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ORIGINAL ARTICLE

Mycobacterium tuberculosis and *M. bovis* infection in Feedlot Deer (*Cervus unicolor swinhoei* and *C. nippon taiouanus*) in Taiwan

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Sequence analysis

Background/purpose: Mycobacterium bovis frequently infects wild and farm deer species with tuberculosis. This study investigated mycobacterial infection in two native deer species *Cervus unicolor swinhoei* (Formosan Sambar, Sambar) and *C. nippon taiouanus* (Formasan Sika, Sika).
Methods: Based on different sampling sources of 19 intradermal tuberculin test (ITT) Sambar, mycobacterial infection and/or species were detected by acid-fast stain, duplex polymerase chain reaction (PCR) and multiplex nested PCR (mnPCR) methods, traditional mycobacterial culture and gross lesion. Blood samples of 167 Sambar deer and 147 Sika deer were then tested by duplex PCR and mnPCR methods to investigate the prevalence of mycobacterial infection. Sequence variations of these mycobacterial species were analyzed as well.
Results: Duplex PCR and mnPCR assays could differentiate between MTBC (*M. bovis* and *M. tuberculosis*) and *M. avium*, as well as between *M. bovis* and *M. tuberculosis*, respectively. These PCR methods showed a higher detection rate than traditional culture and matched the gross lesions examined in 19 ITT-examined Sambar. Therefore, the mycobacterial infection in blood samples of 314 deer samples was detected using these PCR methods. Duplex PCR and mnPCR showed an identical prevalence of 16.1% in Sambar and 8.2% in Sika and a significant difference in prevalence between these two deer species. *M. bovis* and *M. tuberculosis* were the species detected in feedlot Sambar and Sika. *M. tuberculosis* was found only and first in Sambar fed in central Taiwan. Sequence analysis revealed diverse genetic variations in *M. bovis* and *M. tuberculosis* associated with deer subspecies.

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Conclusion: Multiplex PCR methods were established, and *M. bovis* and *M. tuberculosis* were identified in feedlot deer in Taiwan. Sequence variations indicated diverse sources of both mycobacterial species.

Introduction

Unlike *Mycobacterium tuberculosis,* which only causes human tuberculosis (TB), *M. bovis* is a zoonotic pathogen that infects humans and domestic animals. This infection causes bovine TB, thus posing a major economic and public health problem for the animal husbandry industry. In North America, bovine tuberculosis is a major infectious disease in cervids.[1] In Taiwan, *Cervus unicolor swinhoei* (Sambar) and *C. nippon taiouanus* (Sika) are farmed for health and medicine (antlers and blood), food (venison), and leather products (hide). Although infection rates of *M. bovis* in deer declined from 4.1% in 1996 to 0.2% in 2006,[2] *M. tuberculosis* has not yet been reported in deer.

As a relatively slow-growing bacteria, *M. bovis* can be identified by acid-fast bacilli (AFB) staining, traditional mycobacterial culture, and intradermal tuberculin test (ITT). Of these methods, AFB staining is the fastest, yet requires more than 10^4 /mL bacteria in a clinical sample.[3] Although traditional culture method is golden standard, 8 to 12 weeks are required[4] and inappropriate sampling produces a false negative outcome.[5] As the conventional test for diagnosing TB,[6] ITT method has a high sensitivity (84%) and specificity (80%) in detecting Mycobacteria from infected animals, yet lacks the ability to differentiate *M. bovis* from *M. avium* and other Runyon groups III and IV mycobacteria.[7] Additionally, thin skins (1−3 mm)[8] or commonly mixed infection with *M. avium*[9,10] or *M. avium* ssp. *paratuberculosis*[11] in deer significantly lowers the sensitivity of the ITT test. In addition to the *M. tuberculosis* complex (MTBC), infection with nontuberculous mycobacteria (NTM) has also been described.[12] Therefore, a molecular diagnostic method such as polymerase chain reaction (PCR) has been developed to differentiate MTBC from NTM.[13,14] Aimed at controlling TB annually to eliminate ITT-positive animals, a preventative program in Taiwan from 1996 to 2006 gradually reduced mycobacterial infection for dairy cattle, dairy goats and deer from 0.37% to 0.16%, 0.5% to 0.2% and 4.1% to 0.2%, respectively.[2] However, ITT-positive deer are not forced to be eliminated from the field and sanitation strongly affects mycobacterial infection. This study investigates *M. bovis* and *M. tuberculosis* infection in Sambar and Sika deer collected from several farms by using ITT, mycobacterial culture, and PCR methods. The phylogenetic relation of *M. bovis* and *M. tuberculosis* associated with deer subspecies is also analyzed.

Materials and methods

Samples and experimental design

In Trial 1, 16 positive and three negative ITT *Cervus unicolor swinhoei* (Sambar) were sampled from five feedlot farms. The deer were examined by necropsy for gross lesion, and their blood and tissues were sampled for bacterial culture, acid-fast stain and PCR identification as well as histopathological diagnosis. Specimens were then placed in a saturated sodium-borate solution to inhibit overgrowth of other microorganisms before shipment for culture. Next, representative gross lesions of retropharyngeal, mediastinal, tracheobronchial, and mesenteric lymph nodes, as well as the lung were determined. Finally, DNA templates of blood, lung tissues, and lymph nodes were used for PCR amplification.

In Trial 2, 167 Sambar deer and 147 Sika deer were randomly sampled from 15 Sambar and six Sika deer farms in central and southern Taiwan from May 2005 to May 2008. Twenty mL of blood were taken from the jugular vein, placed in a tube containing EDTA, and stored at 4°C for DNA purification. Finally, mycobacterial infection of both species was determined by PCR amplification of purified blood DNA.

Mycobacterial identification

Conventional method

a. Culture methods

Ten mL of sampled blood were added into BACTEC™ Myco/F Lytic Culture Vials, which were then normally placed in the BACTEC 9000 Blood Culture System and incubated at 35°C with continuous agitation for 42 days. The identification period was shortened by measuring the fluorescence of each vial at 10-minute intervals by the BACTEC fluorescent series instrument (Becton, Dickinson and Company, Sparks, MD, USA). Mycobacterial infection of each blood sample was then verified using the rate of oxygen reduction. Additionally, isolation and identification of *Mycobacterium* were followed according to the method of Thoen.[15]

b. Intradermal tuberculin test (ITT)

ITT skin test was performed by intradermal injection of 0.1 mL of 2000 IU (0.3 mg of protein) *M. bovis* PPD into lateral cervical region for each individual. Skin thickness was measured at the injection site before and 72 hours (i.e., the optimal timing to detect maximal dermal reactivity in cattle[16]) after injection by calipers. Moreover, test sensitivity was maximized by considering visible, measurable, or palpable responses as positive indications of TB infection.

PCR amplification

a. Preparation of DNA template

DNA templates of reference *M. bovis* and *M. tuberculosis* were kindly donated by the Animal Health Research Institute, Council of Agriculture, Executive Yuan, Republic of China, Taiwan. Chromosomal DNA of other tested bacteria was prepared by centrifugation at 20,000*g* for 2 minutes of

overnight broth first. Pellets were then mixed with 180 μL of lysozyme buffer (AMRESCO) (20mM Tris-HCl, 2mM EDTA, 1.2% Triton X-100; 20 mg/mL lysozme) and reacted at 37°C for 30 minutes Next, mycobacterial chromosomal DNA was purified by the Tissue and Cell Genomic DNA purification kit (Gene Mark, Taipei, Taiwan). For the blood sample, 10 mL of blood were mixed with 0.2% EDTA (pH 7.2), centrifuged at 3300*g* for 10 minutes and incubated with RBC lysis buffer for 5 minutes at room temperature. Finally, genomic DNA was purified by a blood genomic DNA purification kit (Gene Mark, Taiwan).

b. PCR amplification

A 50 μL PCR reaction mixture contained 5—10 ng DNA template, 1× PCR reaction buffer, 0.2 mM dNTPs, 1.6 μM of each primer and 1.25 U Taq DNA polymerase. Table 1 lists all primers.

i) *Duplex PCR*. MTBC and NTM were differentiated by a duplex PCR amplification containing two primer sets Tbc1/TbcR5 and M5/RM3[17] designed to amplify the 235- and 136-bp DNA fragments of gene *rpoB* encoding RNA polymerase β-subunit for MTBC (*M. bovis* and *M. tuberculosis*) and *M. avium*, respectively. The PCR condition was then processed with an initial denaturation at 95°C for 5 minutes, 35 cycles of 30 seconds at 95°C, 30 seconds at 68°C, 60 seconds at 72°C, and a final elongation at 72°C for 10 minutes. Finally, the PCR products were separated by 1.5% agarose.

ii) *Multiplex nested PCR (mnPCR)*. The multiplex nested PCR method was designed not only to differentiate *M. bovis* from *M. tuberculosis*, but also to increase PCR sensitivity. First PCR amplification was performed by using primer sets TBF/R and Rv3618F/R, followed by second PCR amplification with second primer sets of NTBF/R and NRv3618F/R. The PCR condition was described previously.[18,19]

iii) *Sensitivity and specificity of each PCR reaction*. Spiked blood samples were prepared by spiking about 10^3 CFU of bacteria into a normal blood sample and using a positive control. Additionally, an internal DNA control was used by amplification of a partial *spnI* DNA fragment with SI-1 and SI-2 primers.[20] Based on 10-fold serial dilution of known mycobacterial DNA, the sensitivity of each PCR set was evaluated to detect four *Mycobacterium* species, which were *M. tuberculosis, M. bovis, M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *avium* (BCRC15441). Moreover, PCR specificity was tested using *Staphylococcus aureus* (BCRC10781), *Streptococcus agalactiae* (BCRC 10787), *Streptococcus uberis* (BCRC12579), *Streptococcus dysagalactiae*, *Escherichia coli*, and *Pasteurella multocida*. Furthermore, sensitivity and specificity of PCR method towards the conventional culture method to identify mycrobacterial infection were evaluated.[21]

Sequence analysis of PCR products amplified from *M. bovis* and *M. tuberculosis*

Following purification of PCR products for second PCR amplification of multiplex nested PCR by the Promega PCR purification kit, the purified products were sequenced and aligned by the MegAlign program of Lasergene software (DNASRAR, Madison, WI, USA). Accession numbers are from FJ593676 to FJ593692 for *M. tuberculosis* and from FJ409086 to FJ409111 for *M. bovis*.

Statistical analysis

Differences in mycobacterial infection rates among the three detection methods and samples from the two deer species were analyzed based on Chi-square testing.

Results

Detection of mycobacterial infection by using Duplex PCR and mnPCR methods

The two PCR methods differed in their objectives. Duplex PCR could differentiate *M. bovis* and *M. tuberculosis* from *M. avium*, while mnPCR could differentiate *M. bovis* from *M. tuberculosis* (Tables 1 and 2). The sensitivity of mnPCR was 10-fold higher than that of duplex PCR (100 fg for mnPCR vs. 1 pg for duplex PCR) (Table 2). Owing to its high sensitivity and specificity, *M. bovis* and *M. tuberculosis* in deer were detected using mnPCR.

Table 1 Primers used for Duplex and Multiplex nested PCR (mnPCR) amplification

PCR method	PCR Step	*Mycobacterium* spp.	Primer set	Primer sequence (5′—3′)	Product size (bp)	Reference
Duplex PCR	1	*M. bovis* and *M. tuberculosis*	Tbc1	CGTACGGTCGGCGAGCTGATCCAA	235	17
			TbcR5	CCACCAGTCGGCGCTTGTGGGTCAA		
		M. avium	M5	GGAGCGGATGACCACCCAGGACGTC	136	
			RM3	CAGCGGGTTGTTCTGGTCCATGAAC		
mnPCR	1	*M. bovis*	TBF	GACCACGGTGGTCCGCG	636	19
			TBR	CATGACCCCGCCTACCG		
		M. tuberculosis	Rv3618F	ATTGCACATCCGCCCC	326	18
			Rv3618R	GGACAAACCCTGCCGC		
	2	*M. bovis*	NTBF	CCCGCTGATGCAAGTGCC	471	19
			NTBR	CCCGCACATCCCAACACC		
		M. tuberculosis	NRv3618F	GCTCAACACCCGCCAATC	224	18
			NRv3618R	ACATCCGCCCCTACACC		

Table 2 The specific PCR detection of *Mycobacterium tuberculosis*, *M. bovis* and *M. avium*

Reference strains	Duplex PCR		Multiplex nested PCR	
	Tbc1-TbcR5	M5-RM3	TBF-TBR	Rv3618F-Rv3618R
Sensitivity	1 pg	1 pg	100 fg	100 fg
Mycobacterium tuberculosis complex				
M. tuberculosis[a]	+	-	-	+
M. bovis[a]	+	-	+	-
Nontuberculous mycobacteria				
M. avium subsp. *paratuberculosis*[a]	-	+	-	-
M. avium subsp. *avium* (BCRC15441)[b]	-	+	-	-
Non-mycobacteria				
Staphylococcus aureus (BCRC10781)[b]	-	-	-	-
Streptococcus agalactiae (BCRC10787)[b]	-	-	-	-
Streptococcus uberis (BCRC 12579)[b]	-	-	-	-
Streptococcus dysagalactiae[c]	-	-	-	-
Escherichia coli[c]	-	-	-	-
Pasteurella multocida[c]	-	-	-	-

[a] *M. tuberculosis* was kindly provided by Animal Health Research Institute (AHRI), Council of Agriculture Executive Yuan in Taiwan.
[b] BCRC stands for Bioresource Collection and Research Center in Taiwan.
[c] Clinical strains were identified by biochemical assays.

Comparison of different detection methods for *Mycobacterium*

Necrospy examination revealed tubercle in the lung, caseous necrosis exudates in the retropharygeal lymph node and confluent tubercles in the lymph node (Fig. 1). Additionally, the granulomatous and acid-fast bacteria were also observed in the histopathologic section (Fig. 1). Moreover, mycobacterial infection in 19 ITT-positive and negative samples was determined using ITT, traditional

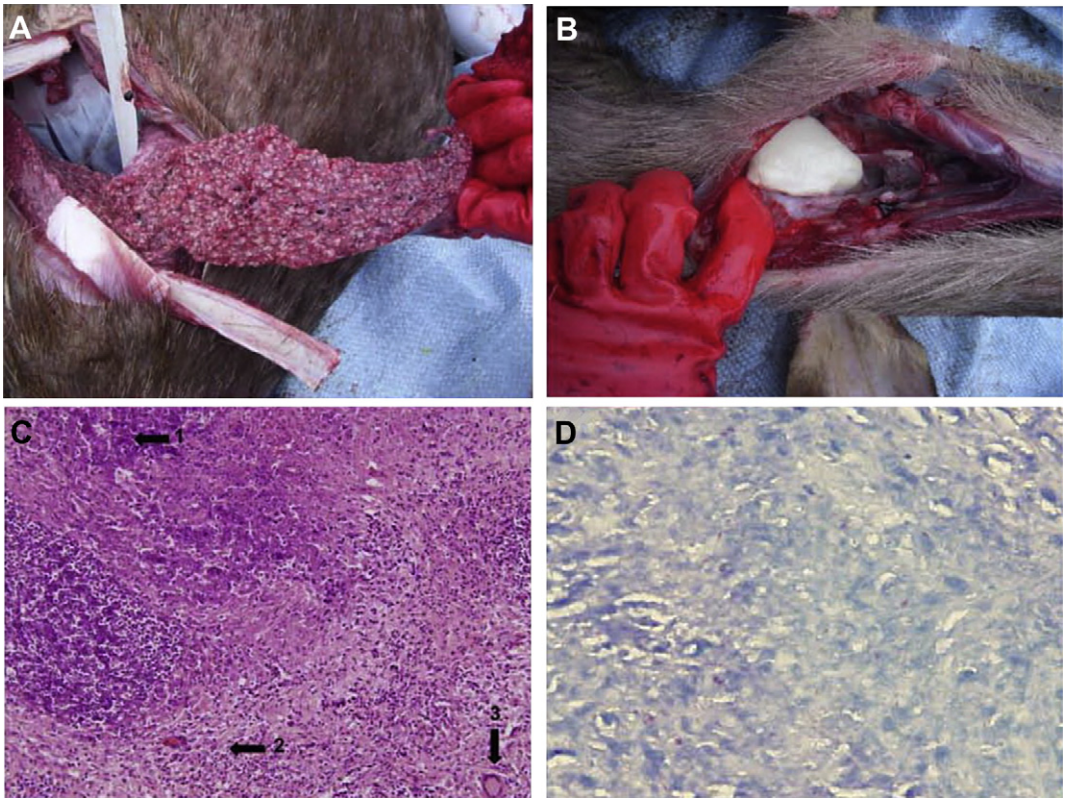

Figure 1. Necropsy examination of mycobacterial infected Sambar Deer. (A) Tubercle in the lung. (B) Caseous necrosis exudates in the retropharyngeal lymph node. (C) Confluent tubercles in the lymph node (200×). Arrow 1: caseous necrosis, Arrow 2: epithelioid cells, and Arrow 3: Langhans' giant cell. (D) Acid-fast stained bacteria in the lung (600×).

Table 3 Comparison of different methods in identifying mycobacterial infection in *C. unicolor swinhoei*

Farm	Number	ITT	Acid-fast stain	Culture[a]	Multiplex-nest-PCR (mn-PCR)[b]	Gross lesion
A	960311	-	+	*M. bovis*	*M. bovis*	Necrotic and caseous granulomas and enlarged lymph node
	960312	+	-	-	*M. bovis*	-
	960313	+	+	*M. bovis*	*M. bovis*	Necrotic and caseous granulomas and enlarged lymph node
	960314	+	-	*Mycobacterium* spp.	-	-
	960316	+	-	-	*M. bovis*	Necrotic and caseous granulomas
	960320	+	-	*Mycobacterium* spp.	-	-
	960343	+	+	*M. bovis*	*M. bovis*	Necrotic and caseous granulomas and enlarged lymph node
	960344	+	+	*M. bovis*	*M. bovis*	-
	960348	-	-	-	*M. bovis*	Necrotic and caseous granulomas
B	96048	+	-	-	-	-
	96066	+	+	*M. bovis*	*M. bovis*	Lung necrotic and caseous granulomas
	96074	-	-	-	-	-
C	98B585	+	-	*M. bovis*	*M. bovis*	-
D	93H224	+	+	-	*M. bovis*	-
	95J502	+	-	-	*M. bovis*	-
E	95J474	+	+	*M. bovis*	*M. bovis*	Lung necrotic and caseous granulomas
	95J115	+	+	-	*M. bovis*	Necrotic and caseous granulomas
	95J118	+	-	*M. bovis*	*M. bovis*	-
	95J121	+	-	-	*M. bovis*	-
Total,%	19	16	42.1 (8/19)	52.6 (10/19)	78.9 (15/19)	42.1 (8/19)

[a] Culture samples were lymph node and lung.
[b] mn-PCR samples were blood, lymph node and lung.

culture, acid-fast stain, PCR amplification and gross lesion in the lungs. The prevalence of mycobacterial infection was 84.2% (16/19) for ITT, 52.6% (10/19) for culture method (8 *M. bovis* isolates and 2 unknown *Mycobacterium* spp. isolates from lymph node and lung), 42.1% (8/19) for acid-fast stain, 78.9% (15/19) for PCR amplification of blood, lung, and lymph node samples (3 ITT-negative deer were dead before blood collection), and 42.1% (8/19) for gross lesion in the lung and lymph node (Table 3). Above results were not consistent with each other for any two methods. In comparison with gold standard (infection) results, sensitivity and specificity were 83.3% and 100% for culture method, 83.3% and 28.6% for PCR amplification, and 66.7% and 100% for acid-fast stain, respectively (Table 4).

In comparison with the culture method, mnPCR revealed a higher sensitivity and lower specificity in mnPCR. Due to time-consuming nature of the culture method, PCR method is rapid, simple and inexpensive. Therefore, mycobacterial infection in the blood samples of two feedlot deer was identified using PCR methods. Among 314 samples, Duplex PCR and mnPCR revealed an identical prevalence of 16.1% in Sambar and 8.2% in Sika, as well as a significant difference in prevalence between these two deer species (Table 5).

Infection of *M. bovis* and *M. tuberculosis* in both deer species

An attempt was made to eliminate possible contamination by positive samples during PCR amplification by performing each test three times and sequencing directly all PCR

Table 4 The comparison of positive infection with Culture, mnPCR and acid-fast stain detecting method

Positive infection[a]	Culture		mnPCR		Acid-fast stain	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	10	2	10	2	8	4
Negative	0	7	5	2	0	7
Total	10	9	15	4	8	11
Sensitivity	83.33% (10/12)		83.33% (10/12)		66.67% (8/12)	
Specificity	100% (7/7)		28.57% (2/7)		100% (7/7)	
Kappa	0.78652		0.13072		0.59574	

[a] Infection definition: deer was positive in acid-fast stain, culture ,and gross lesion/histopathology.

Table 5 Prevalence of *Mycobacterium tuberculosis* complex (MTBC) determined by two PCR methods in two deer species

Cervus species	Duplex PCR (%)			Multiplex nested PCR (%)
	Male	Female	Total	
C. unicolor swinhoei (Sambar)	11.4 (13/114)	26.4 (14/53)	16.1[x] (27/167)	16.1[x] (27/167)
C. nippon taiouanus (Sika)	9.9 (8/81)	6.1 (4/66)	8.2[y] (12/147)	8.2[y] (12/147)
Total	10.8 (21/195)	15.1 (18/119)	12.4 (39/314)	12.4 (39/314)

[x,y] in the same column indicate significant difference between the two groups determined by the Chi square test ($p < 0.001$).

products of *M. bovis* and *M. tuberculosis*. Mycobacterial infection was equally prevalent in Sambar farms (5/15; 33%) and Sika farms (2/5; 40%). *M. tuberculosis* was also identified in both deer from five farms (Table 6). Compared to *M. tuberculosis*, *M. bovis* was more prevalent in Sambar (29.3% for *M. bovis* vs. 15.5% for *M. tuberculosis*) and equally prevalent in Sika. In Sambar, mycobacterial infection was significantly higher in central Taiwan (57.5% in

Table 6 Prevalence of *M. bovis* and *M. tuberculosis* in Sambar and Sika from 7 ITT positive deer farms in central and south Taiwan

Deer Species	Region	Farm	Total deer	Sample number	*Mycobacterium* species	Infected number	Sex	
							F	M
Sambar	Central Taiwan	NT-A	35	25 (71.4%)	MB	6 (24%)	6	0
					MT	3 (12%)	2	1
					Both	1 (4%)	1	0
					Total	10 (40%)	9	1
		NT-B	25	9 (36%)	MB	5 (55.6%)	0	5
					MT	2 (22.2%)	1	1
					Total	7 (77.8%)	1	6
		NB-C	6	6 (100%)	MB	2 (33.3%)	1	1
					MT	4 (66.7%)	0	4
					Total	6 (100%)	1	5
		Total	66	40 (60.6%)	MB	13 (32.5%)	7	6
					MT	9 (22.5%)	3	6
					Both	1 (2.5%)	1	0
					Total	23 (57.5%)	11	12
	South Taiwan	TL-A	30	13 (43.3%)	MB	2 (15.4%)	2	0
					MT	0 (0%)	0	0
					Total	2 (15.4%)	2	0
		TL-B	40	5 (12.5%)	MB	2 (40%)	1	1
					MT	0 (0%)	0	0
					Total	2 (40%)	1	1
		Total	70	18 (25.7%)	MB	4 (22.2%)	3	1
					MT	0 (0%)	0	0
					Total	4 (22.2%)	3	1
	Sum		136	58 (42.6%)	MB	17 (29.3%)	10	7
					MT	9 (15.5%)	3	6
					Both	1 (1.7%)	1	0
					Total	27 (46.55%)	14	13
Sika	South Taiwan	TS-A	79	79 (100%)	MB	4 (5.1%)	2	2
					MT	2 (2.5%)	0	2
					Total	6 (7.6%)	2	4
		TS-B	60	8 (13.3%)	MB	2 (25%)	0	2
					MT	3 (37.5%)	2	1
					Both	1 (12,5%)	0	1
					Total	6 (75%)	2	2
		Total	139	87 (62.6%)	MB	6 (6.9%)	2	4
					MT	5 (5.7%)	2	3
					Both	1 (1.1%)	0	1
					Total	12 (13.8%)	4	8

average) than in southern Taiwan (an average of 22.2%). Such a difference in prevalence among farms was also found in Sika from 7.6% in TS-A farm to 75% in TS-B farm. Mycobacterial infection was generally higher in Sambar (46.6%) than in Sika (13.8%). Additionally, *M. bovis* and *M. tuberculosis* from the same deer were isolated for both deer species.

Nucleotide variations were found mainly in 16 bp out of 401 bp (4.0%) for 25 PCR products of *M. bovis* and 9 bp out of 171 bp (5.3%) for 16 PCR products of *M. tuberculosis*. Notably, most mutations were either point mutation or deletion. Phylogenetic study of *M. bovis* and *M. tuberculosis* sequences demonstrated. Two main clusters in *M. bovis* and *M. tuberculosis*, respectively. In *M. bovis*, cluster I was separated into 11 subtypes, and the reference strain was in I-8 type with two other sequences (Fig. 3A). In *M. tuberculosis*, cluster I was categorized into two main subtypes I-1 and I-2; in addition, reference strains and the other 11 strains belonged to I-1 subtypes (Fig. 3B). The sequences from two deer species were mixed together in cluster I for both *Mycobacterium* species, except Sik2 for *M. bovis* and SamI of *M. tuberculosis*, which were independent of the other sequences.

Discussion

The conventional culture method and ITT methods are time consuming and dependent of experienced users. ITT examination of TB infection is performed based on cell-mediated immune response induced by injection of PPD under skin. With the single mid-cervical skin test, ITT has a sensitivity of approximately 80% for TB diagnosis in deer.[7,8,22] However, delayed-type hypersensitivity response developed at 2 to 4 weeks after infection;[23] this manifests itself as a positive response to PPD, yet can be a negative result in a culture assay. According to our results, ITT-negative deer died of *M. bovis* infection (Table 3). The conventional culture method is limited in that 8 to 12 weeks[4] are required for culture, and a false negative is given due to sampling and minimal bacterial dose.[5] Deer and cattle can be infected with *M. bovis* at 500 CFU, and a low infection rate was found in sentinel node of deer.[24] To increase the detection rate and shorten the examination period of mycobacteria in clinical samples, PCR assays have been developed[25] by targeting different genes, such as 16S rRNA,[26] IS*6110*,[27] *mtp40* (target gene of Rv3618/NRv3618 primer set) ,[28–30] RD9 and RD10.[31] In this study, both duplex PCR method and mnPCR method could identify mycobacterial infection by using blood samples with a higher sensitivity than that achieved by the ITT method (Tables 3 and 4). ITT and PCR methods may differ owing to the booster phenomenon, such as PCR assays, and errors in PPD administration and reading the results,[32] failure to produce an i.d. bleb, or the pseudo-negative immune response common in the early or chronic phases of TB.

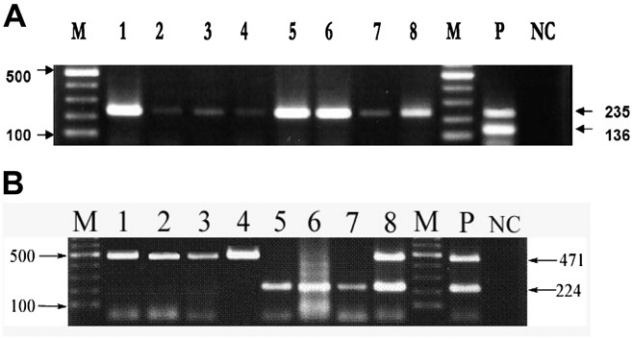

Figure 2. (A) Gel electrophoresis of PCR products of MTBC species amplified from animal blood or nasal swab specimens by Duplex PCR assay for identification of *M. bovis* and *M. tuberculosis* as well as *M. avium* subsp. *avium* (lanes 1–8 = positive MTBC specimens; M = 100 bp DNA ladder marker; NC = negative control; P = reference strain of *M. bovis* and *M. avium* subsp. *avium*, 235-bp product stands for *M. bovis* and *M. tuberculosis*. 136-bp PCR product represents *M. avium*)and (B) multiplex nested PCR for identification of *M. bovis* and *M. tuberculosis* (M: 100 bp DNA ladder marker, lanes 1–4: Positive specimens of *M. bovis*, lanes 5–7: *M. tuberculosis*, lane 8: positive specimens of *M. bovis* and *M. tuberculosis* detected in the same specimen, P: reference strains of *M. bovis* and *M. tuberculosis*, NC: negative control. The size of PCR product was 471 bp for *M. bovis* and 224 bp for *M. tuberculosis*).

In contrast to the duplex PCR method for differentiation of MTBC (including *M. tuberculosis* and *M. bovis*) and *M. avium*,[17] this study developed mnPCR to differentiate *M. bovis* from *M. tuberculosis* (Fig. 2, Tables 2 and 5). *M. bovis* has been reported in farm and wild deer species, with prevalence ranging from 0% in red deer (*C. elaphus*), to 2.1% in sika deer *(C. nippon)*, and up to 18.5% in fallow deer *(Dama dama)*.[33] The highest prevalence (89%) was observed in the mixture of *C. nippon* and *D. dama*.[7] Although previous studies have found PCR amplification of *M. tuberculosis* in humans,[34,35] this study described for the first time *M. tuberculosis* infection in both deer species by mnPCR amplification of blood samples (Table 5). In the present study, both mycobacterial species were also identified from blood, lung and lymph node (Tables 3–5), suggesting that *M. bovis* and *M. tuberculosis* in an activated state can enter into the lymph node, the blood stream and other tissues in the infectious state.

This study also attempted to eliminate the possible contamination of positive control in PCR amplification, with sequence analysis results demonstrating that *M. bovis* and *M. tuberculosis* could infect both deer species and are derived from diverse sources (Fig. 3, Table 5). Sequence information also revealed that *M. bovis* and *M. tuberculosis* evolved differently more faster in Sika than in Sambar; in addition, sequence variations of *M. tuberculosis* were quicker than that of *M. bovis* (Fig. 3). Although prevalence of *M. bovis* and *M. tuberculosis* were equal in Sika, *M. tuberculosis* was identified in Sambar in central Taiwan (Table 5), which has high humidity year-round. Importantly, our results demonstrate that deer may be the reservoir for *M. tuberculosis*, posing a potential public health threat by transmission of *M. tuberculosis* between deer and humans.

In conclusion, *M. bovis* and *M. tuberculosis* were identified in feedlots Sambar and Sika by mnPCR amplification from blood samples. Additionally, prevalence of both mycobacterial species differed between two deer species and between regions in Sambar. Furthermore, sequence

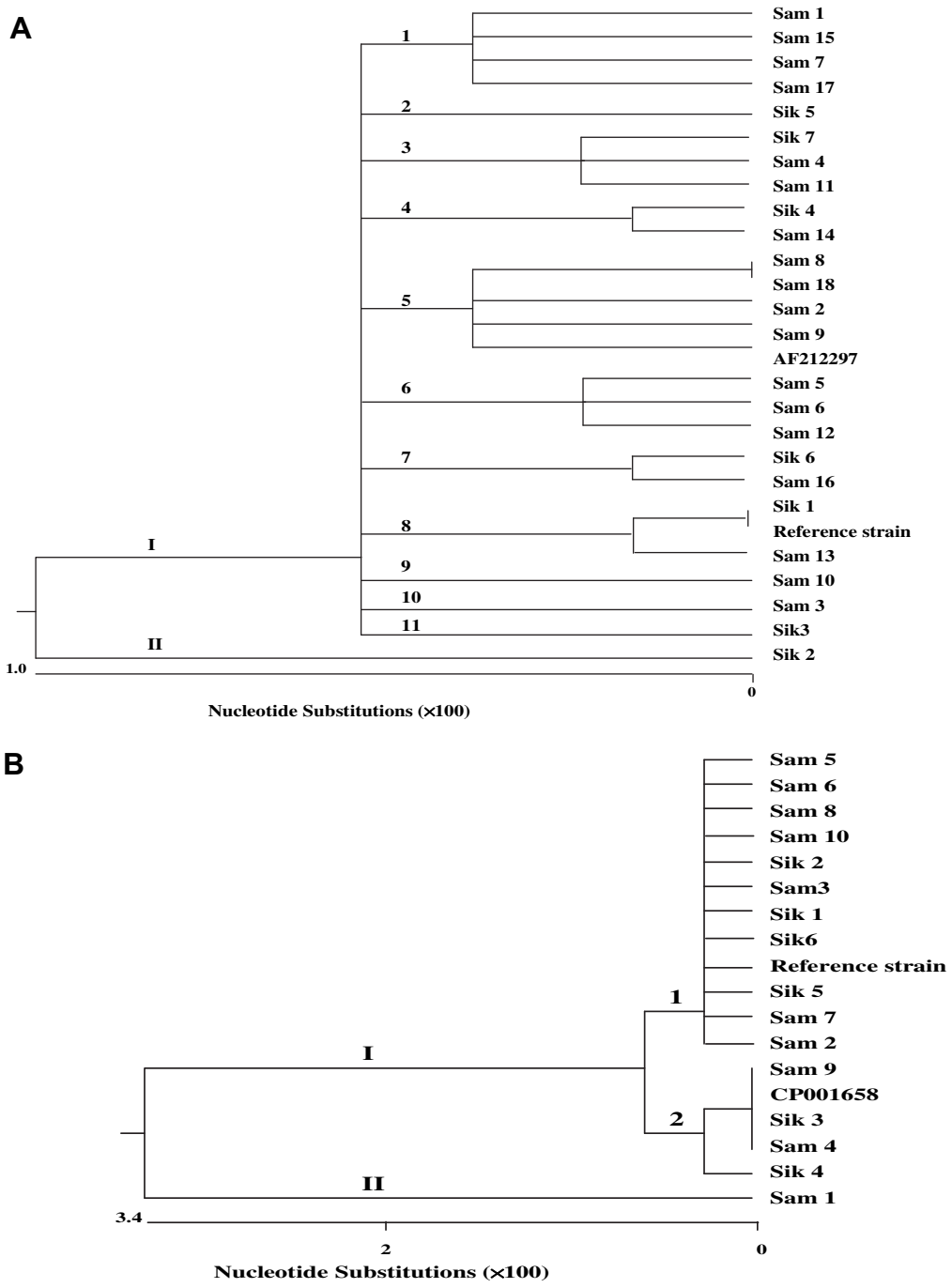

Figure 3. Dendrograms of *Mycobacterium bovis* (A) and *M. tuberculosis* (B) was constructed by using MegAlign Cluster W method of Lasergene v7.1 software (DNASRAR, Madison, Wisconsin, USA) to analyze the sequences of PCR products amplified from blood of two feedlot deer with parameter of gap penalty of 15.00 and gap length penalty of 6.66.

variations of mycobacterial species were associated with deer species.

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References

1. Conner MM, Ebinger MR, Blanchong JA, Cross PC. Infectious disease in cervids of North America: data, models, and management challenges. *Ann N Y Acad Sci* 2008;**1134**:146—72.
2. Council of Agriculture, Executive Yuan, R.O.C (Taiwan). In: *Yearly Report of Taiwan's Agriculture*. Taipei: Council of Agriculture; 2006.
3. Yeager H, Lacy J, Smith LR, LeMaistre CA. Quantitative studies of mycobacterial populations in sputum and saliva. *Am Rev Respir Dis* 1967;**95**:998—1004.

4. Morgan MA, Horstmeier CD, DeYoung DR, Roberts GD. Comparison of a radiometric method (BACTEC) and conventional culture media for recovery of mycobacteria from smear-negative specimens. *J Clin Microbiol* 1983;**18**:384—8.
5. Caws M, Wilson SM, Clough C, Drobniewski F. Role of IS*6110*-targeted PCR, culture, biochemical, clinical, and immunological criteria for diagnosis of tuberculous meningitis. *J Clin Microbiol* 2000;**38**:3150—5.
6. Thom M, Morgan JH, Hope JC, Villarreal-Ramos B, Martin M, Howard CJ. The effect of repeated tuberculin skin testing of cattle on immune responses and disease following experimental infection with *Mycobacterium bovis*. *Vet Immunol and Immunopathol* 2004;**102**:399—412.
7. Kollias GV, Thoen CO, Fowler ME. Evaluation of comparative cervical tuberculin skin testing in cervids naturally exposed to mycobacteria. *JAVMA* 1982;**181**:1257—62.
8. Corrin KC, Carter CE, Kissling RC, de Lisle GW. Short interval intradermal skin testing in farmed red deer (*Cervus elaphus*) inoculated with *Mycobacterium bovis*. *NZ Vet J* 1987;**35**:204—7.
9. de Lisle GW, Havill PF. Mycobacteria isolated from deer in New Zealand from 1970—1983. *NZ Vet J* 1985;**33**:138—40.
10. Quigley FC, Costello E, Flynn O, Gogarty A, McGuirk J, Murphy A, et al. Isolation of *Mycobacteria* from lymph node lesions in deer. *Vet Rec* 1997;**141**:516—8.
11. Mackintosh CG. Deer health and disease. *Acta Vet Hungarica* 1998;**46**:381—94.
12. Libanore M, Bicocchi R, Ghinelli F. Mixed bronchial infection due to *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare* in an AIDS patient. *Infection* 1992;**20**:298—9.
13. Soo PC, Horng YT, Hsueh PR, Shen BJ, Wang JY, Tu HH, et al. Direct and simultaneous identification of *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium tuberculosis* (MTB) by rapid multiplex nested PCR-ICT assay. *J Microbiol Methods* 2006;**66**:440—8.
14. Sweeney FP, Courtenay O, Hibberd V, Hewinson RG, Reilly LA, Gaze WH, et al. Environmental monitoring of *Mycobacterium bovis* in badger faeces and badger sett soil using real-time PCR, confirmed by immunofluorescence, immunocapture and cultivation. *Appl Environ Microbiol* 2007;**73**:7471—3.
15. Thoen CO. Isolation and identification of mycobacteria from tissues of exotic animals. In: Montali RJ, editor. *Mycobacterial infections of zoo animals*. Washington, DC: Smithsonian Institute Press; 1978. p. 99—101.
16. Francis J, Sieler RJ, Wilkie IW. The sensitivity and specificity of various tuberculin tests using bovine PPD and other tuberculins. *Vet Rec* 1978;**103**:420—5.
17. Kim BJ, Hong SK, Lee KH, Yun YJ, Kim EC, Park YG, et al. Differential identification of *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria by duplex PCR assay using the RNA polymerase gene (*rpoB*). *J Clin Microbiol* 2004;**42**:1308—12.
18. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Sci NY* 1999;**284**:398—401.
19. Romero RE, Garzon DL, Mejia GA, Monroy W, Patarroyo ME, Murillo LA. Identification of *Mycobacterium bovis* in bovine clinical samples by PCR species-specific primers. *Can J Vet Res* 1999;**63**:101—6.
20. Horng YT, Deng SC, Daykin M, Soo PC, Wei JR, Luh KT, et al. The LuxR family protein SpnR functions as a negative regulator of N-acylhomoserine lactone-dependent quorum sensing in Serratia marcescens. *Mol Microbiol* 2002;**45**:1655—71.
21. Martin EHJ, William TH, Harry VH. Role of the laboratory in zoonoses recognition. In: *Zoonoses*. 8th ed. Ames, IA: Iowa State University Press; 1995. p. 49—71.
22. Griffin JFT, Buchan GS. Etiology, pathogenesis and diagnosis of *Mycobacterium bovis* in deer. *Vet Microbiol* 1994;**40**:193—205.
23. Tsicopoulos A, Hamid Q, Varney V, Ying S, Moqbel R, Durham SR, et al. Preferential messenger RNA expression of Th1-type cells (IFN-gamma^{+}, IL-2^{+}) in classical delayed-type (tuberculin) hypersensitivity reactions in human skin. *J Immunol* 1992;**148**:2058—61.
24. Griffin JFT, Rodgers CR, Liggett S, Mackintosh CG. Tuberculosis in ruminants: characteristics of intra-tonsilar *Mycobacterium bovis* infection models in cattle and deer. *Tuberculosis* 2006; **86**:404—18.
25. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Sci NY* 1988;**239**:487—91.
26. Takakura S, Tsuchiya S, Isawa Y, Yasukawa K, Hayashi T, Tomita M, et al. Rapid detection of *Mycobacterium tuberculosis* in respiratory samples by transcription-reverse transcription concerted reaction with an automated system. *J Clin Microbiol* 2005;**43**:5435—9.
27. Kent L, McHugh TD, Billington O, Dale JW, Gillespie SH. Demonstration of homology between IS6110 of *Mycobacterium tuberculosis* and DNAs of other *Mycobacterium* spp. *J Clin Microbiol* 1995;**33**:2290—3.
28. Del Portillo P, Murillo LA, Patarroyo ME. Amplification of a species-specific DNA fragment of *Mycobacterium tuberculosis* and its possible use in diagnosis. *J Clin Microbiol* 1991;**29**: 2163—8.
29. Liebana E, Aranaz A, Francis B, Cousins D. Assessment of genetic markers for species differentiation within the *Mycobacterium tuberculosis* complex. *J Clin Microbiol* 1996;**34**: 933—8.
30. Rodriguez JG, Mejia GA, Del Portillo P, Patarroyo ME, Murillo LA. Species-specific identification of *Mycobacterium bovis* by PCR. *Microbiol (Reading, England)* 1995;**141**:2131—8.
31. Parsons LM, Brosch R, Cole ST, Somoskövi A, Loder A, Bretzel G, et al. Rapid and simple approach for identification of *Mycobacterium tuberculosis* complex isolates by PCR-based genomic deletion analysis. *J Clin Microbiol* 2002;**40**:2339—45.
32. O'Reilly LM, Daborn CJ. The epidemiology of *Mycobacterium bovis* infections in animals and man: a review. *Tuber Lung Dis* 1995;**76**(suppl. 1):1—46.
33. Delahay RJ, Deleeuw AN, Barlow AM, Clifton-Hadley RS, Cheeseman CL. The status of *Mycobacterium bovis* infection in UK wild mammals: a review. *Vet J* 2002;**164**:90—105.
34. De Wit D, Steyn L, Shoemaker S, Sogin M. Direct detection of *Mycobacterium tuberculosis* in clinical specimens by DNA amplification. *J Clin Microbiol* 1990;**28**:2437—41.
35. Eisenach KD, Cave MD, Bates JH, Crawford JT. Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. *J Infect Dis* 1990;**161**: 977—81.