Immunodiagnostic Assays and Molecular Epidemiology of Bovine Tuberculosis

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Immunodiagnostic Assays and Molecular Epidemiology of Bovine Tuberculosis

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The Doctoral Dissertation submitted to
the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the
degree of Doctor of Philosophy

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December 2013

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December 2013

Acknowledgements

박사 학위 논문을 작성하면서 지난 학위과정을 다시 한 번 떠올리며 도움을 주신 분들께 감사의 인사를 드립니다.

바쁘신 가운데서도 부족한 저를 끝까지 지도해주신 조상래 교수님께 감사 드리며, 함께 미생물학교실에서 사랑과 배려로 학생들을 지도해주신 교수님들께 감사의 말씀을 드립니다. 또 바쁘신 가운데 논문을 심사해주신 신전수 교수님, 최인홍 교수님, 이경원 교수님, 강영애 교수님께도 진심으로 감사 드립니다.

오랫동안 함께 실험실에서 연구해온 박사님들과 선생님들께 감사의 인사를 드립니다. 또한 논문을 위해 함께 오랜 기간 동안 필드에서 수고해주신 경기도 축산위생연구소분들께도 감사 드립니다.

그리고 항상 저를 응원해주시고 기도해주신 사랑하고 존경하는 부모님께 감사드립니다. 또한 항상 사랑으로 격려해주시고 기도해주신 온누리교회 초등부 선생님들께도 진심으로 감사의 말씀 드립니다.

마지막으로 저를 구원하시고 인도하셔서 이 모든 일들을 이루어주신 창조주 하나님께 감사드립니다.

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CHAPTER II. MOLECULAR EPIDEMIOLOGY OF BOVINE TUBERCULOSIS

ABBREVIATIONS

TB, Tuberculosis

TST, Tuberculin skin test

***M. bovis*,** *Mycobacterium bovis*

ELISA, Enzyme-linked immunosorbent assay

ESAT6, 6 kDa early secreted antigenic target

CFP10, 10 kDa culture filtrate protein

MPB70, Mycobacterial protein from species *bovis*-70

MPB83, Mycobacterial protein from species *bovis*-83

HIV, Human immunodeficiency virus

PPD, Purified protein derivative

BCG, Bacillus Calmette Guérin

DTH, Delayed type hypersensitivity

PDR, Positive duplicate rate

VNTR, Variable number tandem repeat

MIRU, Mycobacterial interspersed repetitive units

ETR, Exact tandem repeats

QUB, Queen's University Belfast

ABSTACT

Immunodiagnostic Assays and Molecular Epidemiology of Bovine Tuberculosis

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(Directed by Professor Sang-Nae Cho)

Bovine tuberculosis (TB) is a highly prevalent infectious disease of dairy herds worldwide and is a major issue of both human public health and economics. Annual testing of bovine TB and culling of *Mycobacterium bovis*-infected cattle has been a back-bone of the bovine TB control programs in Korea as well as many other countries in the world. Tuberculin skin test (TST) has been the international standard for diagnosis of bovine TB in dairy cattle. Recently, to improve sensitivity and specificity of diagnosis for bovine TB, new immunodiagnostic assays using recombinant mycobacterial antigens were introduced in many countries. In this study, an in-house IFN-γ assay using an 6 kDa early secreted antigenic target (ESAT6) and 10 kDa culture filtrate protein (CFP10) cocktail antigen and an ELISA-based antibody test

using mycobacterial protein from species *bovis*-70 (MPB70) and mycobacterial protein from species *bovis*-83 (MPB83) antigens were evaluated for immunodiagnosis of bovine TB in dairy cattle in Korea. Both the IFN-γ assay and antibody tests showed clear distinction between TST-reactors and non-reactors from bovine TB-free herds with sensitivity of 87.4% (118/135) and specificity of 100% (100/100) for IFN-γ assay and sensitivity of 77.0% (137/178) and specificity of 95.7% (111/116) for antibody tests. However, many TST-negative cattle were positive by immunological assays in dairy cattle herds with recent bovine TB outbreaks within 12 months showing positivity of 36.8% by IFN-γ assay and 23.7%–29.8% by antibody tests. A majority of such cattle (11/14) were confirmed to be *M. bovis*-infected by post-mortem examinations followed by culture and molecular detection of *M. bovis*. Each *M. bovis*-infected cattle, however, showed various patterns of immune responses in immunodiagnostic tests. These results suggested that besides TST, other ancillary immunological tests such as IFN-γ assay and antibody tests are required for more effective detection of *M. bovis* infection in cattle in Korea.

In addition, a molecular typing method was established for distinguishing *M. bovis* strains as an effort to understand transmission of *M. bovis* among different animal species, particularly between cattle and deer. A total of 133 *M. bovis* clinical isolates from 59 Holstein dairy cattle, 40 Korean beef cattle, and 34 deer with bovine TB-like lesions were analyzed in this study. Thirty published variable-number tandem repeat (VNTR) markers were applied to these isolates, and 16 of 30 markers showed allelic diversity. The most discriminatory locus for *M. bovis* isolates in Korea was VNTR 3336 (h = 0.59). Queen's University Belfast (QUB) 26, mycobacterial interspersed repetitive units (MIRU) 31, VNTR 2401, and VNTR 3171 also showed high discriminatory power (h = 0.42). The combined VNTR loci had an allelic

diversity of 0.84. On the basis of the VNTR profiles of 30 VNTR loci, 26 genotypes (A–Z) were identified in Korea. Two genotypes, designated as K and L, were prevalent among all *M. bovis* isolates (33.1% and 18.8%, respectively). Six genotypes–I, J, Q, R, S, and T–were also common in 2 out of the 3 species. These results suggest that *M. bovis* interspecies transmission may occur frequently in Korea.

In summary, the immunological assays developed in this study were useful in identifying animals infected with *M. bovis* in substantial portion of TST-negative cattle in the herds with bovine TB outbreaks, thus applicable to the control and eradication programs of bovine TB as ancillary tests to TST. In addition, MIRU-VNTR typing was useful for differentiation of *M. bovis* molecular types in Korea. Molecular typing data showed a clear evidence of *M. bovis* inter-herd and interspecies transmission, thus highlighting the importance of bovine TB control programs in deer as well as in dairy and beef cattle.

Key words : *Mycobacterium bovis*, IFN-γ assay, antibody test, VNTR typing

Immunodiagnostic Assays and Molecular Epidemiology of Bovine Tuberculosis

Chapter I. Immunodiagnostic Assays of Bovine tuberculosis

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I. Introduction

Bovine TB is chronic wasting disease caused by *M. bovis*, a member of the *M. tuberculosis* complex. *M. bovis* can infect cattle, deer, wild life, and also human through respiratory routes [1]. So bovine TB has been spreading worldwide and is a major issue of both human public health and economics in large areas of the world [2]. In developing country, bovine TB affects people by raw milk and raw milk products, known to be major source of human infection. In some countries, *M. bovis* is responsible for 5–10% of human TB. Such infection is more exacerbated in the countries where not applying veterinary surveillance policy [3]. In developed countries, the proportion of *M. bovis* infection in human TB cases is very low, however, the incidence of human TB caused by *M. bovis* is higher in rural than urban regions.

Agricultural workers may acquire the disease through respiratory routes from *M. bovis*-infected cattle. Recently, in addition, the potential impact of human immunodeficiency virus (HIV) prevalence on the incidence of human TB due to *M. bovis* has caused some concern [4].

The diagnosis of bovine TB is primarily based on the measurement of *M. bovis*-specific cell mediated immune (CMI) responses. The primary screening test used for surveillance is TST. TST relies on the response of the animal to the injection of tuberculin with assessment of the test site for the presence of induration or swelling, or the measurement of these reactions in millimetres at 48–72 hours following the injection. Application of TST resulted in a rapid decrease in the incidence of bovine tuberculosis.

Recently, it was reported TST have low sensitivity and specificity in detection of individual animal infected with *M. bovis*. In addition, TST requires a "desensitization" period of 60 days or more before retesting because of its suppression of reactivity to the subsequent TST in the animals that are infected with *M. bovis* [5]. Nonspecific reaction to TST may also occur when non-infected animals are exposed to antigens of environmental mycobacteria [6]. Other alternative test has been, therefore, desirable for diagnosis of *M. bovis* infection to overcome such limitation of TST above.

IFN-γ assay has been reported to be a potential diagnostic test measuring *M. bovis*-specific cell-mediated immune responses. It is a rapid whole blood assay, which is based on the release of gamma-interferon from sensitized lymphocytes during an overnight incubation with *M. bovis*-specific antigen [7]. This assay was useful in detecting *M. bovis*-infected cattle during the initial stages of infection when cellular immune responses dominate [8]. So, it will detect a substantial proportion of *M. bovis*-infected cattle that have no reaction to the TST [9]. IFN-γ assay allows for more rapid repeat testing because no tuberculin is injected and there is no interference with the host's

immune system. However, the earlier version of IFN-γ assay employed purified protein derivative (PPD) antigens of *M. bovis* and *M. avium* in order to improve specificity of the assay. On the other hand, *M. tuberculosis* complex-specific antigens such as ESAT6 and CFP10 antigens have been widely used for detecting latent TB infection in humans. Therefore, it would be of great interest to evaluate usefulness of such ESAT6 and CFP10 antigens in the IFN-γ assay for detecting *M. bovis* infection in cattle.

Both the IFN-γ assay and TST have a low probability of detecting *M. bovis*-infected cattle having depressed cell-mediated immune response. However, such unresponsive animals, which are almost chronically infected, may have an antibody response [10]. Therefore, antibody response test, like ELISA, may be useful in detecting chronically-infected cattle in the advanced stages of infection. In this study, in order to overcome low sensitivity by single antigen-based antibody test during the initial stages of infection, an antibody response test using two antigens, namely MPB70 and MPB83, has been evaluated and established in diagnosis of cattle, deer and wild life [11, 12, 13].

In Korea, to control bovine TB, annual test for dairy herds has been performed once per year by using the TST. Recently, antibody test was introduced partially to screen Korean beef cattle herds. But confirmatory diagnosis depends on only TST. In this study, therefore, IFN-γ assay and antibody test were established and evaluated as new diagnostic tools for identifying *M. bovis*-infected and non-infected dairy herds in Korea.

II. Materials and Methods

Preparation and purification of mycobacterial antigen

The recombinant proteins ESAT6, CFP10, MPB70 and MPB83 were prepared and purified as previously described [14, 15, 16, 17]. Briefly, ESAT6, CFP10, MPB70 and MPB83 sequences of *M. tuberculosis* genomic DNA are amplified by polymerase chain reaction (PCR). The PCR product was purified and digested by restriction enzyme *Bam*HI and *Nde*I enzymes (NEB, Ipswich, MA, USA). DNA insert containing a 6 × histidine tag were inserted into ampicillin-resistant pET11a_KB vector (NEB, Ipswich, MA, USA). Completely ligated DNA vector was transferred to *E. coli* DH5 by heat shock. Transformed *E. coli* DH5 was selected on LB agar plate with 100 μg/mL ampicillin (Sigma-Aldrich, Dorset, United Kingdom) and incubated. Then, plasmids were extracted and transferred to *E. coli* BL21 (DE3) for protein expression. *E. coli* BL21 (DE3) were grown in Luria-Bertrani (LB) broth containing 100 μg/mL ampicillin at 37°C for 3 h until an OD_{600} of 0.6–0.7. Cells were then induced with 1 mM isopropyl β-D-1-thiogalactoside (IPTG) and incubated again for 4 h, after which cells were harvested and the pellet was sonicated in a 20 mM Tris buffer (pH8.0). After centrifugation at 12,000 rpm for 30 min, Ni-NTA resin (Qiagen, Hilden, Germany) for histidine affinity purification was added to the supernatant and allowed to bind overnight at 4°C. For insoluble protein, ESAT6, MPB70 and MPB83, the remaining inclusion bodies were prepared in a 20 mM Tris-HCl (pH 8.0) buffer containing 300 mM NaCl, 5 mM imidazole and 6 M urea, and allowed to bind to Ni-NTA resin. Then, the resin was packed and washed with an identical buffer containing 30 mM imidazole. Final elution was performed using a similar Tris buffer containing 200 mM imidazole. After affinity purification with Ni-NTA resin, the recombinant proteins were further purified

by fast protein liquid chromatography (FPLC) using an ÄKTA FPLC (GE, Pittsburgh, PA, USA) and a MonoQ anion exchange column (GE, Pittsburgh, PA, USA). Purified recombinant proteins were confirmed by SDS-PAGE analysis.

Animals for immunodiagnostic assays

Diagnostic tests were performed in Holstein Friesian cattle aged more than 1 year in Gyeonggi-do, South Korea. The cattle have been tested with TST annually. For TST, Cattle were injected with 100 μl (2 mg/mL) of bovine PPD in caudal fold. Result was determined as skin thickness 48–72 h after injection. The animals were considered positive if there was an increase of 5 mm or more in skin thickness, dubious if the increase in skin thickness was more than 3 mm and less than 5 mm, and negative if an increase of no more than 3 mm in skin thickness was observed. The herd with bovine TB outbreak was acquired by reaction to TST. *M. bovis*-infected cattle are slaughtered and the herd is tested repeatedly 2–3 months later. Herd having shown no reaction to TST for 5 years was considered as non-infected herd.

Blood collection and IFN-γ assay

Heparinized blood samples were collected from each animal. The samples were delivered to the laboratory within 8–10 h of blood collection. Whole blood cultures were performed in 96-well plates in aliquots of 200 μL/well. Each aliquot was stimulated with pokeweed mitogen (PWM) (Sigma-Aldrich, Dorset, United Kingdom), a mixture of recombinant ESAT6 and CFP10 antigens, and reconstituted in phosphate buffered saline (PBS). PWM and PBS were used as positive and negative controls, respectively. The final concentration of the antigen mixture and PWM was 5 μg/mL. Supernatants were harvested after incubating the plates at 37ºC in a

humidified 5% CO_2 incubator for 18–24 h. IFN-γ was then determined by a sandwich enzyme-linked immunosorbent assay (ELISA) technique using a commercially available pair of monoclonal antibodies. Briefly, the ELISA plate wells were coated overnight at 4ºC with 1 μg/mL anti-bovine IFN-γ antibody (AbD Serotec, Oxford, United Kingdom) in 50mM carbonate buffer (pH 9.5, 100 μL per well). Unbound antibody was removed by washing with PBS containing 0.05% Tween (PBS-T), and the wells were blocked with 10% fetal calf serum (FBS) in PBS-T (assay diluent). Culture supernatants were added to the wells and incubated at 4ºC overnight. After the plates were washed, 100 μL of 1 μg/mL biotin-conjugated anti-bovine IFN-γ antibody (AbD Serotec, Oxford, United Kingdom) diluted in assay diluent were added and the plates were incubated for 60 min. After further washing, 100 μL of streptavidin-horseradish peroxidase (HRP) (AbD Serotec, Oxford, United Kingdom) diluted 1:10,000 in assay diluent were added to the wells and the plates were incubated for 30 min. The plates were washed again and then tetramethylbenzidine (KPL, Gaithersburg, MD, USA) was added to the wells. After 25 min, the reaction was stopped by the addition of 50 μL of 2.5 N H_2SO_4, and the absorbance of each well was read at 450 nm. Recombinant bovine IFN-γ (AbD Serotec, Oxford, United Kingdom) was used for generating a standard curve. IFN-γ levels are given as picograms of protein per milliliter of supernatant. Before analysis, the mean absorbance value from medium control wells was subtracted from that of antigen-stimulation wells. Positive result was defined by IFN-γ value of [antigen-stimulated well – PBS-stimulated well] ≥ 200 pg/mL, and IFN-γ value of [antigen-stimulated well – PBS-stimulated well] ≥ 15 % IFN-γ value of PBS-stimulated well. Blood culture and IFN-γ ELISA was run in duplicates.

ELISA for antibody detection

Microtiter plates were coated overnight at 4ºC in a humidified incubator with MPB70, MPB83 at 1 μg/mL, 2 μg/mL in 50 mM carbonate buffer (pH 9.5, 100 μl per well), respectively. The plates were washed with PBS-T, and blocked with PBS-T containing 5% normal goat serum (PBST-NGS) for 1 h at 37ºC. Serums diluted 1:300 in PBS-NGS were added and incubated for 2 h at 37ºC. The plates were washed with PBS-T and bound antibody was detected with 100 μL of 1:10,000 HRP - conjugated rabbit anti-bovine IgG (H+L) (KPL, Gaithersburg, MD, USA) diluted in PBST-NGS. After incubation for 1 h at 37ºC, the plates were washed with PBS-T, and substrate (orthophenylenediamine in 0.05 M citrate phosphate buffer (pH 5.0) containing 30 % [v/v] H_2O_2) was added to each well. Following incubation at room temperature for 25 min, 100 μl of stopping solution 2.5 N H_2SO_4 was added, and the absorbance of each wells were then read at 490 nm.

***M. bovis* culture and DNA extraction from hilar lymph node**

Hilar lymph node were homogenized and treated with 2 % NaOH for 15 min, and then centrifuged for 15 min at 4000 rpm. The supernatant was discarded, and tissue homogenates were resuspended in Phosphate-buffered solution. After centrifugation step was repeated, supernatant was discarded. The residues were inoculated onto slopes of ogawa medium containing 0.05 % pyruvate and the medium were incubated for 12 weeks at 37ºC. For DNA extraction, lymph node homogenates were prepared using DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturers' instructions.

Polymerase chain reaction (PCR) for IS*1081*

Smart Taq Pre-Mix (Solgent, Daejeon, Korea) was used for the PCR amplification. DNA prepared as described above and the oligonucleotides 5'-CTG CTC TCG ACG TTC ATC GCC G-3' and 5'-TGG CGG TAG CCG TTG CGC-3' specific for a 113 bp IS*1081* amplicon, were used [18]. PCR reaction was performed with initial denaturation at 95ºC for 7 min was followed by 35 cycles of 30 sec at 94ºC, 60 sec at 58ºC, 30 sec at 72ºC, and final extension at 5 min at 72ºC. The PCR products were analyzed by performing electrophoresis with using 1.5 % agarose gels in 1 × Tris-acetic acid-EDTA buffer (pH 7.2). The 100 bp DNA ladder (Bioneer, Daejeon, Korea) was used for estimating the size of the PCR products.

Statistics and definition

Data were analyzed using Prism Graphpad v5 (Graphpad software, La Jolla, CA, USA). A receiver operating characteristic (ROC) curve was generated for each tested antigen to select the cutoff values that more effectively discriminate positive from negative samples. An unpaired t-test was used to evaluate the differences in IFN-γ and antibody responses. The Mann-Whitney U test was used to compare nonparametric unpaired data. Correlation between MPB70 and MPB83 ELISA was assessed using Pearson's correlation coefficient. Agreement between dichotomized results of each assay was evaluated by the kappa statistic and positive concordance rate. To compare positive reactors of each test, the positive duplicate rate (PDR) was defined as 2 times the number of concordant positive results (C) divided by the sum of all positive results of each assay (X_1+X_2), $PDR = \frac{2*C}{X_1 + X_2}$. PDR is useful to assess rates of cattle having positive responses between each assay, excluding non-reactors. A P value <0.05 was considered to be significant.

III. Results

1. Interferon-gamma assay for detection of *M. bovis* infection

A. IFN-γ responses in TST-positive and TST-negative cattle

A whole blood assay was established in a 96-well culture plate format to determine IFN-γ production after stimulation with the recombinant ESAT6 and CFP10 cocktail antigen. The IFN-γ concentration in each well was determined by comparing the optical density (O.D.) values with those of wells containing recombinant IFN-γ. Standard curves were derived from 8 concentrations ranging from 0.078 ng/mL to 10 ng/mL (Fig. 1), and each plate included its own standard curve. As the samples were diluted 1:2 before the assay, the maximum detectable concentration was 20 ng/mL. To compare IFN-γ secretion of TST-positive cattle with those of TST-negative cattle, blood samples obtained from TST-positive cattle from herds with multiple reactors or having strong positive responses (induration diameter >10 mm) (n = 135). TST-negative blood samples were originated from known bovine TB-free herds for more than 5 years (n = 100). The majority of the 135 TST-positive cattle produced more than 0.5 ng/mL of IFN-γ, compared to none of 100 TST-negative cattle from bovine TB-free herds (Fig. 2).

B. Sensitivity and specificity of the IFN-γ assay for detection of *M. bovis* infection

Using the results of IFN-γ concentration of TST-positive and negative cattle, a receiver operating characteristics (ROC) curve was generated for the IFN-γ assay using the ESAT6 and CFP10 cocktail antigen in order to determine the cutoff value for an IFN-γ-positive result (Fig. 3). The most appropriate cutoff was defined as the point from the ROC curve with the

greatest distance from the diagonal line (sensitivity = 1 – specificity). On this basis, the appropriate cutoff IFN-γ concentration was 0.125 ng/mL; however, 0.2 ng/mL of IFN-γ was used instead in order to improve the specificity of the IFN-γ assay without losing much sensitivity. When this cutoff was applied, 118 (87.4%) of the 135 TST-positive animals gave IFN-γ assay-positive results indicating that the assay sensitivity was 87.4%, and none of the 100 TST-negative cattle gave IFN-γ-positive results, indicating that its specificity was 100% (Table 1). Therefore, the IFN-γ-positive results may be indicative of TST-positive results or *M. bovis* infection in cattle with a high specificity.

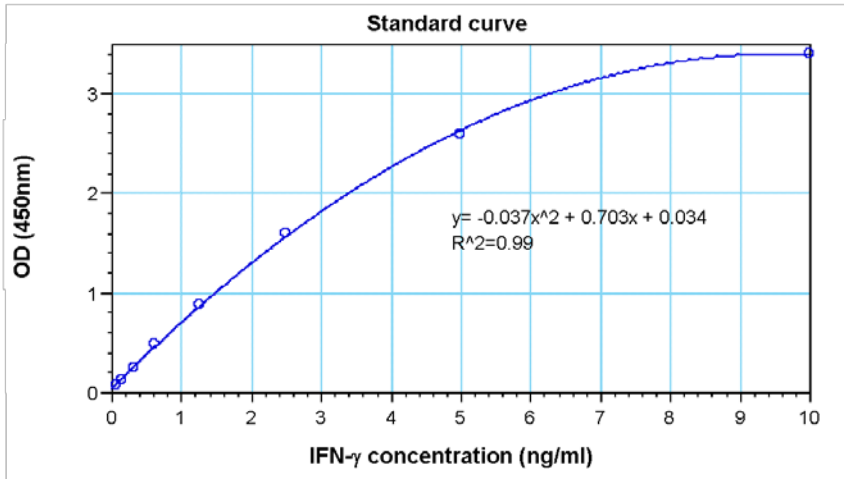

Figure 1. Standard curve for IFN-γ ELISA showing measurement of recombinant bovine IFN-γ. Using sandwich ELISA, the bovine IFN-γ standard curve was determined by comparing the concentrations with O.D. values of wells containing recombinant IFN-γ. Linearity was determined at 8 concentration points ranging from 0.078 to 10 ng/mL.

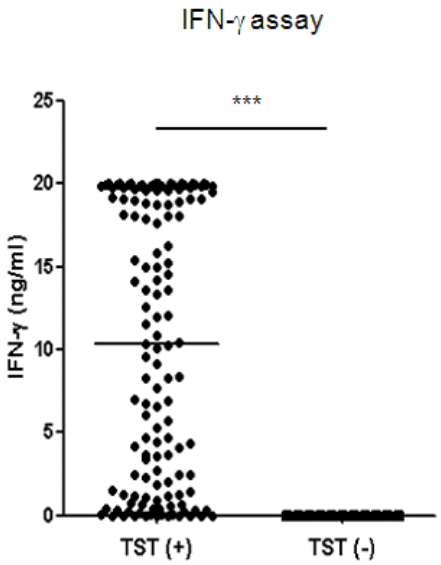

Figure 2. IFN-γ secretion to the ESAT6 and CFP10 cocktail antigen in TST-positive and -negative cattle. The blood samples of 135 TST-positive and 100 TST-negative cattle were stimulated by ESAT6+CFP10 cocktail antigen and the levels of IFN-γ secretion were determined by using sandwich ELISA. ***, $p < 0.0001$.

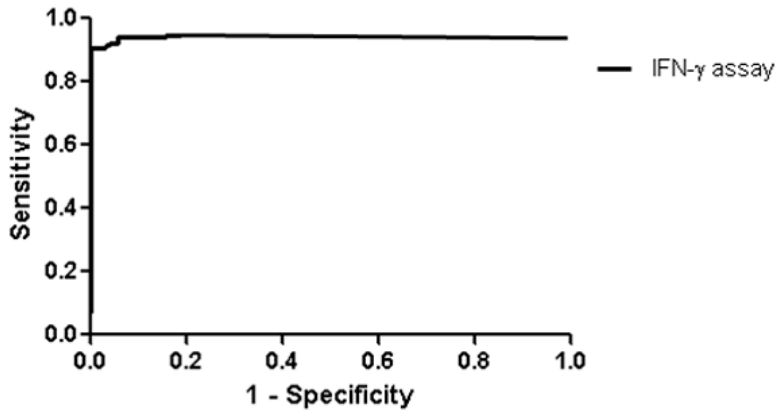

Figure 3. Calculated ROC curves for IFN-γ assay using the ESAT6 and CFP10 cocktail antigen. Using the results of IFN-γ concentration of 135 TST-positive and 100 negative cattle, ROC curve was generated for the IFN-γ assay using the ESAT6 and CFP10 cocktail antigen to determine the cutoff value. IFN-γ assay AUC = 0.958, 95% confidence interval (CI) = 0.930 to 0.985.

Table 1. Comparison between TST and IFN-γ assay for detection of *M. bovis* infection in cattle

Cattle	IFN-γ assay (%)	
	Positive	Negative
TST-positive (n = 135) [a]	118 (87.4)	17 (12.6)
TST-negative (n = 100) [b]	0 (0)	100 (100)

[a] Tuberculin skin test (TST)-positive cattle from herds with multiple reactors or having strong positive response (induration diameter >10 mm).

[b] TST-negative cattle from bovine TB-free herds for more than 5 years.

C. Extent of *M. bovis* infection determined using the IFN-γ assay in the herds with bovine TB outbreaks

Only TST-positive animals were selectively culled during annual screening, and we therefore wished to determine the extent of undetected *M. bovis* infection in the remaining cattle using the IFN-γ assay described above. We tested a total of 374 animals from two groups of the herds with bovine TB outbreaks; one consisting of 260 TST-negative cattle from 11 dairy herds that suffered bovine TB outbreaks as determined by the most recent annual test (recent outbreak), and the other consisted of 114 TST-negative cattle from 4 herds with a history of bovine TB outbreaks but in which all of the animals tested negative in the most recent annual test (remote outbreak). The mean IFN-γ concentration of the animals from the herds with remote bovine TB outbreaks was significantly higher than that of the animals from the herds with recent bovine TB outbreaks ($p < 0.05$, Student's *t* test) (data not shown). When the cutoff criteria set by this study was applied for the IFN-γ assay, 84 (32.3%) of the 260 TST-negative cattle from the herds with recent bovine TB outbreaks and 42 (36.8%) of 114 TST-negative animals from the herds with bovine TB outbreaks that occurred at least 2 months previously tested positive for IFN-γ, respectively (Table 2). These results indicate that a substantial portion of animals had an infection that was not detected by the annual TST screen. In addition, although there was no statistical difference between the two groups, there seemed to be a trend towards a greater number of *M. bovis* infections over time. However, there was a marked variation in *M. bovis* infection rates among the dairy cattle herds regardless of the number of TST-positive animals (Table 3). In B herd, only one (3.7%) of 27 cattle was TST-positive while 20 (74.1%) were IFN-γ-positive, whilst in another, thus missing 19 animals with *M. bovis* infection by TST. On the other hand, 6 (16.2%) of 37cattle in the herd H were TST-positive while 7 (18.9%) of 37 cattle were

IFN-γ-positive, thus missing only one animal by TST. Based on the results above, therefore, the total depopulation of animals in herd with bovine TB outbreak would be more justifiable as a bovine TB control practice.

Table 2. The results of IFN-γ assay for the cattle in the herds with bovine TB outbreaks recently and remotely

Cattle herds	IFN-γ assay (%)	
	Positive	Negative
Recent outbreaks (n = 260)[a]	84 (32.3)	176 (67.7)
Remote outbreaks (n = 114)[b]	42 (36.8)	72 (63.2)

[a] TST-negative cattle in the herds with recent bovine TB outbreaks excluding TST-positive cattle.

[b] TST-negative cattle in the herds with remote bovine TB outbreaks occurred 2–12 months previously.

Table 3. The results of TST and IFN-γ assay in the herds with bovine TB outbreaks

Herds[a]	No. (%) of Positive/ Tested		
	TST	IFN-γ assay	IFN-γ assay excluding TST (+)[b]
A	1 (2.4) /41	15 (36.6) /41	14 (35.0) /40
B	1 (3.7) /27	20 (74.1) /27	19 (73.1) /26
C	1 (3.7) /27	0 (0.0) /27	0 (0.0) /26
D	1 (4.0) /25	15 (60.0) /25	14 (58.3) /24
E	2 (4.3) /46	9 (19.6) /46	8 (18.2) /44
F	1 (9.1) /11	4 (36.4) /11	3 (30.0) /10
G	5 (14.3) /35	15 (42.9) /35	10 (33.3) /30
H	6 (16.2) /37	7 (18.9) /37	1 (3.2) /31
I	8 (47.1) /17	8 (47.1) /17	1 (11.1) /9
J	19 (54.3) /35	25 (71.4) /35	11 (68.8) /16
K	11 (73.3) /15	13 (86.7) /15	3 (75.0) /4
Total	56 (17.7) /316	131 (41.5) /316	84 (32.3) /260

[a] Herds with recent bovine TB outbreaks.

[b] TST-negative cattle in the herds with bovine TB outbreaks excluding TST-positive cattle.

D. Post-mortem examination for confirmation of *M. bovis* infection

In order to confirm *M. bovis* infection among the TST-negative but IFN-γ-positive cattle, we slaughtered 14 animals in order to examine them for the presence of visible lesions and removed the hilar lymph nodes for culture tests and the molecular detection of *M. bovis* (Fig. 4). No visible lesion was found in the internal organs (including the lung, spleen, liver, and kidney), but 6 cattle had granuloma lesions in their hilar lymph nodes. *M. bovis* was isolated from the hilar lymph nodes of 5 cattle, 4 of which had a caseous lesion. Eleven cattle, including 6 cattle with caseous lesions, were *M. bovis*-specific IS*1081* PCR positive, thus confirming that the IFN-γ assay used in this study can detect *M. bovis* in a portion of dairy cattle that were TST negative (Table 4).

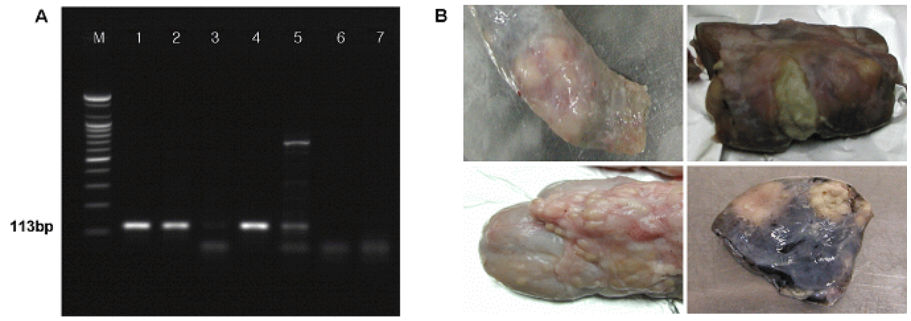

Figure 4. PCR analysis and visible lesion of hilar lymph nodes of TST-negative cattle.

A. Electrophoresis on 1.5 % agarose gel showing the 113 bp PCR products after amplification with a set of primers IS*1081* F/R. Lanes M: 100 bp DNA size marker. Lanes 1: *M. bovis* DNA. Lanes 2−7: samples of hilar lymph nodes. B. Visible lesions of hilar lymph nodes from cattle showing positive response to IFN-γ assay but negative to TST.

Table 4. The results of post-mortem examination of IFN-γ assay-positive but TST-negative cattle

Cattle	Visible lesion	Culture	PCR (IS*1081*)
1	+	+	+
2	-	-	+
3	+	+	+
4	-	+	+
5	-	-	-
6	+	+	+
7	+	+	+
8	+	-	+
9	-	-	+
10	+	-	+
11	-	-	+
12	-	-	-
13	-	-	-
14	-	-	+
Total [a]	6/14	5/14	11/14

[a] The number of cattle with positive results/ the number of cattle tested.

2. Antibody tests for detection of *M. bovis* infection

A. Establishment of antibody test using MPB70 and MPB83

To diagnose chronic infection of bovine TB efficiently, antibody tests were established and evaluated for detection of dairy cattle with *M. bovis* infection in comparison with TST. Mycobacterial antigen MPB70 and MPB83 were prepared and used for ELISA-based antibody test. Blood samples obtained from TST-positive cattle from herds with multiple reactors or having strong positive responses (induration diameter > 10 mm) were used for positive control (n = 178). Negative control blood samples originated from known bovine TB-free herds for more than 5 years (n = 116). Strong antibody responses to MPB70 and to MPB83 were also observed in TST-positive cattle, but not in TST-negative, non-infected cattle ($p < 0.0001$) (Fig. 5). Such antibody responses to mycobacterial antigens used in this study were highly specific and could distinguish between *M. bovis*-infected and non-infected cattle efficiently.

Using the antibody ELISA results, ROC curves were generated for antibody ELISA test using MPB70 and MPB83 antigens (Fig. 6). The most appropriate cut-off was defined as the point from the ROC curve with the largest distance from the diagonal line (sensitivity = 1 - specificity). Based on ROC curves, appropriate cut-off values were determined at O.D. at 490 nm of 0.175 and 0.145 for MPB70 and MPB83 ELISA test, respectively. But a cut-off value of O.D. 0.172 for MPB83 ELISA was used in this study to improve specificity. Relative sensitivities based on TST-positive reactors and specificities of each assay were calculated (Table 5). The recombinant MPB70 antigen alone gave a sensitivity of 73.6% and specificity of 98.3% in antibody test for detection of *M. bovis* infection in cattle when TST results were used as the criteria for its infection. Although the sensitivity of antibody test was improved to 77.0% by combining the results of MPB70 and MPB83

antigens, its specificity decreased to 95.7%. The subsequent analysis of antibody test results was, however, based on the combined results of the two antigens in order to improve its sensitivity in detecting *M. bovis*-infected cattle.

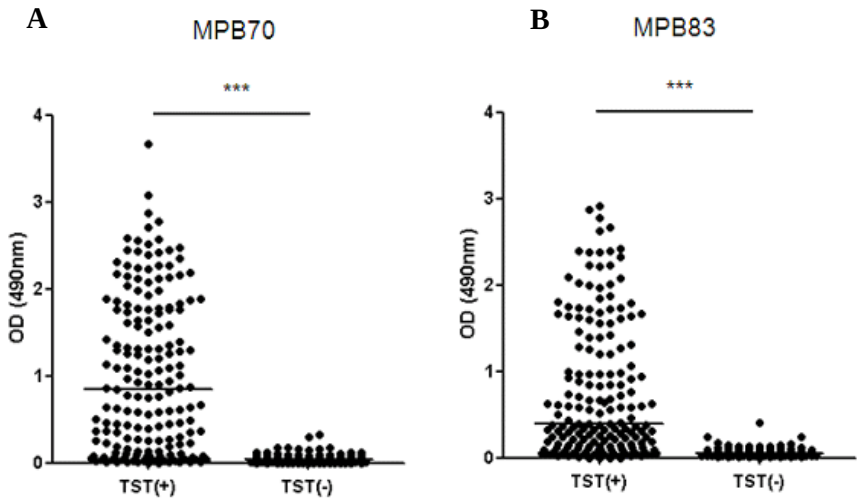

Figure 5. Antibody responses to MPB70 and MPB83 in TST-positive and -negative cattle. The levels of antibody to mycobacterial antigen MPB70 and MPB83 were determined in the 178 TST-positive and 116 TST-negative cattle. A. Antibody responses to MPB70. B. Antibody responses to MPB83. Lines indicate the median level. $***$, $p < 0.0001$.

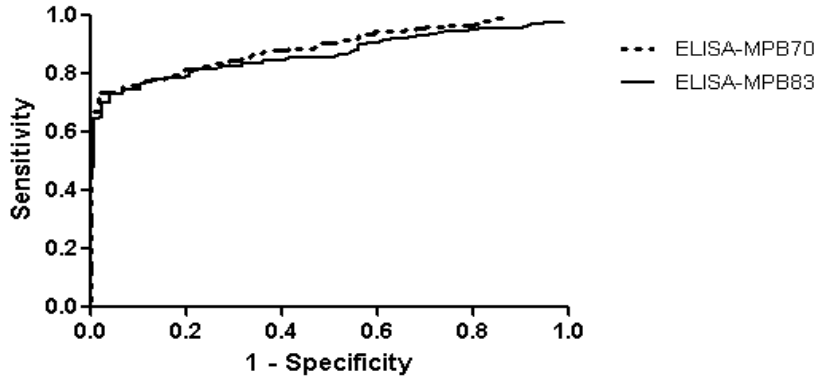

Figure 6. Calculated ROC curves for antibody ELISA using MPB70 and MPB83. Using the antibody ELISA results of the 178 TST-positive and 116 TST-negative cattle, ROC curves were generated for antibody test using MPB70 and MPB83 antigens to determine the cutoff value. ELISA-MPB70 AUC = 0.892, 95% CI = 0.856 to 0.928; ELISA-MPB83 AUC = 0.866, 95% CI = 0.824 to 0.908.

Table 5. TST-based relative sensitivity and specificity of antibody tests

Antigen in ELISA	Sensitivity (No. of Positive / TST-positive[a])	Specificity (No. of Negative / Non-infected)
MPB70	73.6% (131/178)	98.3% (114/116)
MPB83	70.2% (125/178)	97.4% (113/116)
Antibody test[b]	77.0% (137/178)	95.7% (111/116)

[a] TST-positive, multiple reactors from herds with bovine TB outbreaks or having strong positive response (induration diameter > 10 mm).

[b] ELISA results to both MPB70 and MPB83 were combined, i.e., a cattle positive either to MPB70 or MPB83 was considered positive.

B. Extent of *M. bovis* infection determined by antibody test in cattle herds with incomplete culling practice

Antibody test using the MPB70 and MPB83 antigens were evaluated with 374 animals from two groups of the herds with bovine TB outbreaks; one consisting of 260 TST-negative cattle from 11 dairy herds with bovine TB outbreaks as determined by the most recent annual test (recent outbreak), and the other consisted of 114 TST-negative cattle from 4 herds with a history of bovine TB outbreaks but in which all of the animals tested negative in the most recent retest within 2-12 months (remote outbreak) (Table 6). A high level of antibody response to MPB70 and MPB83 was also detected in cattle from herds with recent bovine TB outbreak. Similarly to the results of IFN-γ assay, antibody responses were still detectable in TST-negative cattle from herds with remote bovine TB outbreak. Using the cutoff value of antibody tests for diagnosis, 94 (36.2%) of the 260 TST-negative cattle from the herds with recent bovine TB outbreaks and 41 (36.0%) of 114 TST-negative animals from the herds with bovine TB outbreaks. There was no significant difference in seroreactive rate between the two groups. When the results of 11 herds with bovine TB outbreaks were analyzed, a wide range of positive responses in antibody test was shown (Table 7). In A herd, one (2.4%) of 41 cattle was TST-positive while 14 (31.7%) were positive for antibody test, and two (4.3%) of 46 in E herd were TST-positive but 25 (54.3%) were positive for antibody test. The herds with high TST-positive rates in general had a high level of positive rate for antibody test.

Table 6. The results of antibody test for the cattle in the herds with bovine TB outbreaks recently and remotely

Cattle herds	Antibody test (%)	
	Positive	Negative
Recent outbreaks (n = 260)[a]	94 (36.2)	166 (63.8)
Remote outbreaks (n = 114)[b]	41 (36.0)	73 (64.0)

[a] TST-negative cattle in the herds with recent bovine TB outbreaks excluding TST-positive cattle.

[b] TST-negative cattle in the herds with remote bovine TB outbreaks occurred 2–12 months previously.

Table 7. The results of TST and antibody test in the herds with bovine TB outbreaks

Herds[a]	No. (%) of Positive / Tested		
	TST	Antibody test	Antibody test excluding TST (+)[b]
A	1 (2.4) / 41	13 (31.7) / 41	12 (30.0) / 40
B	1 (3.7) / 27	4 (14.8) / 27	3 (11.5) / 26
C	1 (3.7) / 27	8 (29.6) / 27	7 (26.9) / 26
D	1 (4.0) / 25	6 (24.0) / 25	4 (16.7) / 24
E	2 (4.3) / 46	25 (54.3) / 46	23 (52.3) / 44
F	1 (9.1) / 11	3 (27.3) / 11	3 (30.0) / 10
G	5 (14.3) / 35	25 (71.4) / 35	20 (66.7) / 30
H	6 (16.2) / 37	6 (16.2) / 37	6 (19.4) / 31
I	8 (47.1) / 17	8 (47.1) / 17	0 (0.0) / 9
J	19 (54.3) / 35	32 (91.4) / 35	13 (81,3) / 16
K	11 (73.3) / 15	14 (93.3) / 15	3 (75.0) / 4
Total	56 (17.7) / 316	144 (45.6) / 316	94 (36.2) / 260

[a] Herds with recent bovine TB outbreaks.

[b] TST-negative cattle in the herds with bovine TB outbreaks excluding TST-positive cattle.

3. Comparison of TST, IFN-γ assay, and antibody test for detection of *M. bovis* infection

We analyzed the results of TST, IFN-γ assay and antibody test for cattle in each herd with recent bovine TB outbreak (Table 8). The herds with relatively low TST-positive rates (< 20%) had a wide range of positive rates of IFN-γ assay and antibody test. In herd B with only 3.7% TST-positivity, 20 (74.1%) of 27 cattle had positive responses in the IFN-γ assay, but only 4 (14.8%) of 27 cattle were positive in the antibody test. On the other hand, in herd C with the same 3.7% TST-positivity, all of 27 cattle showed negative responses in the IFN-γ assay, but 8 (29.6%) of 27 cattle were positive in the antibody test. In comparison, the herds with high TST-positive rates (> 50%), like J and K herds, also showed a high level of positive rates in the IFN-γ assay and antibody test (> 70%). In overall, the positive rate of the IFN-γ assay was similar to that of the antibody test, which was significantly higher than that of TST ($p < 0.001$, Chi-square test).

The overall agreement between each assay was determined by using kappa value (κ) and concordance rate (CR) (Table 9). In spite of low positive rates, TST showed higher agreement with IFN-γ assay ($\kappa = 0.39$, CR = 71%) than antibody test ($\kappa = 0.32$, CR = 68%). The agreement between IFN-γ assay and antibody test was the lowest ($\kappa = 0.29$, CR = 65%), which indicates the time of IFN-γ secretion response may be different from that of antibody response after *M. bovis* infection. Since the negative response rate of the assays were more than 60%, the agreement by using kappa value (κ) and concordance rate (CR) had a tendency of higher values than expected. For these reasons, to compare only positive reactors of each assay, we used positive duplicate rate (PDR) as defined in materials and methods (Fig. 7). Among the 316 blood samples from cattle in the herd with recent bovine TB outbreak, 40 samples showed positive responses for all assays. Of 144

samples positive to antibody test, 82 samples also showed positive responses to IFN-γ assay, resulting in PDR of 0.596 (82 x 2 / [131 + 144]) between antibody test and IFN-γ assay. Such value means only 59.6% of positive reactors between two assays were concordant. PDR between TST and IFN-γ assay was 0.502, and PDR between TST and antibody test was 0.48. These results showed only half of positive reactors between each assay were concordant. A total of 56 TST reactors had positive responses to IFN-γ assay or antibody test. The number of cattle showing IFN-γ assay or antibody test-positive but TST-negative results was 137, which indicated TST had the lowest sensitivity for detection of *M. bovis*-infection.

Table 8. Comparison of TST, IFN-γ assay and antibody test in each herd with bovine TB outbreak

Herds[a]	No. (%) of Positive / Tested		
	TST	IFN-γ assay	Antibody test
A	1 (2.4) / 41	15 (36.6) / 41	13 (31.7) / 41
B	1 (3.7) / 27	20 (74.1) / 27	4 (14.8) / 27
C	1 (3.7) / 27	0 (0.0) / 27	8 (29.6) / 27
D	1 (4.0) / 25	15 (60.0) / 25	6 (24.0) / 25
E	2 (4.3) / 46	9 (19.6) / 46	25 (54.3) / 46
F	1 (9.1) / 11	4 (36.4) / 11	3 (27.3) / 11
G	5 (14.3) / 35	15 (42.9) / 35	25 (71.4) / 35
H	6 (16.2) / 37	7 (18.9) / 37	6 (16.2) / 37
I	8 (47.1) / 17	8 (47.1) / 17	8 (47.1) / 17
J	19 (54.3) / 35	25 (71.4) / 35	32 (91.4) / 35
K	11 (73.3) / 15	13 (86.7) / 15	14 (93.3) / 15
Total	56 (17.7) / 316	131 (41.5) / 316	144 (45.6) / 316

[a] Herds with recent bovine TB outbreaks.

Table 9. Agreement between results of different diagnostic assays for the cattle in the herds with bovine TB outbreaks recently (n = 316)

Assay	IFN-γ assay		Antibody test	
	%[a]	κ[b]	%	κ
TST	71	0.39	68	0.32
IFN-γ assay	–	–	65	0.29

[a] Concordance rate (%) : number of cattle with concordant positive or negative result divided by the total number tested.

[b] Level of agreement measured by Cohen's kappa (κ).

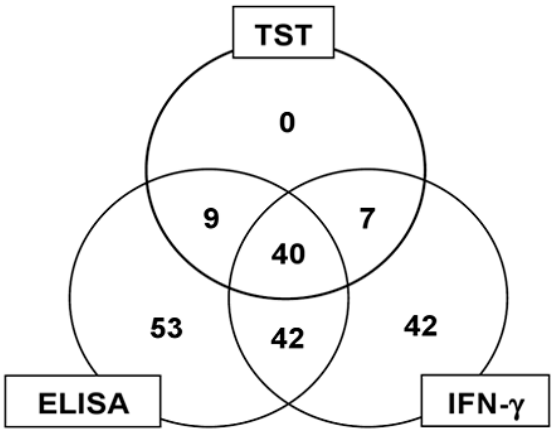

Figure 7. Comparison of positive reactors to TST, IFN-γ assay and antibody test in the herds with bovine TB outbreaks recently. The cattle of herds with recent bovine TB outbreaks were tested by immunodiagnostic assays. Of the 316 cattle tested, 56, 131 and 144 cattle were positive by TST, IFN-γ assay and antibody test, respectively.

IV. Discussion

TST has been the international standard for diagnosis of bovine TB in dairy herds. TST are based on eliciting delayed type hypersensitivity (DTH) response to intradermal injection of PPD from *M. bovis*. Recently, to improve sensitivity and specificity of diagnosis for bovine TB, ancillary methods using IFN-γ secretion and antibody response to mycobacterial antigens are introduced in many countries [19, 20]. In Korea, only TST has been used for ante-mortem diagnosis confirming *M. bovis* infection.

In this study, we determined IFN-γ assay using ESAT6+CFP10 and antibody response ELISA using MPB70, MPB83. Strong IFN-γ secretion and antibody responses to mycobacterial antigens were observed in TST-positive, but not in TST-negative cattle (Fig. 3). IFN-γ assay, antibody test showed high levels of relative sensitivities based on TST positive reactors (Table 1). ESAT6 and CFP10 are dominant IFN-γ-inducing antigens of tuberculous mycobacteria, which are absent from many environmental nontuberculous mycobacteria (NTM) [21, 22]. In house IFN-γ assay using ESAT6+CFP10 displayed high sensitivity and specificity as previously reported [23, 24]. MPB70 and MPB83 are major antigens inducing humoral responses in *M. bovis*-infected animal [25, 26]. Previously, MPB83 was often found to be more reactive to *M. bovis*-infected cattle or deer than MPB70, however, in this study, MPB70 was more sensitive and specific than MPB83 [27].

IFN-γ assay and antibody test were performed in herds with bovine TB outbreaks and non-infected herds. Strong IFN-γ secretion and antibody responses to mycobacterial antigens were shown not only in herds with bovine TB outbreak recently but also remotely. IFN-γ assay or antibody test reactors were 2.4 times more than TST reactors in herds with bovine TB outbreaks. Positive duplicate rates of blood-based assays excluding TST were

more than 50% (Fig. 6). Interestingly, many reactors to blood-based assays were also observed in herds with bovine TB outbreaks remotely within 2–12 months, showing no response to TST recently. After that, such cattle were tested 2–4 times more, but did not react to TST. Post-mortem examination results confirmed such cattle reacting to only blood-based assays were also infected by *M. bovis* (Fig. 7). These results indicated TST had lower discriminatory ability of *M. bovis*-infected cattle in Korea.

Similarly to our results, recent studies reported that cattle with bovine TB can be missed by the TST, most of which will not be detected following a repeat of TST but can be identified by in vitro blood-based assays [28]. Sensitivity of TST ranged from 63.2% to 100%, with a median sensitivity value of 83.9% [20]. However, various factors and host state, like desensitization, anergy and immunosuppressive drug, may lower sensitivity and accuracy of TST. Likewise, TST is often described as good ‘‘herd tests’’, but poor tests for identifying individual infected animals [20]. The results of each herd with bovine TB outbreaks also support such indication. Some herds had 1 or 2 positive reactors to TST, but showed high rates of IFN-γ assay or antibody test positivity. To detect infected cattle of such herds effectively, individual cattle need to be tested by various immunodiagnostic assays after primary screening by TST.

In diagnosis of human TB, TST has been known to have lower specificity in populations with high rate of *M. bovis* bacillus Calmette–Guérin (BCG) vaccination and non-tuberculous mycobacteria exposure, because BCG and NTM have homologous antigens with *M. tuberculosis* PPD and may induce non-specific host response to TST [29, 30]. In Korea, BCG vaccination has not been performed to cattle and there were no well-known NTM infection. Therefore, TST is considered to have high specificity to diagnose bovine TB with a low possibility showing false positive reaction. In many

studies, TST has been reported to have comparable sensitivity to IFN-γ assay for diagnosis for human TB (sensitivity value: 75–90%, 80–90% for TST, IFN-γ assay, respectively) [30, 31]. In immunocompetent populations, the sensitivity of two tests have no significant difference, however, sensitivity of TST may be lower in immunocompromised patients (e.g. HIV infection) than that of IFN-γ assay. Therefore, sequential testing with TST and IFN-γ assay is also used to predict latent tuberculosis infection in humans [32, 33].

This study had limitations. Because of the lack of a gold standard for *M. bovis* infection, we could not determine the sensitivity and specificity of diagnostic assays. To determine the sensitivity accurately, veterinary inspection to all animals tested is necessary and the presence of *M. bovis* must be confirmed by laboratory methods. However, because this study was performed in privately owned cattle herds, all animals tested could not be slaughtered and inspected. For the same reason, we could perform post-mortem examination on only a small number of TST-negative but IFN-γ assay-positive cattle. We need to validate diagnostic assays with post-mortem examination on more animals tested to provide more reliable analysis.

In conclusion, TST having been used for long time in Korea showed low reactivity to *M. bovis*-infected cattle. Previous reports indicated the routine use of a screening test with imperfect sensitivity could result in a substantial pool of undetected infection in the cattle herd [34, 35]. Similarly, in this study, the herds with bovine TB outbreaks were shown to have infected cattle unresponsive to TST. To control and eradicate bovine TB in Korea, in addition to TST, applications of highly sensitive and combinatory diagnostics detecting different sub-populations of infected animals are required.

Chapter II. Molecular Epidemiology of Bovine Tuberculosis

I. Introduction

M. bovis is the causative agent of bovine TB. Bovine TB is a chronic wasting disease that causes significant problems for human public health and agricultural economies [2]. *M. bovis* can infect cattle, deer, wildlife, as well as humans. Therefore, bovine TB is classified as a major zoonosis that threatens human public health worldwide. *M. bovis* is responsible for causing 5–10% of human TB in developing countries where veterinary supervision and food hygiene practices may not be applied properly [3]. In developed countries, the proportion of infection caused by *M. bovis* in human TB cases is very low, ranging from 0.3% to 1.5%. Recently, however, HIV prevalence has been reported to affect the epidemiology of human TB due to *M. bovis* not only in developing countries but also in developed countries [4].

Epidemiological knowledge is important for effective bovine TB control. Epidemiology helps us understand the transmission and sources of *M. bovis* infection. However, conventional trace back approaches cannot precisely identify *M. bovis* or distinguish among the strains. In recent years, molecular epidemiology has provided detailed information, which enables us to trace the source of infection and interspecies transmission more efficiently [36, 37]. In addition, the integration of molecular epidemiology and mathematical modeling helps us to analyze the risk factors of infection and improve surveillance strategies [38].

Standard TB molecular typing techniques include restriction fragment length polymorphism (RFLP), spoligotyping, and variable-number tandem repeat (VNTR) analysis. Analysis with RFLP using polymorphisms of the

insertion sequence *6110* (IS*6110*) has shown high discriminatory power and has been widely used for identification of the *M. tuberculosis* complex. However, *M. bovis* strains usually have a single or few IS*6110* copies; therefore, IS*6110* RFLP has lower sensitivity for typing *M. bovis*. Spoligotyping is based on spacer sequence polymorphisms within direct repeat regions. Spoligotyping is often used for *M. bovis* typing, but has been found to be less discriminatory than RFLP and VNTR [39].

VNTR typing is based on the analysis of multiple genomic sequences, including MIRU, ETR, and QUB, and has been introduced as a tool to study the molecular epidemiology of *M. bovis* [40, 41]. VNTR typing is a PCR-based tool in which results are expressed digitally. MIRU-VNTR typing is a low-cost and easily interpretable method [39]. In addition, owing to the high stability of the markers, MIRU-VNTR typing provides reproducible results [42]. Recently, MIRU-VNTR typing showed better discriminatory power than spoligotyping for *M. bovis* types in Northern Ireland and Chad [43, 44].

In this study, we performed VNTR analysis of 133 *M. bovis* isolates from Korea using 30 previously reported MIRU-VNTR loci. *M. bovis* isolates were collected from dairy cattle, Korean beef cattle, and deer diagnosed with infections. There are no known environmental reservoirs of *M. bovis* in Korea. Therefore, our study focused on the epidemiologic relationships of *M. bovis* in domestic animals. Using VNTR typing, we examined the possibility of interspecies and inter-herd transmission and analyzed the prevalent characteristics and genetic relationships of *M. bovis* strains in Korea.

II. Materials and Methods

M. bovis isolates

The 133 *M. bovis* isolates included in this study were collected by the Veterinary Service Laboratories from 2003 to 2010 from 59 Holstein dairy cattle, 40 Korean beef cattle, and 34 deer showing tuberculosis-like lesions. Hilar lymph node samples from animals suspected to be positive for bovine TB were collected, homogenized in sterile saline solution, and decontaminated with 2% NaOH for 15 min at room temperature. After centrifugation at 4,000 rpm for 15 min, culturing was done on Lowenstein–Jensen (LJ) media (Difco, Detroit, MI, USA) containing 0.05% pyruvate and incubated for 6 to 8 weeks at 37°C. *M. tuberculosis* H37Rv (ATCC 27294) was used as a reference strain; the genomic sequence information is available.

DNA preparation

Genomic DNA was extracted from *M. bovis* isolates as described below [45]. In brief, *M. bovis* isolates obtained from LJ media were grown in Middlebrook 7H9 liquid medium (Difco, Detroit, MI, USA) supplemented with oleic acid-albumin-dextrose-catalase and Tween 80 for 3 to 4 weeks at 37°C. Cultures were collected by centrifugation at 12,000 rpm for 10 min and resuspended in 250 μL of sterile distilled water. The suspended cultures were boiled in a water bath for 5 min, and supernatants collected after removing the cellular debris by centrifugation. DNA concentration was measured at 260 nm with a spectrophotometer (Pharmacia Biotech, Piscataway, NY, USA) and stored at −20°C until use in PCR reactions.

VNTR-PCR analysis

Smart *Taq* Pre-Mix (Solgent, Daejeon, Korea) was used for PCR

amplification. Twelve MIRU, 3 ETR (A to C), 4 QUB (11a, 15, 18, 26), and 11 VNTR (0424, 1895, 1955, 2347, 2401, 2990, 3171, 3232, 3336, 3690, 4156) primer pairs were used (Table 1). The PCR reaction was performed with a 20-μL PCR pre-mixture, contained each primer (forward and reverse) at a final concentration of 0.5 μM and 20 ng of genomic DNA as a template. PCR amplification was performed in a GeneAmp PCR System 2700 (Applied Biosystems, Foster, CA, USA). Initial denaturation at 95°C for 10 min was followed by 35 cycles of elongation at 94°C for 30 s, 58°C for 60 s, and 72°C for 90 s, with a final incubation for 7 min at 72°C. *M. tuberculosis* H37Rv genomic DNA and sterile distilled water were the positive and negative controls, respectively, for each set of reactions. PCR products were analyzed by 1.5% agarose gel electrophoresis in 0.5X Tris-boric acid-ethylenediaminetetraacetic acid buffer (pH 7.2). The TriDye 100-bp DNA ladder (New England Biolabs, Inc., Ipswich, MA, USA) was used to estimate the size of PCR products.

Statistics and definition

The discriminatory power of individual and combined VNTR markers was determined by calculating the allelic diversity (*h*) using the following equation: $h = 1 - \sum x_i^2[n/(n\text{-}1)]$, where n is the number of isolates and x_i is the frequency of the ith allele in the locus [46]. A dendrogram based on MIRU-VNTR profiles recorded as character data was generated using SPSS statistics 18.0 (IBM, Armonk, NY, USA).

Table 1. Primer sequences and repeat unit size of VNTR loci

Locus	Alias	PCR primer sequence (5'-3')[a]	Repeat unit size (bp)
MIRU 2	VNTR 154	TGGACTTGCAGCAATGGACCAACT TACTCGGACGCCGGCTCAAAAT	53
MIRU 4[a]	VNTR 580 ETR-D	CAGGTCACAACGAGAGGAAGAGC GCGGATCGGCCAGCGACTCCTC	77
MIRU 10	VNTR 960	GTTCTTGACCAACTGCAGTCGTCC GCCACCTTGGTGATCAGCTACCT	53
MIRU 16	VNTR 1644	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC	53
MIRU 20	VNTR 2059	GCCCTTCGAGTTAGTATCGTCGGTT CAATCACCGTTACATCGACGTCATC	77
MIRU 23	VNTR 2531	CAGCGAAACGAACTGTGCTATCAC CGTGTCCGAGCAGAAAAGGGTAT	53
MIRU 24	VNTR 2687	CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA	54
MIRU 26	VNTR 2996	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG	51
MIRU 27	VNTR 3007	TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA	53
MIRU 31[a]	VNTR 3192 ETR-E	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT	53
MIRU 39	VNTR 4348	CGCATCGACAAACTGGAGCCAAAC CGGAAACGTCTACGCCCCACACAT	53
MIRU 40	VNTR 802	AAGCGCAAGAGCACCAAG GTGGGCTTGTACTTGCGAAT	54
ETR-A	VNTR 2165	ATTTCGATCGGGATGTTGAT TCGGTCCCATCACCTTCTTA	75
ETR-B	VNTR 2461	GCGAACACCAGGACAGCATCATG GGCATGCCGGTGATCGAGTGG	57
ETR-C	VNTR 0577	GACTTCAATGCGTTGTTGGA GTCTTGACCTCCACGAGTGC	58
QUB 11a	VNTR 2163a	CCCATCCCGCTTAGCACATTCGTA TTCAGGGGGGATCCGGGA	69
QUB 15	VNTR 3155	TACATTCGCGGCCAAAGG AGGGGTTCTCGGTCACCC	54
QUB 18	VNTR 1982	CCGGAATCTGCAATGGCGGCAAATTAAAAG TGATCTGACTCTGCCGCCGCTGCAAATA	78
QUB 26	VNTR 4052	AACGCTCAGCTGTCGGAT GGCCAGGTCCTTCCCGAT	111

VNTR 0424		CTTGGCCGGCATCAAGCGCATTATT GGCAGCAGAGCCCGGGATTCTTC	51
VNTR 1895	QUB 1895	GTGAGCAGGCCCAGCAGACT CCACGAAATGTTCAAACACCTCAAT	57
VNTR 1955		AGATCCCAGTTGTCGTCGTC CAACATCGCCTGGTTCTGTA	57
VNTR 2347		AACCCATGTCAGCCAGGTTA ATGATGGCACACCGAAGAAC	57
VNTR 2401		AGTCACCTTTCCTACCACTCGTAAC ATTAGTAGGGCACTAGCACCTCAAG	58
VNTR 2990		GTGACGTTTACCGTGCTCTATTTC GTCGTCGGACAGTTCTAGCTTT	55
VNTR 3171		GCAGATAACCCGCAGGAATA GGAGAGGATACGTGGATTTGAG	54
VNTR 3232	QUB 3232	TGCCGCCATGTTTCATCAGGATTAA GCAGACGTCGTGCTCATCGATACA	56(57)
VNTR 3336	QUB 3336	ATCCCCGCGGTACCCATC TTCTACGACTTCGCAACCAAGTATC	59
VNTR 3690		AATCACGGTAACTTGGGTTGTTT GATGCATGTTCGACCCGTAG	58
VNTR 4156	QUB 4156	TGGTCGCTACGCATCGTGTCGGCCCGT TACCACCCGGGCAGTTTAC	59

VNTR, variable-number tandem repeat; MIRU, mycobacterial interspersed repetitive units; ETR, exact tandem repeats; QUB, Queen's University Belfast.

[a] Forward and reverse primers, respectively.

III. Results

1. Analysis of MIRU-VNTR loci on *M. bovis* isolates

MIRU-VNTR analysis was performed for 133 *M. bovis* isolates using 30 published markers, including 12 MIRU, 3 ETR, 4 QUB, and 11 VNTR loci. Sixteen of the 30 VNTR markers showed genetic polymorphism (Table 2). Polymorphic loci included 4 MIRUs (4, 26, 27, 31), 2 ETRs (A, B), 3 QUBs (11a, 18, 26), and 7 VNTRs (0424, 1895, 2401, 3171, 3232, 3336, 3690).

Allelic diversity (*h*) differed in individual loci, ranging from 0.00–0.59. The VNTR 3336 locus showed the highest discriminatory power, with *h* = 0.59 (Fig. 1). QUB 26, MIRU 31, VNTR 2401, and VNTR 3171 also showed high allelic diversity (*h* = 0.42), and 11 loci (MIRU 26, VNTR 0424, VNTR 3690, VNTR 1895, ETR B, MIRU 4, MIRU 27, QUB 11a, VNTR 3232, QUB 18, and ETR-A) showed low discriminatory power (*h* = 0.01–0.18).

The allelic diversity (*h*) differed among individual loci in the 59 isolates from Holstein dairy cattle, ranging from 0.00–0.64 (Table 3). The VNTR 3336 locus showed the highest discriminatory power (*h* = 0.64). QUB 26, MIRU 31, VNTR 2401, and VNTR 3171 also showed high allelic diversity (*h* = 0.35), and other 4 loci (MIRU 26, ETR B, VNTR 0424, VNTR 1895) showed low discriminatory power (*h* = 0.02–0.05).

In the 40 *M. bovis* isolates from Korean beef cattle, the discriminatory power of VNTR loci ranged from 0.00–0.45 (Table 4). The VNTR 3336 locus showed the highest discriminatory power (*h* = 0.45). QUB 26, MIRU 31, VNTR 2401, and VNTR 3171 also showed high allelic diversity (*h* = 0.38), and other 8 loci (VNTR 3690, ETR-B, ETR-A, MIRU 4, MIRU 27, QUB 11a, QUB 18, and VNTR 3232) showed low discriminative power (*h* = 0.02–0.12).

Allelic diversity (*h*) of VNTR markers ranged from 0.00–0.50 in 34 *M.*

bovis isolates obtained from deer (Table 5). The VNTR 3336 locus showed the highest discriminatory power (h = 0.50). QUB 26, MIRU 31, VNTR 2401, and VNTR 3171 also showed high allelic diversity (h = 0.48); 6 other loci (ETR-B, VNTR 3232, ETR-A, MIRU 27, MIRU 4, and QUB 11a) showed low discriminatory power (h = 0.03–0.19).

Of 12 MIRU loci, MIRU 31 showed the highest discriminatory power (h = 0.42). Four loci displayed less allelic diversity, and the remaining 8 loci had no allelic diversity. Among the QUB loci, QUB 26 had the highest discriminatory power (h = 0.42), and 3 of the 4 loci showed allelic diversity. In *M. bovis* isolates in Korea, the QUBs and other VNTRs had more polymorphic regions than MIRUs and ETRs.

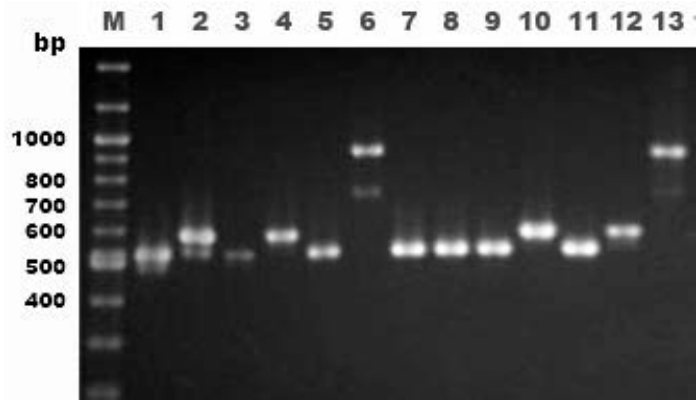

Figure 1. PCR amplification of the VNTR 3336 locus in *M. bovis* isolates.

Locus VNTR 3336 was amplified by PCR and the products resolved by 1.5% agarose gel electrophoresis. Length polymorphisms of various *M. bovis* isolates correspond to multiples of a 59-bp tandem repeat unit. Lane M: 100-bp DNA ladder; lanes 1–13: *M. bovis* isolates.

Table 2. MIRU-VNTR allelic distribution among total 133 isolates

Locus	No. isolates with VNTR allele													Allelic diversity (*h*)
	1	2	3	4	5	6	7	8	9	10	D[a]	S[b]	T[c]	
MIRU 2		133												**0.00**
MIRU 4		1	126		6									**0.09**
MIRU 10		133												**0.00**
MIRU 16		133												**0.00**
MIRU 20		133												**0.00**
MIRU 23				133										**0.00**
MIRU 24		133												**0.00**
MIRU 26				1	132									**0.01**
MIRU 27		7	126											**0.09**
MIRU 31		92	41											**0.42**
MIRU 39		133												**0.00**
MIRU 40		133												**0.00**
ETR-A		1		5		7	120							**0.18**
ETR-B				4	129									**0.05**
ETR-C				133										**0.00**
QUB 11a								6		126		1		**0.09**
QUB 15		133												**0.00**
QUB 18	9	3	121											**0.16**
QUB 26			41	92										**0.42**
VNTR 0424		132	1											**0.01**
VNTR 1895			2	131										**0.02**
VNTR 1955			133											**0.00**
VNTR 2347			133											**0.00**
VNTR 2401				92	41									**0.42**
VNTR 2990			133											**0.00**
VNTR 3171		41	92											**0.42**
VNTR 3232						2	1		3	123	4			**0.14**
VNTR 3336			73	20				1		39				**0.59**
VNTR 3690		132											1	**0.01**
VNTR 4156	133													**0.00**

VNTR, variable-number tandem repeat; MIRU, mycobacterial interspersed repetitive units; ETR, exact tandem repeats; QUB, Queen's University Belfast.

[a]The letter D represents 9, 10 repeats (1 additional copy).

[b]The letter S represents 8, 9, 10 repeats (2 additional copies).

[c]The letter T represents 2, 3, 4 repeats (2 additional copies).

Table 3. MIRU-VNTR allelic distribution among 59 isolates from Holstein dairy cattle

Locus	No. isolates with VNTR allele													Allelic diversity (*h*)
	1	2	3	4	5	6	7	8	9	10	D[a]	S[b]	T[c]	
MIRU 2		59												**0.00**
MIRU 4			59											**0.00**
MIRU 10		59												**0.00**
MIRU 16		59												**0.00**
MIRU 20		59												**0.00**
MIRU 23				59										**0.00**
MIRU 24		59												**0.00**
MIRU 26				1	58									**0.02**
MIRU 27			59											**0.00**
MIRU 31		45	14											**0.35**
MIRU 39		59												**0.00**
MIRU 40		59												**0.00**
ETR-A				5		4	50							**0.26**
ETR-B				1	58									**0.02**
ETR-C				59										**0.00**
QUB 11a										59				**0.00**
QUB 15		59												**0.00**
QUB 18	9		50											**0.25**
QUB 26			14	45										**0.35**
VNTR 0424		58	1											**0.02**
VNTR 1895			2	57										**0.05**
VNTR 1955			59											**0.00**
VNTR 2347			59											**0.00**
VNTR 2401				45	14									**0.35**
VNTR 2990			59											**0.00**
VNTR 3171		14	45											**0.35**
VNTR 3232						2	1		3	53				**0.18**
VNTR 3336			27	18						14				**0.64**
VNTR 3690		59												**0.00**
VNTR 4156	59													**0.00**

VNTR, variable-number tandem repeat; MIRU, mycobacterial interspersed repetitive units; ETR, exact tandem repeats; QUB, Queen's University Belfast.

[a]The letter D represents 9, 10 repeats (1 additional copy).

[b]The letter S represents 8, 9, 10 repeats (2 additional copies).

[c]The letter T represents 2, 3, 4 repeats (2 additional copies).

Table 4. MIRU-VNTR allelic distribution among 40 isolates from Korean beef cattle

Locus	No. isolates with VNTR allele													Allelic diversity (*h*)
	1	2	3	4	5	6	7	8	9	10	D[a]	S[b]	T[c]	
MIRU 2		40												**0.00**
MIRU 4			37		3									**0.12**
MIRU 10		40												**0.00**
MIRU 16		40												**0.00**
MIRU 20		40												**0.00**
MIRU 23				40										**0.00**
MIRU 24		40												**0.00**
MIRU 26					40									**0.00**
MIRU 27		3	37											**0.12**
MIRU 31		29	11											**0.38**
MIRU 39		40												**0.00**
MIRU 40		40												**0.00**
ETR-A		1				1	38							**0.07**
ETR-B				2	38									**0.07**
ETR-C				40										**0.00**
QUB 11a								3		37				**0.12**
QUB 15		40												**0.00**
QUB 18		3	37											**0.12**
QUB 26			11	29										**0.38**
VNTR 0424		40												**0.00**
VNTR 1895				40										**0.00**
VNTR 1955			40											**0.00**
VNTR 2347			40											**0.00**
VNTR 2401				29	11									**0.38**
VNTR 2990			40											**0.00**
VNTR 3171		11	29											**0.38**
VNTR 3232			1							36	3			**0.16**
VNTR 3336			27	2						11				**0.45**
VNTR 3690		39											1	**0.02**
VNTR 4156	40													**0.00**

VNTR, variable-number tandem repeat; MIRU, mycobacterial interspersed repetitive units; ETR, exact tandem repeats; QUB, Queen's University Belfast.

[a]The letter D represents 9, 10 repeats (1 additional copy).

[b]The letter S represents 8, 9, 10 repeats (2 additional copies).

[c]The letter T represents 2, 3, 4 repeats (2 additional copies).

Table 5. MIRU-VNTR allelic distribution among 34 isolates from deer

Locus	No. isolates with VNTR allele													Allelic diversity (*h*)
	1	2	3	4	5	6	7	8	9	10	D[a]	S[b]	T[c]	
MIRU 2		34												**0.00**
MIRU 4		1	30		3									**0.19**
MIRU 10		34												**0.00**
MIRU 16		34												**0.00**
MIRU 20		34												**0.00**
MIRU 23				34										**0.00**
MIRU 24		34												**0.00**
MIRU 26					34									**0.00**
MIRU 27		4	30											**0.18**
MIRU 31		18	16											**0.48**
MIRU 39		34												**0.00**
MIRU 40		34												**0.00**
ETR-A						2	32							**0.08**
ETR-B				1	33									**0.03**
ETR-C				34										**0.00**
QUB 11a								3		30		1		**0.19**
QUB 15		34												**0.00**
QUB 18			34											**0.00**
QUB 26			16	18										**0.48**
VNTR 0424		34												**0.00**
VNTR 1895				34										**0.00**
VNTR 1955			34											**0.00**
VNTR 2347			34											**0.00**
VNTR 2401				18	16									**0.48**
VNTR 2990			34											**0.00**
VNTR 3171		16	18											**0.48**
VNTR 3232										33	1			**0.03**
VNTR 3336			19					1		14				**0.50**
VNTR 3690		34												**0.00**
VNTR 4156	34													**0.00**

VNTR, variable-number tandem repeat; MIRU, mycobacterial interspersed repetitive units; ETR, exact tandem repeats; QUB, Queen's University Belfast.

[a]The letter D represents 9, 10 repeats (1 additional copy).

[b]The letter S represents 8, 9, 10 repeats (2 additional copies).

[c]The letter T represents 2, 3, 4 repeats (2 additional copies).

2. VNTR profiles of the 133 *M. bovis* isolates

The VNTR profiles of *M. bovis* isolates were analyzed on the basis of 16 polymorphic VNTR loci (4 MIRU, 2 ETR, 3 QUB, and 7 VNTR) from the original 30 VNTR loci. Twenty-six genotypes (designated A to Z) were identified from 133 *M. bovis* isolates from Holstein dairy cattle, Korean beef cattle, and deer (Table 6). Two genotypes, K and L, were most frequent in this study. These 2 genotypes were identified in every species of cattle and deer in this study, and constituted 33.1% and 18.8% of the total *M. bovis* isolates, respectively, indicating that *M. bovis* with K and L genotypes have the most common interspecies transmission. Eight genotypes, A, B, C, D, E, F, G, and H, were detected only in *M. bovis* isolates from Holstein dairy cattle. Four genotypes, M, N, O, and P, were identified only in *M. bovis* isolates from Korean beef cattle, and U, V, W, X, Y, and Z genotypes were detected only in deer. Genotypes I, J, Q, R, S, and T were commonly identified in at least 2 species.

To analyze the genetic relation between the isolates, a dendrogram was constructed on the basis of MIRU-VNTR types using a neighbor-joining algorithm (Fig. 2). The dendrogram shows 133 *M. bovis* isolates divided into 2 main genotype groups. The groups were classified on the basis of the copy numbers of MIRU 31, QUB 26, VNTR 2401, and VNTR 3171. Group A has 2-4-4-3 copies, and Group B has 3-3-5-2 copies of MIRU 31, QUB 26, VNTR 2401, and VNTR 3171, respectively. Group A comprised 69.9% of all *M. bovis* isolates (76.3% of Holstein dairy cattle, 72.5% of Korean beef cattle, and 55.9% of deer). Genotype K, showing the highest prevalence in all species, comprised 47.3% of Group A. Group A consisted of 67.3% of Holstein dairy cattle, 24.5% of Korean beef cattle, and 8.2% of deer, after excluding genotype K, indicating that the majority of *M. bovis* isolates from Holstein dairy cattle belonged to Group A. Group B comprised 30.1% of *M. bovis* isolates (23.7% of Holstein dairy cattle, 27.5% of Korean beef cattle,

and 44.1% of deer). The other main genotype, L, comprised 62.5% of Group B. Excluding genotype L, Group B consisted of 20.0% of Holstein dairy cattle, 33.3% of Korean beef cattle, and 46.7% of deer. The rate of *M. bovis* isolates from deer is higher than that from the other species in Group B.

Table 6. Genotype prevalence of the *M. bovis* isolates

Designation index	VNTR allele profiles[a]	No. (%) of isolates	No. (%) of isolates from Holstein dairy cattle	No. (%) of isolates from Korean beef cattle	No. (%) of isolates from deer
A	353275A142443A42	9 (6.8)	9 (15.3)		
B	343275A342443732	1 (0.8)	1 (1.7)		
C	353245A342443A32	5 (3.8)	5 (8.5)		
D	353275A342443632	2 (1.5)	2 (3.4)		
E	353265A342443A32	3 (2.3)	3 (5.1)		
F	353265A343443A32	1 (0.8)	1 (1.7)		
G	353375A3324529A2	3 (2.3)	3 (5.1)		
H	353275A342343A32	2 (3.4)	2 (3.4)		
I	353275A342443A42	11 (8.3)	9 (15.3)	2 (5.0)	
J	353274A342443A32	3 (2.3)	1 (1.7)	2 (5.0)	
K	353275A342443A32	44 (33.1)	12 (20.3)	17 (42.5)	15 (44.1)
L	353375A332452AA2	25 (18.8)	11 (18.6)	6 (15.0)	8 (23.5)
M	353225A342443A32	1 (0.8)		1 (2.5)	
N	3532748342443A32	1 (0.8)		1 (2.5)	
O	353275A242443A32	3 (2.3)		3 (7.5)	
P	353275A342443D3T	1 (0.8)		1 (2.5)	
Q	353275A342443D32	3 (2.3)		2 (5.0)	1 (2.9)
R	353374A332452AA2	2 (1.5)		1 (2.5)	1 (2.9)
S	353365A332452AA2	2 (1.5)		1 (2.5)	1 (2.9)
T	552375A332452AA2	5 (3.8)		3 (7.5)	2 (5.9)
U	353275A342443A82	1 (0.8)			1 (2.9)
V	353275S342443A32	1 (0.8)			1 (2.9)
W	552375A332452A32	1 (0.8)			1 (2.9)
X	252374A332452AA2	1 (0.8)			1 (2.9)
Y	3533658332452AA2	1 (0.8)			1 (2.9)
Z	3533758332452A32	1 (0.8)			1 (2.9)
Total	**26**	**133 (100)**	**59 (100)**	**40 (100)**	**34 (100)**

[a] Order of VNTR loci: MIRU: 4, 26, 27, 31; ETR-A, B; QUB 11a, 18, 26; VNTR 0424, 1895, 2401, 3171, 3232, 3336, 3690. The VNTR allele profiles consist of digits (when 9 or fewer repeats were detected at a particular locus) and letters; the letter A represents 10 repeats; the letter D represents 9, 10 repeats (1 additional copy); the letter S represents 8, 9, 10 repeats (2 additional copies); and the letter T represents 2, 3, 4 repeats (2 additional copies). VNTR, variable-number tandem repeat; MIRU, mycobacterial interspersed repetitive units; ETR, exact tandem repeats; QUB, Queen's University Belfast.

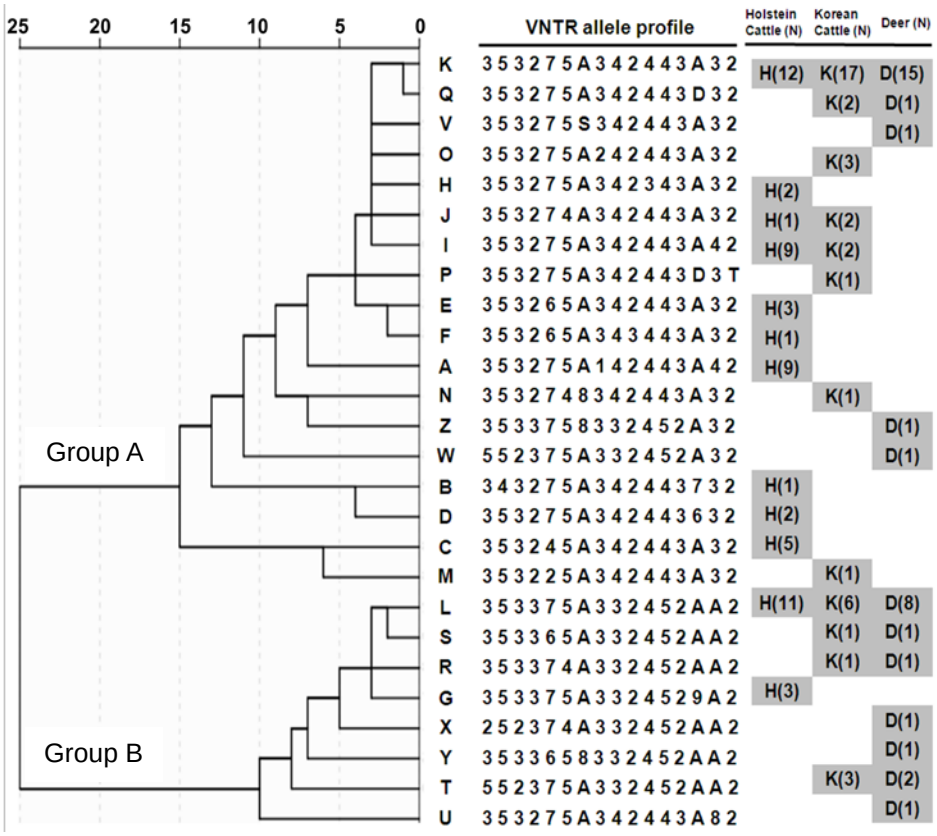

Figure 2. Genetic relationships of *M. bovis* isolates from different animal species. The dendrogram is based on MIRU-VNTR genotypes using a neighbor-joining algorithm. VNTR, variable-number tandem repeat; MIRU, mycobacterial interspersed repetitive units.

IV. Discussion

In Korea, efforts to control bovine TB have continued for more than 3 decades. The incidence of bovine TB in Korea is very low, ranging from 0.1–0.2%; however, the number of cases in cattle has risen annually over the past 2 decades [47]. The problem with the bovine TB control program in Korea is the limited diagnosis policy, which performs annual tests only in dairy cattle herds, but not in Korean beef cattle or deer herds. Recently, the National Veterinary Agency tried to test beef cattle and deer, but tested less than 5% of the total animals. This problem of partial herd diagnosis implicates Korean beef cattle and deer as reservoirs, with the risk of interspecies cross-infection.

In this study, we performed MIRU-VNTR analysis on 133 *M. bovis* strains isolated in Korea, and identified 16 VNTR markers with genetic polymorphism among the 30 tested loci. We used these polymorphic markers to analyze VNTR profiles and identified 26 genotypes. Two of these 26 genotypes—K and L—were isolated from all species of cattle and deer, and comprised over half of the total isolates. This result shows that some genotypes are prevalent in different animal species, which indicates inter-herd and interspecies *M. bovis* transmission. In addition, Korean beef cattle and deer, which have not been tested regularly, may be reservoirs of the organism.

Many countries with cases of bovine TB for a long time may have wildlife reservoirs. In these regions, the wildlife-livestock-human interface is a major risk factor for *M. bovis* transmission. Well-known examples include infections in badgers (*Meles meles*) in the United Kingdom and possums (*Trichosurus vulpecula*) in New Zealand [1, 48]. Wild boar may also be a wildlife host associated with *M. bovis* transmission. Studies have shown *M. bovis* isolates from wild boar and domestic animals to have common genotypes [49, 50]. These studies use molecular typing to provide evidence

for cross infection between wild boar and domestic animals. Such transmission is most likely to occur when wildlife and domestic animals share pasture or territory [51]. Molecular typing methods have recently shown transmission not only between animal species but also between human and animals [52]. In the study, 50 *M. bovis* isolates from humans were typed using spoligotyping and VNTR; 35 isolates shared a genotype with *M. bovis* spoligotypes observed in cattle in the United Kingdom. The study presented epidemiological information that 59% of the patients had some contact with farms, ranging from a Saturday job milking cows, to living on a dairy farm as a child, to working as a farmer. In Korea, there are no known environmental reservoirs of *M. bovis* or officially proven cases of transmission from animals to humans. In our study, however, molecular typing data clearly indicates that inter-herd and interspecies transmission of bovine TB can occur.

Analysis of genetic relationships by MIRU-VNTR typing revealed that 133 *M. bovis* isolates could be classified into 2 main groups (A and B) according to the copy numbers of MIRU 31, QUB 26, VNTR 2401, and VNTR 3171. Group A has 2-4-4-3 copies, and Group B has 3-3-5-2 copies of MIRU 31, QUB 26, VNTR 2401, and VNTR 3171, respectively. The results indicate that 4 markers have strong linkage in Korea, but studies in other countries did not show any marker linkage. We examined the history and infection environment of *M. bovis* strains in each group, but did not find specific differences between the groups. To analyze the characteristic differences between the groups, we need more historical data on their origin as well as clinical studies of samples. However, the predominant genotypes, K and L, of each group were identified in all animal species. This result indicates that cross-infection occurs regardless of *M. bovis* genotype.

Our study has several limitations. Epidemiological information, except for animal species and farm, was not available. For this reason, we could not determine contact-based inter-herds and interspecies transmission.

More detailed epidemiological information, including animal movement among and distance between herds is required to define interspecies transmission. In addition, most of the *M. bovis* isolates in this study originated from the western region of the Korean peninsula. To examine the characteristics and prevalence of *M. bovis* infection in Korea, a larger number of *M. bovis* isolates from other regions and other species, including wildlife, are necessary.

In conclusion, VNTR typing was useful for differentiation of *M. bovis* molecular types in Korea. Domestic animals susceptible to *M. bovis*, but not checked annually using the tuberculin skin test, are implicated as reservoirs for interspecies transmission. Therefore, to efficiently control bovine TB in Korea, Korean beef cattle and deer as well as Holstein dairy cattle should be included in annual testing and culling strategies.

CONCLUSION

1. Bovine TB has been spreading worldwide and is major issue of both human public health and economics in large areas. The bovine TB control program has been performed for more than 50 years in Korea. However, the control strategy has focused on dairy cattle by using TST only. In this study, we tested additional immunodiagnostic assays and molecular epidemiology to find tools to improve bovine TB control programs more efficiently in Korea.

2. We evaluated in-house IFN-γ assay using the ESAT6 and CFP10 cocktail antigen and an ELISA-based antibody tests using MPB70 and MPB83 antigens for immunodiagnosis of bovine TB in dairy cattle. Both the IFN-γ assay and antibody tests showed clear distinction between TST-reactor and non-reactor cattle. However, we found many TST-negative cattle were positive by blood-based assays in dairy cattle herds with bovine TB outbreaks and they were confirmed to be *M. bovis*-infected by post-mortem examinations followed by culture and molecular detection of *M. bovis*. This result indicated that TST shows relatively low sensitivity and that additional diagnostic methods are required for detection of *M. bovis*-infected cattle in Korea.

3. We performed MIRU-VNTR analysis on 133 *M. bovis* strains isolated from Holstein dairy cattle, Korean beef cattle and deer. By using VNTR markers showing polymorphism, we identified 26 genotypes designated A to Z. Of the 26 genotypes, two genotypes, K and L, were isolated from at all species of cattle and deer and occupied over the half of the total *M. bovis* isolates. This result shows that some common genotypes are prevalent among different animal species, thus indicating inter-herds and inter-animal species

transmission of *M. bovis*.

4. The immunological assays employed in this study were useful in identifying animals infected with *M. bovis* in substantial portion of TST-negative cattle in the herds with bovine TB outbreaks, thus applicable to the control and eradication programs of bovine TB as ancillary tests to TST. In addition, MIRU-VNTR molecular typing showed a clear evidence of *M. bovis* transmission among animal species, thus indicating the importance of bovine TB control programs in deer as well as in dairy and beef cattle in Korea.

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ABSTACT (IN KOREAN)

우결핵의 면역진단과 분자역학조사

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제 승 모

우결핵은 동물뿐만 아니라 사람에게도 감염을 일으킬 수 있는 전염성 질병으로, 전세계적으로 널리 퍼져 공중보건 및 경제적 손실을 유발하는 중요한 인수공통전염병이다. 우결핵 검사를 위해서 피내검사법이 여러 나라에서 공식적으로 사용되고 있다. 최근에는 진단의 민감도과 특이도를 높이기 위해 혈액을 이용한 인터페론감마 검사법과 항체검사법이 여러 나라에서 새롭게 도입되었다. 이번 연구에서는 우결핵 진단 개선을 위해 결핵균 특이항원인 ESAT6 와 CFP10 을 이용한 인터페론감마 검사법과 MPB70 과 MPB83 항원을 이용한 항체검사법을 국내 젖소를 대상으로 시행하여 그 유용성을 평가하였다. 시행 결과, 인터페론감마 검사법과 항체검사법 모두 기존의 피내검사법 양성우와 음성우 간에 뚜렷한 반응차이를 나타내었고, 피내검사법을 기준으로 했을

때 인터페론감마 검사법은 87.4%의 민감도과 100% 의 특이도를 보였으며, 항체검사법은 70.2-73.6%의 민감도과 97.4-98.3%의 특이도를 나타내었다. 하지만 현재 우결핵 발생농가와 이전 우결핵 발생농가에서 피내검사법에서는 음성반응을 보이는 많은 소들이 인터페론검사법과 항체검사법에 양성반응을 나타내는 현상이 관찰되었다. 이러한 소들의 결핵감염여부를 진단해내기 위해, 부검 및 균배양과 결핵균 특이 유전자를 대상으로 중합 효소 연쇄반응을 시행하였을 때 대부분의 소에서 양성반응을 나타내었다. 이러한 농가들 중 일부는 피내검사법에 낮은 양성율을 보이지만, 인터페론검사법과 항체검사법에는 높은 양성반응을 나타내었다. 이러한 결과를 통해 현재 국내에서 시행되는 피내검사법의 민감도가 낮은 것으로 분석되며, 보완할 다른 보조 진단법의 도입이 시급한 것으로 판단된다.

한편, 최근에는 각 우결핵균의 유전자 차이를 분석해내는 분자역학조사법이 개발되어 우결핵균의 전파를 효과적으로 파악하고 통제하기 위해서 사용되고 있다. 본 실험에서는 이러한 분자역학조사법 중 연쇄염기서열반복(VNTR) 분석법을 국내에 도입하여 59 두의 젖소와 40 두의 한우, 34 두의 사슴에서 분리한 우결핵 균주에 적용하였다. 사용한 30 개의 연쇄염기서열반복 마커 중 17 개의 마커에서 각 균주의 유전적 다양성이 관찰되었으며, 그 중 가장 높은 다양성을 나타내는 마커는 VNTR 3336 이었다 (h = 0.59). 또한 QUB 26, MIRU 31, VNTR 2401, VNTR 3171 에서도 높은 유전적 다양성이 관찰되었으며, 이러한 마커들을 조합했을 때 133 개의 균주를 26 개의 유전자형(A-Z)으로 분류할 수 있었다. 이 중 K 와 L 형이 전체 균주 중 각각 33.1% 와 18.8% 를 차지해 가장

높은 비율로 관찰되었으며, 각 축종에 공통적으로 분포하고 있는 것이 확인되었다. I, J, Q, R, S, T 형 또한 젖소와 한우, 사슴 중 두 가지 축종에서 공통적으로 확인되었다. 이러한 결과는 국내에 사육되고 있는 각 축종 사이에 우결핵균의 교차감염이 일어나고 있음을 암시한다.

결과를 종합적으로 분석해볼 때, 이번 연구에서 적용된 혈액을 이용한 면역진단법은 피내검사법에 반응하지 않는 우결핵균 감염우를 추가적으로 진단해낼 수 있었으며, 피내검사법을 중심으로 한 국내 우결핵 관리 정책에 보조 진단법으로서 유용성을 지닐 것으로 판단된다. 또한 연쇄염기서열반복 분석법을 이용한 분자 역학조사법을 통해 각 축종 간에 교차감염의 발생 가능성을 확인하였으며, 이러한 결과는 국내에서 젖소를 중심으로 시행되는 우결핵 진단을 한우 및 사슴 등 다른 축종에도 확대 강화해야 할 필요성을 제시해주고 있다.

핵심되는 말 : 우결핵, 인터페론감마 검사, 항체검사, 연쇄염기서열반복 분석법

PUBLICATION LIST

1. Jeon B, Je S, Park J, Kim Y, Lee E, Lee H, et al. Variable number tandem repeat analysis of *Mycobacterium bovis* isolates from Gyeonggi-do, Korea. J Vet Sci 2008;9:145-53.

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(The manuscript was completely accepted, and final publication is pending)