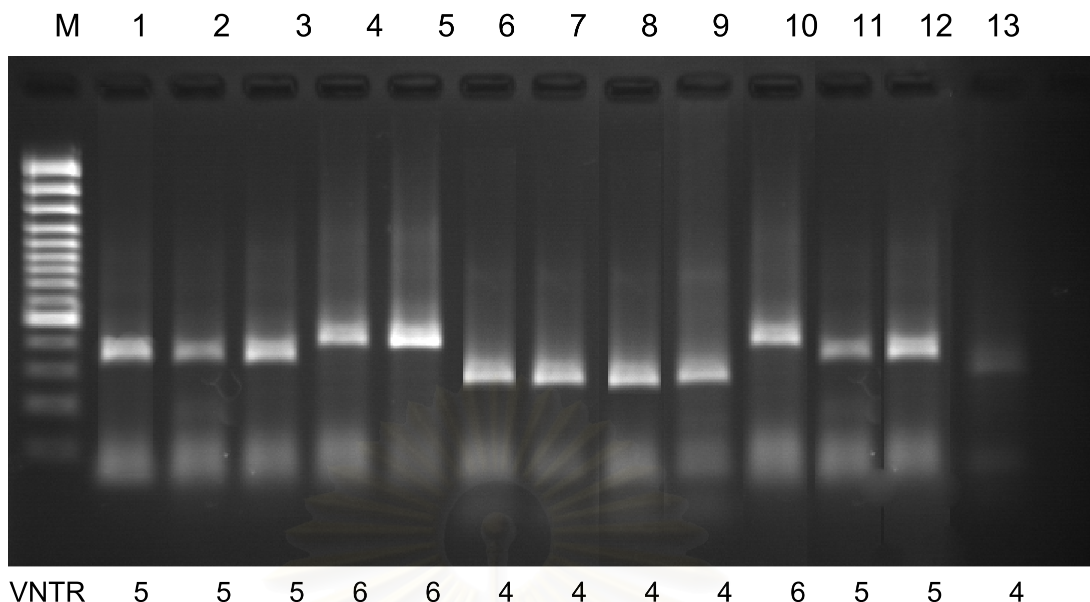

Figure 19. Length polymorphism in *M. bovis* at the ETR-B locus. Each lane (1-13) contains a PCR product from different samples. The number of tandem repeats (VNTR 4,5,6) at locus ETR-B is indicated for each strain. M; the 100-bp marker.

When results for all 5 VNTR loci were combined, 6 allele profiles were identified in the 18 *M. bovis* strains (Table 9), and only one allele profiles were identified in the 12 *M. tuberculosis* strains (Table 10). The VNTR allele profiles of *M. bovis* strains were rather similar to each other, differing at only one to three loci. Examples and comparison of allele profiles of two *M. bovis* strain are shown in Figure 20. The VNTR allele profiles of *M. tuberculosis* strains were identical to each other, but there were only three strains that could not be amplified in ETR-E locus because of DNA insufficiency.

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Table 9. Allele profiles of 18 samples of the *M. bovis* analyzed by VNTR typing

ID Number	No. of tandem repeat units at locus					Allele Profile*
	ETR-A	ETR-B	ETR-C	ETR-D	ETR-E	
M. bovis (ATCC 19210)	8	5	4	3	3	8-5-4-3-3
C2	8	5	5	4	3	8-5-5-4-3
E1	8	5	5	4	3	8-5-5-4-3
L1	8	5	5	4	3	8-5-5-4-3
B2	8	5	4	3	3	8-5-4-3-3
B4	8	5	4	3	3	8-5-4-3-3
D1	8	5	4	3	3	8-5-4-3-3
E2	8	5	4	3	3	8-5-4-3-3
P1	8	5	4	3	3	8-5-4-3-3
Q1	8	5	4	3	3	8-5-4-3-3
A1	8	4	4	3	2	8-4-4-3-2
I1	8	4	4	3	2	8-4-4-3-2
A4	7	6	4	3	2	7-6-4-3-2
D2	7	6	4	3	2	7-6-4-3-2
G1	7	6	4	3	2	7-6-4-3-2
R1	7	6	4	3	2	7-6-4-3-2
D3	7	5	4	3	3	7-5-4-3-3
A3	7	4	4	3	2	7-4-4-3-2
S1	7	4	4	3	2	7-4-4-3-2

*Each digit of the five-digit allele profile represents the number of copies at one of the five VNTR loci.

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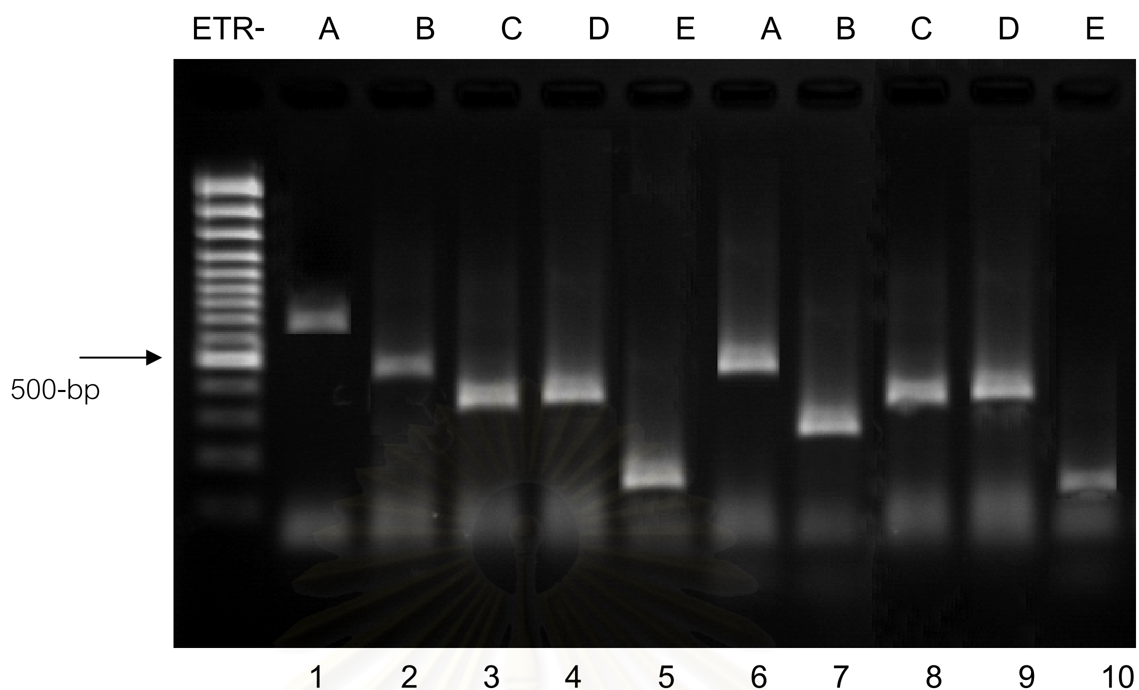

Figure 20. Comparison of the allele profiles of *M. bovis*. The two strains have an identical numbers of tandem repeats at two loci (ETR-C and –D) and vary at three loci (ETR-B, -A and –E). Lanes 1-5, *M. bovis* isolated ID: B2 (8-5-4-3-3); Lanes 6-10 *M. bovis* isolated ID: A3 (7-4-4-3-2)

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Table 10. Allele profiles of 12 samples of the *M. tuberculosis* analyzed by VNTR typing

ID Number	No. of tandem repeat units at locus					Allele Profile*
	ETR-A	ETR-B	ETR-C	ETR-D	ETR-E	
	3	3	3	3	2	3-3-3-3-2
B1	3	3	3	3	2	3-3-3-3-2
C1	3	3	3	3	2	3-3-3-3-2
F2	3	3	3	3	2	3-3-3-3-2
H1	3	3	3	3	2	3-3-3-3-2
J1	3	3	3	3	2	3-3-3-3-2
M1	3	3	3	3	2	3-3-3-3-2
N1	3	3	3	3	2	3-3-3-3-2
U1	3	3	3	3	2	3-3-3-3-2
W1	3	3	3	3	2	3-3-3-3-2
O1	3	3	3	3	N/A	3-3-3-3-0
T1	3	3	3	3	N/A	3-3-3-3-0
F1	3	3	3	3	N/A	3-3-3-3-0

*=Each digit of the five-digit allele profile represents the number of copies at one of the five VNTR loci.

N/A= Not available.

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CHAPTER V

DISCUSSION

Bovine tuberculosis, caused by *M. bovis*, has been on the increase in developed countries and continues to occur in developing countries (Cosivi et al., 1998; Grange et al., 2001). The epidemiological impact of *M. bovis* infection in humans is a problem in developing countries. *M. bovis* has been known to spread to humans from infected cattle (zoonotic tuberculosis) by aerosol or by consumption of contaminated dairy products (prasad et al., 2005).

Tuberculosis caused by *M. bovis* is clinically indistinguishable from that caused by *M. tuberculosis.* In Thailand, reliable diagnosis of bovine tuberculosis in live animals remains a problem. The tuberculin test has been used commonly to identify infected animals. Nevertheless, there are a disadvantage of sensitivity and specificity, the currently intradermal tuberculin test has been highly use. A negative tuberculin test does not rule out infection, especially in countries where the disease is highly endemic, whereas a positive reaction can also reflects a hypersensitivity reaction owing to previous exposure to mycobacteria

Traditionally, culture followed by a panel of biochemical tests has been used for identification of mycobacteria. Most of clinical important mycobacteria are slow growers and hence are difficult to isolate and cultivate. The time required for primary isolation ranges from 4 to 6 weeks. Moreover, the small amount of clinical samples also accounts for the low efficiency of isolation of pathogenic mycobacteria (Kidane et al., 2002; Singh et al., 2000). In addition, particularly *M. bovis* and *M. tuberculosis*, there have been reports of difficulty in identifying these closely related mycobacterial species (Mishra et al., 2005). Therefore, there is a need to develop molecular biological tools such as PCR-based assays for reliable, early detection and identification of mycobacteria in clinical samples and, as a consequence, for determination of the

disease burden caused by diverse pathogenic mycobacteria, especially *M. bovis* and *M. tuberculosis*.

The success of isolation and detection of *M. bovis* and/or *M. tuberculosis* by culture or PCR-based assays are difficult. Culture techniques depend on viable bacteria and therefore reflect ongoing infection. In the case of PCR, the assay can detect both alive and dead mycobacteria. PCR can also detect non-viable mycobacteria that cannot be grown by culture. Though, PCR-based methods are useful for rapid detection of target DNA suspected clinical samples. The efficacy of PCR assays can be influenced by the quality of target DNA extracted from appropriate samples and the presence of PCR inhibitors. Singh et al. (2000) have reported that inhibitors were detected more frequently in extrapulmonary than in pulmonary tissue. Solid specimens such as skin and lymph nodes also cause difficulties in DNA extraction and discordant results in different methods may be caused by non-homogenous distribution of AFB in the specimen. There problems have been address by several researchers (Chakravorty and tyagi, 2001; Mishra et al., 2005). Thus, spiked controls have been applied in the present study to overcome these problems.

To establish the usefulness of a PCR reaction in the diagnostic tests it was nwssary to compare the results of the PCR with results obtained by some standard methods. In our study PCR test was compared with histopathology, AFB staining for detection of *Mycobacterium* in different specimens. AFB positivity in histological specimens depends on the bacillary load of the specimen. Different studies have reported a wide rage of AFB positively ranging from as low as 0% to as high as 75% (Mahaisavariya et al., 2005). In this study, AFB from histological specimens was positive only 58% of the cases and all of AFB positive samples showed caseating granulomas on histopathological examination. Even if AFB examination is as sensitive as a PCR, the differences may still occur. The low detection rate was a problem in the present study. AFB finding require skilled personnel and a large number of organisms in the sample. It has been described in the literature that the effectiveness of AFB with formalin-fixed and paraffin-embedded tissue is impaired by multiple interacting factors, including the type

of fixative and the fixation time (Mahaisavariya et al., 2005). Whereas only qualitatively preserved DNA are required for PCR.

The panel PCR-based assays described in the present study are based on 1*6SrRNA*, Rv*1970* and JB fragment of mycobacteria. The specificity of each PCR assay to detect and identify *M. bovis* and *M. tuberculosis* was determined by using a variety of mycobacterial microorganisms. Further, the reliability of the assay was demonstrated by using *M. bovis* and *M. tuberculosis* strains from different geographical region (Hughes et al., 1993; Huard et al., 2003; Romero et al., 1999; Shah et al., 2002).

According to type of tissue tested, it has been reported that in some tissue types such as the lung and spleen, DNA could be amplified in every cases. An amplification sensitivity of 75% was obtained in lymph node samples. Mycobacterial DNA in liver samples could not be amplified at all (Singh et al., 2000). Kidane et al. (2002) and Shah et al. (2002) reported that the sensitivity of the PCR assays was limited to detect 10 - 20 pg DNA of the tubercle bacilli. Therefore, the sensitivity of the PCR technique may lead to false positives (Wang et al., 2004). Hence, the results of the PCR could potentially point to infection rather than disease. In this study, the probability of false positive results being generated by the panel PCR technique is low because there are three specific primers to confirm each other. Furthermore, the high correlation between tuberculin reactivity status and the results of AFB staining and PCR was found among the tissue samples examined.

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In this study, we report the utility of the panel PCR assay for the direct detection of *M. bovis* and *M. tuberculosis* in bovine samples. This feature of the panel PCR assay has a distinct advantage over the convention methods. Thirty of 36 clinical tissue samples (83.33%) were positive for 16S rRNA amplification, 18 from 30 (60%) were identified in *M. bovis* strains and surprisingly, 12 from 30 (40%) were positive for *M. tuberculosis*. We also used *oxyR* primers to distinguish *M. bovis* and *M. tuberculosis*. DNA sequencing has shown that the *oxyR* genes of *M. bovis* strains differ from those of other members of the *M. tuberculosis* complex at sequence position 285, where, in *M.*

bovis, adenine is replaced by guanine. Our results with the *oxyR* polymorphisms confirm the 285 assumption and are in agreement with two target primers, Rv1970 and JB21/JB22.

However, there is no evidence of mixed infection by *M. bovis* and *M. tuberculosis* in this present study. Mixed infection, presence of both *M. tuberculosis* and *M. bovis* in the same sample, was reported in an individual human sample of tuberculous lymphadenitis patient (Kidane et al., 2002). In addition, mixed infection by *M. bovis* and *M. tuberculosis*, several other mycobacteria, such as *M. avium* complex, has been reported to occur along with *M. tuberculosis* (Leite et al., 1998). The detection of both pathogens in the cattle suggests a double danger of infected cattle being a source of mycobacterial pathogens transmitting to animals and humans. Contact between farm animals and humans would naturally facilitate a bi-directional infection of mycobacterial pathogens.

The presence of *M. tuberculosis* in bovine samples is indicative of reverse zoonosis, as has been reported for animals in contact with humans (Erwin et al., 2004; Fritsche et al., 2004). It has been documented that animal can be infected by exposure to any individual shedding the organism. Prasad et al. (2005) reported a few examples of human-to-cattle transmission and stressed the potential danger that patients with smear-positive pulmonary tuberculosis pose to animals. Grange and Yates (1994) reported that farm workers urinating in cowsheds might represent a source of infection for animals. Fritsche et al. (2004) has also reported the transmission of *M. bovis* from animal to man and back to animal. Therefore, there is a necessary to detect tuberculosis among cattle handlers and farmers, as human are the major reservoir of *M. tuberculosis*, unlike with *M. bovis*. Identification of *M. tuberculosis* and *M. bovis* would help the formulation of public health strategies to interrupt the transmission chain not only among cattle but also between humans and cattle (Mishra et al., 2005). This demands the strict policies regarding the detection of tuberculosis among animals and cattle handlers. Besides mycobacteria identification from cattle, molecular differentiation (molecular

subtyping) need to be included to establish the clearly relationship between strains isolated from humans and those isolated from cattle.

Until recently, most of the *M. bovis* typing techniques permitted a satisfactory differentiation of isolates (Haddad et al., 2004). With the development of molecular techniques, molecular typing has become possible for mycobacteria, especially for those belonging to the *M. tuberculosis* complex. The availability of different techniques, such as spoligotyping, VNTR typing, and RFLP-IS*6110* allows a wide range of applications, and provides increased discrimination power, when independent and complementary techniques are used together. VNTR typing is becoming a very important tool with highly reliable accuracy. On a practical point of view, in veterinary medicine, typing is particularly useful for helping to trace the spread of *M. bovis* between herds, and for evaluating the role of wild and human reservoirs.

The discrimination of the VNTR loci was compared by using set of exact tandem repeats (ETR-A, -B, -C, -D and –E). When results for all 5 VNTR loci were combined, 6 allele profiles were identified in the 18 *M. bovis* strains, and only one allele profile were identified in the 12 *M. tuberculosis* strains. Surprisingly, the VNTR allele profiles of the *M. tuberculosis* strains were similar or identical to each other. Although VNTR was able to discriminate *M. bovis* isolates, it was subsequently shown to lack discrimination with isolates of the *M. tuberculosis*, or else *M.tuberculosis* isolates in this study are the same clones.

VNTR typing provides a convenient and high–resolution typing method. VNTR approach has provided a means for the rapid and clear differentiation of members of MTBC. It is possible that different degrees of discrimination may be appropriate for different studies, with some loci being useful for larger population-based studies. More discriminating loci may be applicable to outbreak analysis. The flexibility of the VNTR typing technique is high, as is the degree of discrimination and the ease of application. Future molecular epidemiological studies should benefit from the application of this technique, as greater discriminating data will be available to facilitate the study of

transmission, contact tracing and the identification of strains with particular phenotypic traits, which may be relevant for improving diagnosis or vaccination strategies.

Our findings indicated that *M. bovis* infected dairy cattle in Nakhon Pathom, Thailand, belong to different or diverse clones of mycobacteria. On the other hand *M. tuberculosis* caused tuberculosis in cattle are from only one simple clone. However, this finding needs further investigation by including more sample numbers and sources as well as applying more than one molecular subtyping techniques.

In conclusion, panel PCR amplification techniques may present a rapid and useful tool for detecting mycobacterial infection. In addition, we propose that the VNTR PCR-based approach, which is simple to perform, can be incorporated routine into the general laboratory for mycobacterial differentiation.

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Conclusion and suggestion

In this study, we focus on a rapid and simple PCR-based method for identification and differentiation of mycobacteria isolated directly from bovine clinical sample. Our study showed

1. A panel of Polymerase Chain Reaction (PCR) based on 3 specific genes, 1*6SrRNA*, Rv*1970*, JB fragments and *oxyR* gene sequencing can be used to confirm the identity of the *Mycobacterium* species in bovine clinical samples. This PCR technique can be directly applied on DNA extracted from the clinical sample without prior long-time culturing.

2. PCR-based VNTR analysis has provided the molecular epidemiological information of bovine tuberculosis in Thailand. VNTR discriminates well between *M. bovis* strains but lack discrimination ability with isolates of the *M. tuberculosis.*

3. Bovine tuberculosis infection in dairy cattle in Thailand is caused by both *M. bovis* and *M. tuberculosis*. The occurrence of *M. tuberculosis* may be as a result of human-to-cattle transmission or reverse zoonosis. This data can be of benefit to the diagnosis, prevention and control of this zoonotic disease in the future.

4. By VNTR typing, *M. bovis* infected dairy cattle in Nakhon Pathom, Thailand, belong to the different or diverse clones of mycobacteria. On the other hand, *M. tuberculosis* caused tuberculosis in cattle are from only one simple clone. However, this finding needs further investigation by including more sample numbers and sources as well as applies more than one molecular subtyping techniques.

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APPENDICES

สถาบันวิทยบริการ
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APPENDIX A

Reagents and preparations

1. 0.5 M EDTA, pH 8.0

Disodium ethylene diamine tetraacetate. $2H_2O$	186.1 g
DDW	800.0 ml
Adjust pH to 8.0	
Adjust volume to 1,000 ml	

2. 1 M.Tris-HCL, pH 8.0

Tris (ultrapure)	121.1 g
DDW	800.0 ml
Adjust pH to 8.0 by adding conc. HCL	42.0 ml
Sterilize by autoclaveing	

3. 50X Tris-Borate buffer (TBE)

Tris	242.0 g
Boric acid	57.1 g
0.5 M EDTA pH 8.0	100.0 g
Adjust the volume to 1,000 ml with DDW	
Sterilize by autoclaving	

4. 3M Sodium acetate

Sodium acetate. $3H_2O$	408.1 g
DDW	800 ml
Adjust pH to 5 with glacial acid	
Adjust volume to 1,000 ml	

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APPENDIX B

Reagents for agarose gel electrophoresis

1. 10 MG/ML Ethidium bromide

Ethidium bromide	1	g
DDW	1,000	ml

Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer to a dark bottle and store at 4 °C.

2. 2 % Agarose gel

Agarose (ultrapure)	0.3	g
1 X TBE	20.0	ml
10 mg/ml Ethidium bromide	1.0	μ1

APPENDIX C

Reagents for AFB staining

1. Carbol fuchsin

Basic fuchin, strong

Basic Fuchsin, powder	15	g
Ethanol, absolute	250	ml
Phenol	85	g
Deionized water	1250	ml

Mix the phenol and fuchsin and if necessary heat gently to dissolve the phenol. Add the ethanol and distilled water and filter into a stoppered bottle. Propanol may be substituted for ethanol.

Carbol fuchsin, weak (10%)

Dilute one volume of strong carbol fuchsin with 10-20 volumes of distilled water.

2. 3% acid alcohol

Conc. HCl	3	ml
Ethanol, 95%	97	ml

Mix well before use.

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Acid-fast Staining Procedures for Mycobacteria

1. Flame slides to heat fix.
2. Flood the entire slides with strong carbol fuchsin and heat until steam rises (but do not boil).
3. After 5 min apply more heat until steam rises again; do not be let stain dry on slide.
4. About 5-7 min after the first application of heat wash the thoroughly under running water.
5. Decolorize in 3 % acid-alcohol until all traces of red have disappeared from the film. Decolorization should not be attempted in one stage; there should be intermittent washings in water and re-application of acid-alcohol.
6. Wash well in water when decolorization is complete.
7. Counterstain with Loeffler's methylene blue. Keep on the slides for 1 minute. Rinse the slide thoroughly with water.

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BIOGRAPHY

Mr. Suphasawatt Puranaveja was born on August 24, 1974 in Bangkok, Thailand. He graduated from the Faculty of Veterinary Medicine, Chulalongkorn University, Thailand in 1998. Then he has been a veterinarian at Diagnostic Laboratory, Faculty of Veterinary Medicine, Chulalongkorn University since 1999. After that, he enrolled the degree of Master of Science in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University since 2004.

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