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Evaluation of Different Diagnostic Methods of Mycobacterial Infection in Intradermal Tuberculin Testing-positive Feedlot Cattle and Deer

[1]Chishih Chu, [2]Hengching Lin and [2]Yaochi Su

[1]Department of Microbiology, Immunology and Biopharmaceuticals, National Chiayi University, No.300 Syuefu Road, Chiayi City 60004, Republic of China, Taiwan, China

[2]Department of Veterinary Medicine, National Chiayi University, No.580 Xing Ming Road, Chiayi City 60004, Republic of China, Taiwan, China

Corresponding Author: Yaochi Su, Department of Veterinary Medicine, National Chiayi University, No. 580, Xing Ming Road, Chiayi, 60004, Republic of China, Taiwan, China

ABSTRACT

Tuberculosis (TB) is a zoonotic disease affecting mammals worldwide. Herbivore TB is caused by *Mycobacterium bovis* and other Nontuberculous Mycobacteria (NTM). This study aimed to investigate the prevalence of mycobacterial species and to evaluate the diagnostic methods in detecting mycobacterial infection in Intradermal Tuberculin Test (ITT) positive animal. Samples were collected from ITT positive animals (151 cattle and 36 deer) for mycobacterial isolation, pathological examination, acid-fast stain, molecular diagnosis and ELISA. The detection rate of mycobacterial infection in tissues differed among methods from highest in gross lesion (105, 69.5%), Mn-PCR (89, 64.2%), histopathology (89, 58.9%) to acid fast acid (84, 55.6%) in cattle and from Mn-PCR (28, 77.8%), gross lesion and histopathology (19, 52.8%), to acid fast stain (47.2%) in deer. Among 64 culture positive, 48 samples were infected by *M. bovis* (30 in cow vs. 18 in deer). ELISA examination found high level of *M. paratuberculosis* infection (67, 44.4%) in cattle and *M. bovis* (18, 52.8%) in deer. Among gross lesion, histopathology, acid fast stain and Mn-PCR methods, the most prevalent tissues of mycobacterial infection were retropharynx lymph node and mediastinal lymph nodes in cattle and retropharynx lymph node and lung in deer. Farm with only once ITT in routine test may be not true *M. bovis* infected and ITT was overestimated in national MTB clearance program and the reliability in Taiwan. DNA sequence analysis of *M. bovis* demonstrated identical genotypes between deer and cattle from Yulin and diverse origins among cattle from three counties.

Key words: ELISA, intradermal tuberculin test, molecular diagnosis, *Mycobacterium* spp. cattle and deer

INTRODUCTION

Tuberculosis (TB) is an important zoonosis and a significant public health and economic issue. Humans may be infected through contact with infected animals or consumption of contaminated raw milk or meat (Ayele *et al*., 2004). Human TB is primarily caused by *Mycobacterium tuberculosis* complex (MTBC), including *M. tuberculosis* (most commonly), *M. bovis*, *M. africanum* and *M. microti*. In a study of human TB cases during 2004~2005, *M. tuberculosis* was the main

pathogen and accounted for 99.5% (3,306/3,321) cases, followed by *M. bovis* (0.5%, 15/3,321) (Jou *et al*., 2008). In contrast, *M. bovis* is the predominant species causing bovine TB, though occasionally *M. tuberculosis*, *M. avium* and other atypical *Mycobacterium* can also cause the disease with mainly local lesions or non-gross lesions (Jones *et al*., 1997; Pfyffer *et al*., 2007). However, these *Mycobacterium* species exhibit positive reaction in Intradermal Tuberculin Test (ITT) in cattle. Traditionally, acid-fast stain with Ziehl-Neelsen, ITT, bacteria culture and histopathology are commonly used in detecting TB. However, the sensitivity and specificity of ITT to detect bovine TB were just 54.1 and 76.8%, respectively in Taiwan (Jiang, 1993). Necropsy analysis of ITT-positive cattle found predominantly lesions in the retropharyngeal lymph nodes (38.5%), followed by the mesenteric lymph nodes (25%), lung (15%), tracheal and bronchial lymph nodes (10%) and the mediastinal and hilar lymph nodes (2%), respectively (Wu, 1989). Recent molecular methods, such as Polymerase Chain Reaction (PCR), DNA probe, Loop-mediated Isothermal Amplification (LAMP), demonstrates rapid detection of mycobacterial infection and high sensitivity to TB diagnostics (Iwamoto *et al*., 2003). Additionally, Enzyme-linked Immunosorbent Assay (ELISA), interferon-γ (IFN-γ) production and peripheral blood lymphocyte proliferation has been applied to diagnose mycobacterial infection (Rothel *et al*.,1990; Jeon *et al*., 2010). Although, IFN-γ reaction and peripheral blood lymphocyte proliferation test have revealed better detection rate than the ITT method (Jiang, 1993; Chiu, 2004), both methods are time-consuming and operationally difficult. However, serum ELISA is fast, low cost and operationally simple.

The TB clearance program has been executed in Taiwan. In this study, traditional methods (necropsy, microbial culture, histopathology section and acid-fast stain), PCR and ELISA methods were performed on diagnosis of mycobacterial infection in blood and tissue samples of ITT-positive bovine and deer in Taiwan. Furthermore, we investigated the *Mycobacterium* spp. in different organs and phylogenetic analysis of *M. bovis*.

MATERIALS AND METHODS

Sample collections: The ITT-positive cattle and deer were farmed in central and southern Taiwan from January, 2008-December, 2011. One hundred and fifty one ITT-positive cattle from 12 farms and 36 ITT-positive deer from six farms were performed necropsy and analyzed. Following euthanasia, 50 mL of whole blood were collected into tube with EDTA anticoagulant and retropharyngeal, hilar, mediastinal, mesenteric and inguinal lymph nodes and lungs were sampled. All blood and serum collection and sampling was performed in coordination with the relevant county/city Animal Health Research Institute.

Reference strains: *Mycobacterium bovis*, *M. avium* subsp. *avium* (*M. avium*) and *M. avium* subsp. *paratuberculosis* (*M. paratuberculosis*) were kindly provided by the Animal Health Research Institute, Council of Agriculture, Executive Yuan and inactivated *M. tuberculosis* and DNA were provided by the Reference Medical Laboratory Center.

Intradermal Tuberculin Test (ITT): All ITT were operated by officers of the local county/city Animal Health Research Institute by injection of 0.1 mL PPD-b tuberculin (50,000 IU mL^{-1}) into cattle tail. Based on the criteria of the Animal Health Research Institute, Council of Agriculture, Executive Yuan, visual examination and palpation were conducted 72 h after injection. Any swelling at the injection site is defined as a positive reaction. Furthermore, any farms with positive animals were detected every three month and farms without positive animals were tested annually.

Comparative Intra-dermal Tuberculin Testing (CITT): CITT testing was effected by intradermal injection of 0.1 mL of PPD-B (50,000 IU mL^{-1}) and 0.1 mL of avian PPD (PPD-A, 25,000 IU mL^{-1}) on 2 separate sites of the previously shaved mid-neck. The skin swelling measured with a caliper at 72 h later. The outcomes were interpreted as recommended by the OIE (Council Directive 64/432/EEC (International Office of Epizootics (OIE, 2009): Positive (+) for difference skin thickness between the PPD-B injection site and PPD-A injection site more than 4 mm (>4 mm), inconclusive (±) difference between 1 and 4 mm and negative (-) for equal or less at both sites.

Bacterial culture: Fresh tissue block was placed in 50 mL centrifuge tube, sealed with paraffin wax and stored at 4°C. All samples were sent to the Animal Health Research Institute, Council of Agriculture, Executive Yuan for mycobacterial culture and identification.

Histopathological diagostics and acid-fast stain: The tissues and organs of necropsy animals were fixed in 10% neutral formalin and paraffin-embedded at the Animal technology institute Taiwan and stained with hematoxylene and eosine. Histopathological examination of these sections was performed. Simultaneously, the fresh tissues were examined via acid-fast stain.

ELISA detection of mycobacterial infection: *M. bovis* antibody in serum was detected using AniGen BTB Ab ELISA (BioNote, Korea) following the procedures described by the manufacturer. *M. paratuberculosis* was detected using Pourquier® ELISA Paratuberculosis Antibody Verification kit (Institute. Pourquier, Montpellier, France) for bovine and ID Screen®Paratuberculosis indirect (Montpellier, France) for deer. The absorbance of 450 nm (OD_{450}) of each sample recorded within 1 h following the assay by a bichromatic spectrophotometer. The S/P value was calculated as:

$$S/P = \frac{\text{Sample } OD_{450}\text{-average } OD_{450} \text{ of negative control serum}}{\text{Average } OD_{450} \text{ of positive control serum-average } OD_{450} \text{ of negative control serum}}$$

If S/P values is equal and larger than 0.5 (≥0.5), the sample is positive. The sample is negative for S/P values smaller than 0.5 (<0.5).

PCR identification: Total blood sample DNAs were purified using MasterPure™ DNA Purification Kit for Blood Version II (EPICENTRE® Biotechnologies, USA). AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen Biosciences, USA) was used to purify total DNA from tissue and organ samples. Table 1 lists primers used to identify mycobacterial species (Kunze *et al*., 1991; Romero *et al*., 1999; Kim *et al*., 2004; Vansnick *et al*., 2004; Soo *et al*., 2009). Primer set I could identify MTBC and NTM. Primer set II is a multiplex nested PCR (Mn-PCR) to diagnose *M. bovis* and *M. tuberculosis*. Primer set III could identify *M. avium* and *M. paratuberculosis*. 50 μL PCR reagents contained five μL of DNA template, 20 μM of forward and reverse primer, 2.5 mM of dNTPs, 5 μL of 10×PCR reaction buffer and 1.4 U of *Taq* DNA Polymerase and distilled water. Table 2 lists all PCR conditions for three PCR sets. All PCR products were separated by 2% agarose gel in 0.5 ×TAE buffer at 100 V for 1.5 h. The image was recorded following staining with ethidium bromide (0.5 μg mL^{-1}) and UV illumination.

Phylogenetic analysis of *M. bovis*: Purified PCR products of 471 bp *M. bovis* were sequenced by a biotech-company and a phylogenetic tree was constructed using the Megalign program of DNASTAR software.

Table 1: Primer sequences and the size of PCR products

Primer set	Primers	Target	Primer sequence (5'-3')	Product size (bp)	Reference
I	Tbc1	MTBC	CGTACGGTCGGCGAGCTGATCCAA	235	Kim *et al*. (2004)
	TbcR5		CCACCAGTCGGCGCTTGTGGGTCAA		
	M5	NTM	GGAGCGGATGACCACCCAGGACGTC	136	
	RM5		CAGCGGGTTGTTCTGGTCCATGAAC		
II	TBF	*M. bovis*	GACCACGGTGGTCCGCG	636	Romero *et al*. (1999)
	TBR		CATGACCCCGCCTACCG		
	NTBF		CCCGCTGATGCAAGTGCC	471	
	NTBR		CCCGCACATCCCAACACC		
	Rv3618F	*M. tuberculosis*	ATTGCACATCCGCCCC	326	Soo *et al*. (2009)
	Rv3618R		GGACAAACCCTGCCGC		
	NRv3618F		GCTCAACACCCGCCAATC	224	
	NRv3618R		ACATCCGCCCCTACACC		
III	IS901F	*M. avium*	GCAACGGTTGTTGCTTGAAAGGAAT	213	Kunze *et al*. (1991)
	IS901R		GCGCACGCATGATGAGTGGACTTAC		
	IS900F	*M. paratuberculosis*	GGGTTGATCTGGACAATGACGGTTA	572	Vansnick *et al*. (2004)
	IS900R		AGCGCGGCACGGCTCTTGTT		

Table 2: Polymerase chain reaction conditions of each primer pairs

		Initial		Denaturation		Annealing		Extension			Final extension	
Primer set	Primer pair	°C	min	°C	min	°C	min	°C	min	No. of cycle	°C	min
I	Tbc1/TbcR5 M5/RM3	95	5	95	0.5	68	1	-	-	35	72	10
II	TBF/R Rv3618F/R	94	5	94	1	63	1	72	1	35	72	10
	NTBF/R Nrv3618F/R	94	5	94	1	64	1	72	1	35	72	10
III	IS900F/R IS901F/R	94	5	94	1	67	1	72	1	40	72	10

RESULTS

TB prevalence among different methods: Mycobacterial infection in 151 ITT-positive cattle and 36 ITT-positive deer varied among six methods and two animal species (Table 3, Fig. 1-2). The prevalent mycobacterial infection was found highest in gross lesion (69.5%), followed by Mn-PCR (64.2%), histopathology (58.9%), acid fast stain (55.6%), ELISA (51%) and traditional culture (29.2%) in cattle and highest in Mn-PCR and ELISA (77.8%), followed by traditional culture (55.6%), gross lesion and histopathology (52.8%) and acid fast stain (47.2%) in deer.

Prevalence of mycobacterial infection in different tissues: Following, we evaluated the gross lesion, histopathology, acid fast stain and Mn-PCR methods to identify mycobacterial infection in retropharyngeal, hilar, mediastinal, mesenteric and inguinal lymph nodes and lungs. The prevalence of mycobacterial infection differed among six tissues and four detection methods associated with hosts. In cattle, retroppharynx lymph nodes from 27.8% in Mn-PCR to 43.7% in gross lesion and mediastinal lymph nodes from 27.8% in Mn-PCR to 31.1% in gross lesion revealed highest mycobacterial infection and other four tissues revealed lower than 25% mycobacterial infection, except Mn-PCR also detected highest infection in hilar lymph nodes (Table 3). In deer, all four methods identified highest mycobacterial infection in lung from 36.1% in acid fast stain to 47.2% in Mn-PCR and retropharynx lymph node from 25.0% in acid fast stain to 44.4% in MnPCR and lower than 11% of mycobacterial infection rate in other four tissues (Table 3).

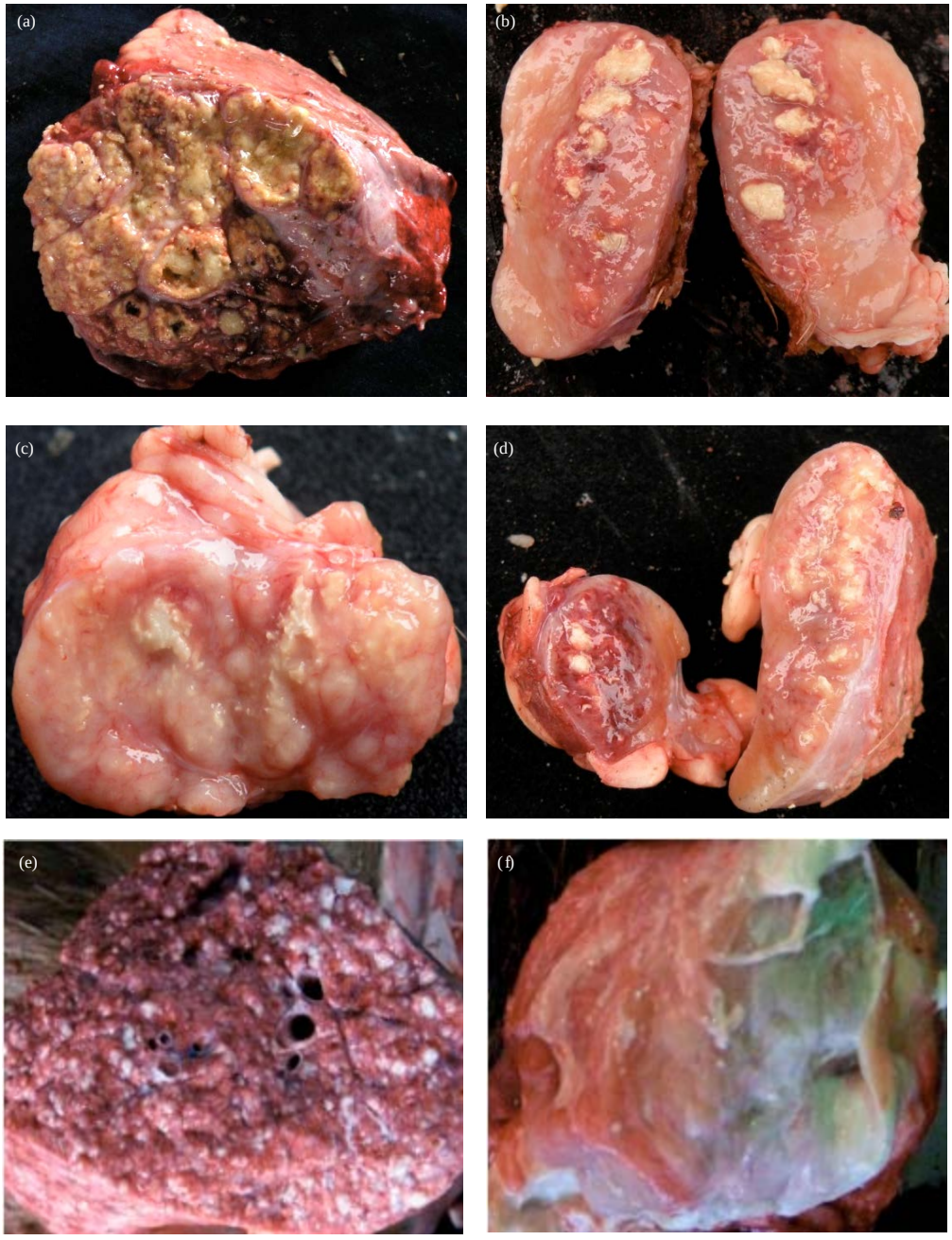

Fig. 1(a-f): The gross lesion of sampling tissues from ITT-positive herbivores. Gross lesion included hemorrhage, enlargement and focal to multifocal yellow to gray nodule of lung (a), retropharynx lymph node (b), hilar lymph nodes (c) and mediastinal lymph nodes (d) of cattle as well as lung (e) and retropharynx lymph node (f) of deer.

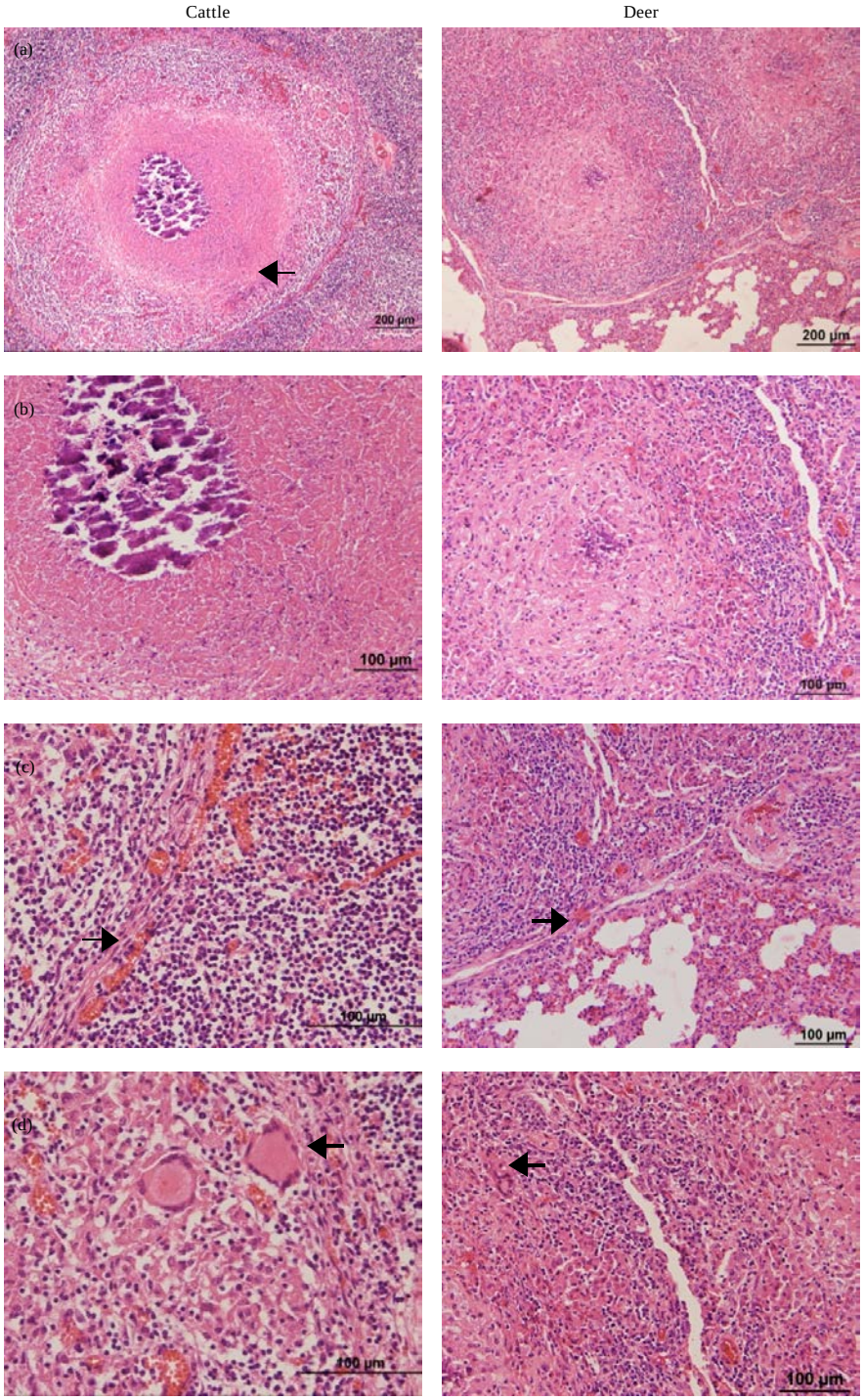

Fig. 2(a-d): Histopathological examination of the lymph node of ITT-positive cattle and deer (a) Arrow indicates the granuloma at 100x, (b) Caseous necrosis in the center of granuloma at 200x, (c) Connective tissue surround granuloma at 400x and (d) Langhan's giant cell at 400x

Table 3: Tuberculosis in different tissue samples of the ITT-positive cattle and deer determined by different methods

Animal species	Total number	Detection	Positive	Lung	Retropharynx lymph node	Hilar lymph node	Mediastinal lymph node	Mesenteric lymph node	Inguinal lymph node
Cattle	151	Gross lesion	105 (69.5)	11 (10.5)	66 (43.7)	24 (15.9)	47 (31.1)	20 (13.2)	2 (1.3)
		Histopathology	89 (58.9)	10 (6.6)	48 (31.8)	22 (14.6)	45 (29.8)	16 (10.6)	2 (1.3)
		Acid fast stain	84 (55.6)	10 (6.6)	48 (31.8)	22 (14.6)	45 (29.8)	13 (8.6)	0 (0)
		Mn-PCR	97 (64.2)	16 (10.6)	42 (27.8)	43 (28.5)	42 (27.8)	20 (13.2)	5 (3.3)
Deer	36	Gross lesion	19 (52.8)	15 (41.6)	13 (36.1)	02 (10.5)	00 (0)	02 (5.6)	0 (0)
		Histopathology	19 (52.8)	14 (38.9)	10 (27.8)	01 (2.8)	00 (0)	00 (0)	0 (0)
		Acid fast stain	17 (47.2)	13 (36.1)	09 (25.0)	01 (2.8)	00 (0)	00 (0)	0 (0)
		Mn-PCR	28 (77.8)	17 (47.2)	16 (44.4)	02 (5.6)	00 (0)	00 (0)	0 (0)

Other detection methods		Bacterial culture	Positive	Serum sample	Positive
Cattle	151	*M. bovis*	30 (19.9)	Mb ELISA	6 (4.0)
		Other species	14 (9.3)	Mpt ELISA	67 (44.4)
Deer	36	*M. bovis*	18 (50.0)	Mb ELISA	22 (61.1)
		Other species	02 (5.6)	Mpt ELISA	6 (16.7)

Comparison of traditional culture and ELISA method: Mycobacterial culture for all lymph node tissues mixed together identified 48 *M. bovis* strains and 16 strains of other *Mycobacterium* spp. (Table 3). The prevalence of *M. bovis* infection was higher in deer (50.0%) than in cattle (19.9%). Therefore, ratio of *M. bovis* and other *Mycobacterium* spp. were found in 2:1 (30 vs. 14) in cattle and 9:1 (18 vs. 2) in deer. Although, ELISA results showed higher mycobacerial infection rate than traditional culture in both animal species, ELISA analysis identified *M. bovis* infection in serum less than in traditional culture and more *M. paratuberculosis* infection in cattle (Table 3). However, such high *M. paratuberculosis* infection was not found in deer. Additionally, we found that five cattle and four deer were infected by both mycobacterial species.

CITT classification associated with *M. bovis* infection and gross lesion: CITT-positive group was only observed in Farm D with ITT-positive animals more than once in routine examination and lacked in other Farms A, B, C, E, F, G with ITT positive animals once (Table 4). A strong correlation was obtained between CITT-positive (+) and negative (-) group and *M. bovis* infection and gross lesion. In CITT-positive (+) group, all animals were infected by *M. bovis* and showed TB gross lesion. In contrast, animals in CITT-negative (-) group did not showed any *M. bovis* infection and any gross lesion. However, parts of animals in inconclusive (+) group was infected by *M. bovis* and showed gross lesion. Additionally, PCR amplification of soil/feces DNAs identified the presence of *M. paratuberculosism*, *M. avium* and other mycobacterial species.

Sequence analysis of *M. bovis*: Sequence analysis of 471 bp PCR products of *M. bovis* sequences and reference strain AF2122/97 (GeneBank U87961) revealed 99.3~100% nucleotide sequence homology. Phylogenetic information indicated that *M. bovis* from five deer and four cattle from Yulin were identical in sequence (Fig. 3), suggesting possibly transfer of *M. bovis* between cattle and deer. Other *M. bovis* from cattle were clustered in three other clusters, indicating diverse origins of *M. bovis* in cattle.

Table 4: CITT classification associated with *Mycobacterium bovis* infection and gross lesion

Farm[a]	CITT[b]	Number	*M. bovis* (%)		Gross lesion (%)		Soil/feces samples
A	±	2	0	0	0	0	*M. paratuberculosis,*
	-	2	0	0	0	0	Other mycobacterial species
B	-	1	0	0	0	0	Other mycobacterial species
C	±	1	0	0	0	0	Other mycobacterial species
	-	1	0	0	0	0	Other mycobacterial species
D	+	2	2	100	2	100	*M. avium*
	±	4	1	25	3	75	
	-	5	0	0	0	0	
E	±	1	0	0	0	0	*M. avium, M. paratuberculosis*
	-	1	0	0	0	0	
F	±	2	0	0	0	0	*M. avium, M. paratuberculosis*
	-	3	0	0	0	0	
G	-	2	0	0	0	0	*M. paratuberculosis*
Total	+	2	2	100	2	100	
	±	10	1	10	3	30	
	-	15	0	0	0	0	

[a]ITT: Positive animals was confirmed more than once in Farm D and once for other farms in routine examination, [b]+: Positive, ±: Inclusive and -: Negative

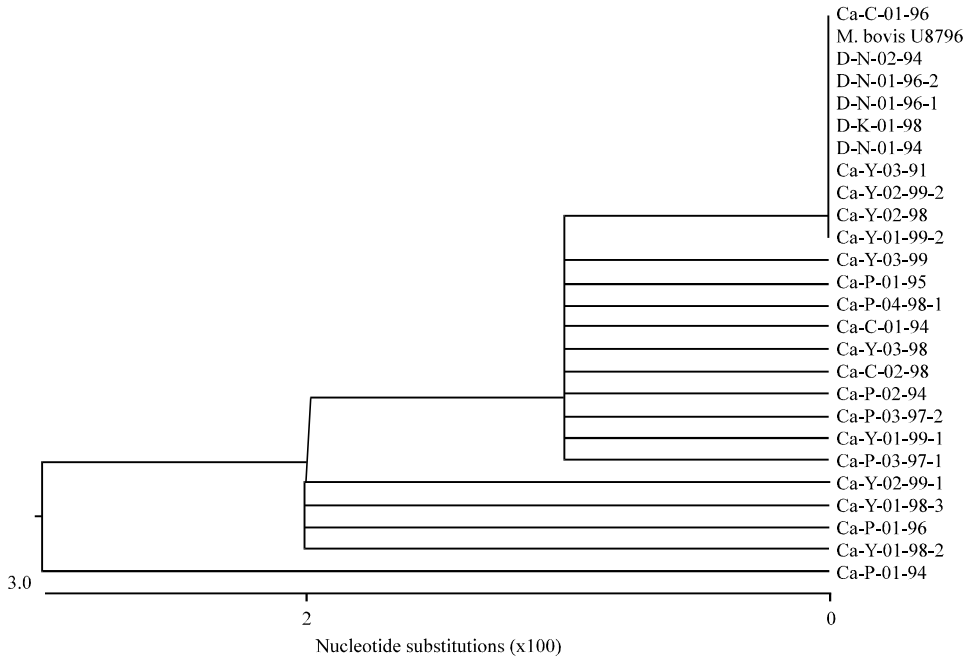

Fig. 3: Phylogenic tree constructed by analyzing the nucleotide sequences of 25 *M. bovis* isolates with the Clustal program of DNASTAR software

DISCUSSION

Tuberculosis prevalence is strongly associated with identification methods and mycobacterial species. ITT is a standard diagnostic method and has been used in Office International des E'pizooties (OIE) and the European Union (OIE, 2009). Although, ITT is easy to perform and

reveals high specificity, ITT frequently gives low sensitivity (Jiang, 1993; Ngandolo *et al*., 2009). In this study, ITT-positive samples still revealed negative results determined by isolation, culture, acid-fast stain and Mn-PCR detection. Although, *M. bovis* is the main pathogen causing TB in herbivores, *M. paratuberculosis* and *M. avium* can cause cross-reaction among ITT and other examination methods (Lauzi *et al*., 2000; Aranaz *et al*., 2006). Our investigation also determined that *M. avium*, *M. paratuberculosis* and other species presents in the soil and feces (Table 4), which give false positives for the ITT. However, ITT results showed higher identification rate in deer than in cattle in this study.

Early study reported that retropharyngeal lymph nodes was most prevalent site with gross lesion and mediastinal lymph nodes and hilar lymph nodes increased in gross lesion (Wu, 1989), suggesting that macrophage carrying *Mycobacterium* enters those organs early. Histopathological diagnostics demonstrated that the highest gross lesions occurred in retropharyngeal and mediastinal lymph nodes for cattle and in lung and retropharyngeal lymph nodes for deer (Table 3). *M. bovis* mainly infects the respiratory tract with the digestive tract little affected (Rhyan *et al*., 1992; Romero *et al*., 1999). In our study, *M. bovis* was the main pathogen causing TB in deer (50%), not in cattle (19.9%) (Table 3). In cattle, the main pathogen may be *M. paratuberculosis* and *M. avium* (Table 3, 4). Furthermore, traditional culture may yield negative results because of the low quantity of live bacteria (1 mL minimum requirement of 10,000 bacteria), transportation and processing (Wards *et al*., 1995; Araujo *et al*., 2005).

Secreted antigens of *M. bovis* are associated with different stages of disease development in cattle (Fifis *et al*., 1994). Therefore, ELISA analysis using MPB70 protein differed in sensitivity from 18.1-89.7% and specificity from 96.4-100% (Wood *et al*., 1991; Cho, 1998). In our study, sensitivity and specificity was 4.5-96.8%, respectively. However, antigens may not be associated with disease development (De la Rua-Domenech *et al*., 2006). Seroconversion and interference of other mycobacterial species may yield unsatisfactory results (Amadori *et al*., 2002). Therefore, mixing two or more TB-specific antigens may obtain better diagnostic data for identifying TB in cattle by ELISA analysis (Whelan *et al*., 2008). The present study also identified *M. paratuberculosis* infection in ITT-positive cattle (Table 3). Early study demonstrated that *M. avium* spp. and vaccination of live *M. paratuberculosis* interferes with TB diagnosis and displays false positive results (Aranaz *et al*., 2006; Varges *et al*., 2009).

Mn-PCR can successfully differentiate *M. tuberculosis* and *M. bovis* (Chu *et al*., 2012) and in this study, determined that the prevalence of *M. bovis* differed among the lymph nodes and lung with the highest prevalence (ca. 28%) in hilar lymph nodes, mediastinal lymph nodes and retropharyngeal lymph node; suggesting that these three lymph nodes near the respiratory tract are the main target for TB spread and the main site for pathogen isolation and sample collection (Liebana *et al*.,1999; Araujo *et al*., 2005). Although, Mn-PCR outperforms ELISA for tuberculosis detection, PCR detection of mycobacteria in blood may require the presence of pathogens in monocyte or macrophage, or free in plasma (Chia, 2001). Sequence analysis demonstrates that *M. bovis* may originate from the same sources and transmit infection between these two animals (Fig. 3).

CONCLUSION

In the ITT-positive cattle and deer, *M. bovis* was the major pathogen followed by *M. paratuberculosis* and other mycobacterial species. Among four methods, highest infection rate was found in the retropharynx and mediastinal lymph nodes of cattle and in the lung and

retropharynx lymph node in deer. CITT analysis could be used to evaluate *M. bovis* infection and TB gross lesion. Identical *M. bovis* strain infects cattle and deer in Yulin county and different origins of *M. bovis* were observed in three counties.

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