

Investigation of bovine tuberculosis outbreaks by using a trace-back system and molecular typing in Korean Hanwoo beef cattle

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Bovine tuberculosis is a chronic contagious disease responsible for major agricultural economic losses. Abattoir monitoring and trace-back systems are an appropriate method to control bovine tuberculosis, particularly in beef cattle. In the present study, a trace-back system was applied to bovine tuberculosis cases in Korean native Hanwoo beef cattle. Bovine tuberculosis was detected in three index beef cattle during abattoir monitoring in Jeonbuk Province, Korea, and the original herds were traced back from each index cow. All cattle in each original herd were subjected to tuberculin skin test. The positive rates in the tuberculin skin test were 64.6% (62 of 96), 4.8% (2 of 42), and 8.1% (3 of 37) at farms A, B, and C, respectively. On post-mortem examination of 56 tuberculin-positive cattle, 62% had granulomatous lesions, and *Mycobacterium bovis* was cultured from 40 (71.4%) of the cattle. Molecular typing by spoligotyping and the mycobacterial interspersed repetitive unit-variable-number tandem repeat assay revealed the genotype of the *M. bovis* strains from the index cattle were same as the *M. bovis* genotype in each original herd. The results suggest that tracing back from index cattle to the original herd is an effective method to control bovine tuberculosis in beef cattle.

Keywords: Korean Hanwoo beef cattle, bovine tuberculosis, index beef cattle, molecular typing, track-back system

Introduction

Bovine tuberculosis is a chronic contagious disease caused by *Mycobacterium bovis* that has been detected in a wide variety of animals [16]. Bovine tuberculosis is responsible for economic losses in agricultural herds. Many countries have adopted bovine tuberculosis control programs and testing, and culling has been used to control and eradicate bovine tuberculosis [15].

Bovine tuberculosis was first reported in 1913 in Korea, and the estimated incidence of bovine tuberculosis in the 1940s was 15% [14]. The National Bovine Tuberculosis Control Program was implemented in 1964 in Korea, and it relies on testing and culling [5]. Bovine tuberculosis is detected by using the

intradermal tuberculin skin test with a purified protein derivative (PPD). Through implementation of the National Bovine Tuberculosis Control Program, the incidence of bovine tuberculosis in Korea was only 0.15% in 2005 [12,17].

However, the National Bovine Tuberculosis Control Program has been instituted mainly in dairy cattle, whereas bovine tuberculosis control remains unresolved in beef cattle. About three million head of Korean native Hanwoo beef cattle are present in Korea [13]. Nationwide annual bovine tuberculosis testing with the intradermal tuberculin skin test has been performed on all dairy cattle, but only a limited number of beef cattle have been tested because, compared to the number of cattle that need to be tested, there are few authorized and trained

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personnel available to perform the testing.

A trace-back system could be an alternative to the tuberculin skin test. In such a system, if lesions suggesting bovine tuberculosis are detected on gross inspection at slaughter, all animals in the trace-back herd would be subjected to the tuberculin skin test [7,8]. A few reports have documented outbreaks of bovine tuberculosis among Korean beef cattle [2,9]; however, post-mortem examinations and mycobacterial cultures are rarely performed to confirm an *M. bovis* infection in the original herd traced back from the index cattle.

In the present study, suspected original herds were traced back from the index beef cattle, and the tuberculin skin test was performed on all cattle in that herd. Tuberculin-positive beef cattle were subjected to post-mortem and mycobacterial culture examination to confirm the presence of an *M. bovis* infection. Spoligotyping and a mycobacterial interspersed repetitive unit-variable-number tandem repeats (MIRU-VNTR) assay were conducted to identify the *M. bovis* transmission source.

Materials and Methods

Index beef cattle, trace-back system, and tuberculin skin testing

Three index beef cattle were identified via abattoir monitoring in Jeonbuk Province, Korea from July 2011 to October 2012. The index beef cattle had granulomatous lesions in the laryngopharyngeal lymph nodes, and acid-fast bacilli were identified via Ziehl-Neelsen staining. The original herds were traced back by applying the Animal Products Traceability System of the Korean Ministry of Agriculture, Food and Rural Affairs, and a tuberculin skin test was performed on all beef cattle in the original herds. The tuberculin skin test was performed by using the caudal fold tuberculin test according to official Korean national guidelines. In brief, 0.1 mL of bovine PPD was injected intradermally in the skin of the caudal tail fold, and skin thickness of the area where the PPD was injected was measured approximately 72 h later. The reaction was interpreted to be tuberculin-positive if skin thickness increased by > 5 mm, inconclusive if the increase in skin thickness was 1 to 4 mm, and negative if the increase in skin thickness was < 1 mm.

Post-mortem examination and mycobacterial culture

To determine whether the tuberculin-positive beef cattle were infected with *M. bovis*, they were subjected to post-mortem examination, which was performed by authorized personnel according to the guidelines of the Korean National Bovine Tuberculosis Eradication Program. The laryngopharyngeal lymph node, septal lymph node, diaphragmatic lymph node, lungs, and other tissues were collected for gross observation, histopathological examination, and mycobacterial culture at necropsy. Tissue samples for histopathology were fixed in 10%

neutral buffered formalin, and sections were stained with hematoxylin and eosin and Ziehl-Neelsen stain. The tissues were subjected to mycobacterial culture after homogenization, and the mycobacterial cultures were prepared by using the BD BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 System (Becton-Dickinson, USA). The cultured *M. bovis* isolates were confirmed by using Ziehl-Neelsen stain and SD Bioline TB AgMPT64 Ag Rapid test (Standard Diagnostics, Korea), as described previously [1]. The *M. bovis* isolates were subjected to molecular typing.

Spoligotyping and the MIRU-VNTR assay

Molecular typing to identify the *M. bovis* transmission source was performed by spoligotyping [3] and MIRU-VNTR assay [10]. A dendrogram based on spoligotyping and the MIRU-VNTR profiles was generated by using IBM SPSS (ver. 18.0; IBM, USA).

Results

Index beef cattle and tuberculin skin testing of the original herds

The index beef cattle were identified at a slaughterhouse in Jeonbuk Province in 2011 to 2012, and a trace-back for bovine tuberculosis was conducted to identify the original herds. All three index beef cattle had granulomatous lesions in laryngopharyngeal lymph nodes (Fig. 1), in which acid-fast bacilli were detected, and *M. bovis* was isolated from and identified from all three index cattle. Tuberculin skin testing was performed on all cattle of the remaining original herds at the three identified farms. The tuberculin skin test-positive rates were 64.6% (62 of 96), 4.8% (2 of 42), and 8.1% (3 of 37) at farms A, B, and C, respectively. Interestingly, the tuberculin skin test-positive rate was notably high at farm A.

Post-mortem examination and mycobacterial culture

At each farm, all tuberculin-positive cattle were subject to a

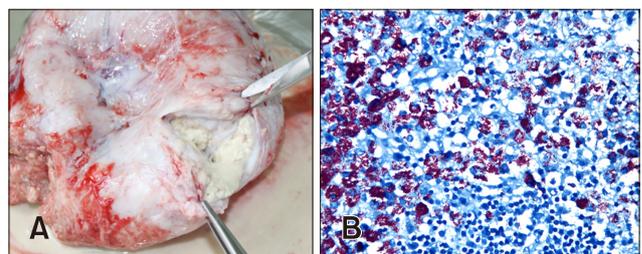


Fig. 1. Visible lesions in and Ziehl-Neelsen staining of the retropharyngeal lymph node of a beef cow. (A) Visible lesion showing caseous granuloma in a lymph node from a beef cow. (B) Ziehl-Neelsen stained portion of a lymph node showing acid-fast bacilli in a granuloma. 400× (B).

post-mortem examination, but that examination was only performed on 51 randomly selected cattle from farm A due to a limitation on the number of examinations that could be completed in a day. Of the 56 cattle examined, 35 (62.5%) had granulomatous lesions in various organs (Tables 1 and 2). The major organ affected was the laryngopharyngeal lymph node (45.7%, 16 of 35), followed by the lung (34.3%, 12 of 35). Other affected organs were the septal lymph node (11.6%, 5 of 35), the diaphragmatic wall (11.4%, 4 of 35), the thoracic wall (8.6%, 3 of 35), and the diaphragmatic lymph node (5.7%, 2 of 35).

Mycobacterial cultures were attempted for the affected organs as well as for corresponding lymph nodes with no visible gross lesions. Among the 56 post-mortem-examined tuberculin-positive cattle, 40 *M. bovis* strains (71.4%) were isolated. Most of the *M. bovis* strains were isolated from granulomatous lesions (85.0%, 34 of 40); the remaining six strains were isolated from laryngopharyngeal lymph nodes without visible lesions.

Spoligotyping and MIRU-VNTR assay

Spoligotyping and MIRU-VNTR assay were performed to investigate the relationships among the isolated *M. bovis* strains. All *M. bovis* strains were designated SB0140 for spoligotyping and were divided into two subgroups (Fig. 2). The major spoligotype subgroup was marked by a lack of spacer types 3, 6, 8–12, 16, and 39–43, which comprised 90.7% (39/43) of the isolated *M. bovis* strains. The other subgroup was marked by the absence of spacers 3, 5–14, 16, 19, and 39–43, to which four *M. bovis* strains (A36, A37, A38, and A39 strains from farm A) belonged.

Each VNTR allele of the *M. bovis* strains from the index cattle was detected in the original herd after undertaking MIRU-VNTR analysis. Interestingly, three additional *M. bovis* genotypes were observed on farm A. The VNTR allele 32531751143411343 and the spoligotype lacking spacers at 3, 6, 8–12, 16, and 39–43 was the major genotype, comprising 87.2% (34/39) of the *M. bovis* strains. The other genotype, accounting for 10.3% (4/39) of the *M. bovis* strains, had the same VNTR allele as the major genotype but a different

spoligotype subgroup lacking spacers at 3, 5–14, 16, 19, and 39–43. Interestingly, only one *M. bovis* strain had a VNTR allele different from the major VNTR genotype at farm A.

The *M. bovis* strains from farms B and C had the same VNTR alleles and spoligotypes as the genotypes of each of the index cows.

Discussion

The Korean National Bovine Tuberculosis Control Program was implemented in 1964 and has contributed to the reduced incidence of bovine tuberculosis in Korean dairy cattle [14]. However, reducing bovine tuberculosis in Korean beef cattle and other animals remains unresolved. Abattoir monitoring has been adopted in many countries to control bovine tuberculosis in beef cattle [15]. In such programs, cattle are inspected for bovine tuberculosis at the slaughterhouse and those with signs of tuberculosis are traced back to the original herd. There are two reports on bovine tuberculosis at slaughterhouses [9,11], but a thorough investigation based on the trace-back system, tuberculin skin test, post-mortem examination, and molecular typing has not been reported in Korea.

In the present study, three index beef cattle were found at a slaughterhouse; subsequent to tracing them back to the original herd, tuberculin skin testing was performed on all cattle in the original herds. Tuberculin-positive beef cattle were detected by skin testing at all three farms. The tuberculin-positive rates at farms A, B, and C were 64.6% (62 of 96), 4.8% (2 of 42) and 8.1% (3 of 37), respectively. The farm B and C results were similar to that presented in a previous report [4]. Interestingly, the tuberculin-positive rate at farm A was notably high. Most of the beef cattle at farm A were self-bred, and the cattle had been exposed to self-breeding for a relatively long period. At farm A, calving cows were infected with bovine tuberculosis, which may have been the tuberculosis transmission route for the entire herd.

Of the 56 tuberculin-positive beef cattle that underwent post-mortem examination, 35 (62.5%) had granulomatous lesions, and the most frequently affected organ was the

Table 1. Results of tuberculin skin testing and post-mortem examination of beef cattle at farms traced back from three index beef cattle

Farm	No. of cattle	No. of index cattle	No. of tuberculin-positive cattle (%)	No. of cattle with gross lesions (%)	No. of <i>M. bovis</i> isolates (%)
A	96	1	62/96 (64.6)	32/51 (62.7)*	38/51 (74.5)*
B	42	1	2/42 (4.8)	1/2 (50.0)	1/2 (50.0)
C	37	1	3/37 (8.1)	2/3 (66.7)	1/3 (33.3)
Total	175	3	67/175 (38.3)	35/56 (62.5)	40/56 (71.4)

*Of the 62 tuberculin-positive cattle, 51 were subjected to post-mortem examination; the percentages of cattle with gross lesions and *Mycobacterium bovis* isolates were calculated based on the cattle that underwent post-mortem examination.

Table 2. Isolated *Mycobacterium bovis* strains from beef cattle and the results of MIRU-VNTR assay and spoligotyping

Isolate	Farm	Origin	VNTR allele profile	Spoligotype
A01*	A	Laryngopharyngeal LN	32531751143411343	SB0140
A02	A	Laryngopharyngeal LN	32531751143411343	SB0140
A03	A	Laryngopharyngeal LN	32531751143411343	SB0140
A04	A	Laryngopharyngeal LN	32531751143411343	SB0140
A05	A	Laryngopharyngeal LN	32531751143411343	SB0140
A06	A	Laryngopharyngeal LN	32531751143411343	SB0140
A07	A	Laryngopharyngeal LN	32531751143411343	SB0140
A08	A	Diaphragm wall	32531751143411343	SB0140
A09	A	Lung	32531751143411343	SB0140
A10	A	Septal LN	32531751143411343	SB0140
A11	A	Lung	32531751143411343	SB0140
A12	A	Lung	32531751143411343	SB0140
A13	A	Laryngopharyngeal LN	32531751143411343	SB0140
A14	A	Diaphragm wall	32531751143411343	SB0140
A15	A	Lung	32531751143411343	SB0140
A16	A	Lung	32531751143411343	SB0140
A17	A	Laryngopharyngeal LN	32531751143411343	SB0140
A18	A	Diaphragm wall	32531751143411343	SB0140
A19	A	Laryngopharyngeal LN	32531751143411343	SB0140
A20	A	Laryngopharyngeal LN	32531751143411343	SB0140
A21	A	Lung	32531751143411343	SB0140
A22	A	Laryngopharyngeal LN	32531751143411343	SB0140
A23	A	Diaphragm wall	32531751143411343	SB0140
A24	A	Laryngopharyngeal LN	32531751143411343	SB0140
A25	A	Laryngopharyngeal LN	32531751143411343	SB0140
A26	A	Lung	32531751143411343	SB0140
A27	A	Laryngopharyngeal LN	32531751143411343	SB0140
A28	A	Lung	32531751143411343	SB0140
A29	A	Lung	32531751143411343	SB0140
A30	A	Thoracic wall	32531751143411343	SB0140
A31	A	Lung	32531751143411343	SB0140
A32	A	Thoracic wall	32531751143411343	SB0140
A33	A	Diaphragm LN	32531751143411343	SB0140
A34	A	Septal LN	32531751143411343	SB0140
A35	A	Lung	32532651143311253	SB0140
A36	A	Septal LN	32531751143411343	SB0140
A37	A	Septal LN	32531751143411343	SB0140
A38	A	Thoracic wall	32531751143411343	SB0140
A39	A	Septal LN	32531751143411343	SB0140
B01*	B	Laryngopharyngeal LN	52523751143311_1053	SB0140
B02	B	Diaphragm LN	52523751143311_1053	SB0140
C01*	C	Laryngopharyngeal LN	42533651143311_1052	SB0140
C02	C	Lung	42533651143311_1052	SB0140

LN, lymph node; MIRU-VNTR, mycobacterial interspersed repetitive unit-variable-number tandem repeat. **M. bovis* strains isolated from the index beef cattle.

laryngopharyngeal lymph node. At the three farms, the proportions of examined cattle with lesions were higher than those in a previous study, in which 20% to 50% of cattle with

tuberculin-positive reactions had pathological lesions [6]. These contrasting results may have been caused by differences in bovine tuberculosis policy implementation. In the report by

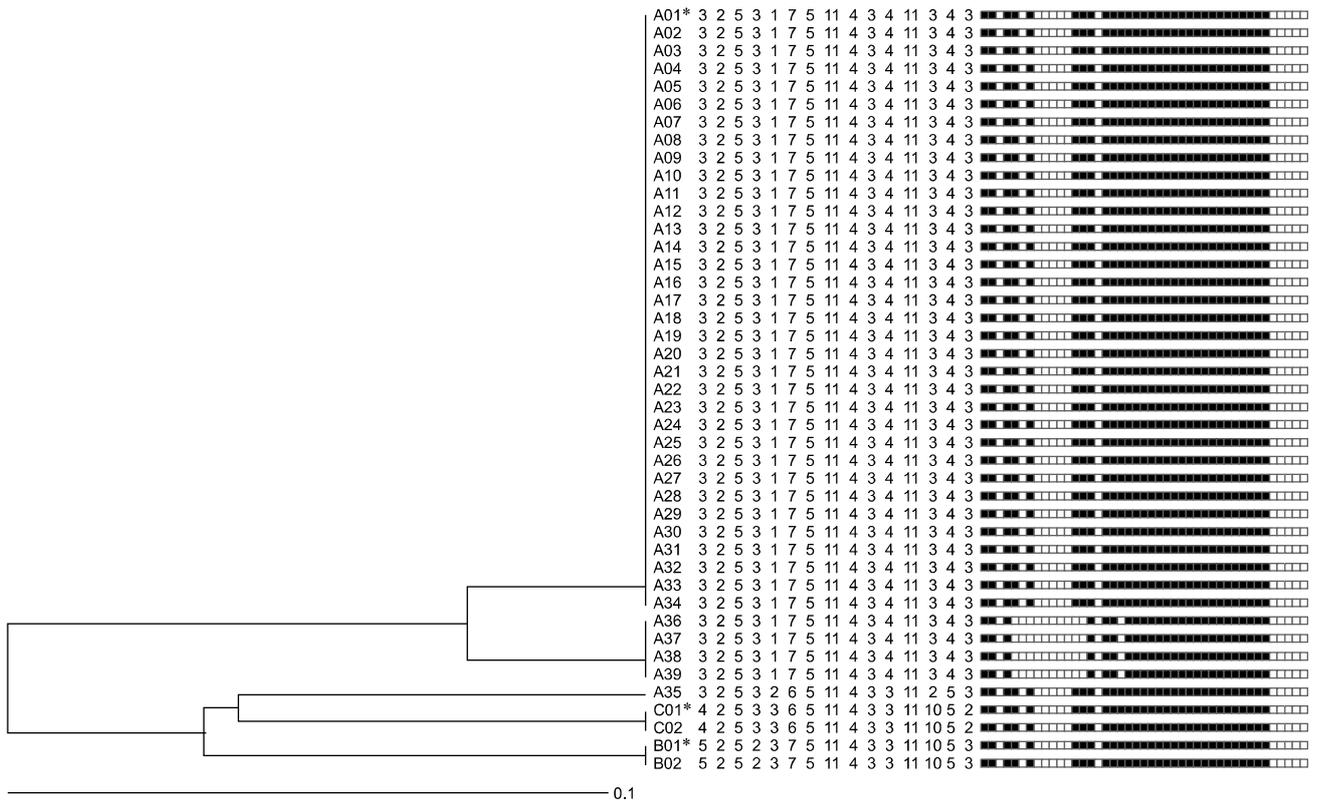


Fig. 2. Genetic relationships among the *Mycobacterium bovis* strains from beef cattle. The dendrogram is based on spoligotyping and MIRU-VNTR genotypes and created using the unweighted pair group method with arithmetic averages algorithm. MIRU-VNTR, mycobacterial interspersed repetitive unit-variable-number tandem repeat. **M. bovis* strains from the index beef cattle.

de la Rúa-Domenech *et al.* [6], post-mortem examination was performed on beef cattle that underwent routine tuberculin testing. In contrast, in this study, the tuberculin testing was performed after detecting tuberculosis in index cattle. Although the tuberculin skin testing is routinely performed on dairy cattle in Korea, routine tuberculin testing is rarely conducted on Korean Hanwoo beef cattle, which may contribute to the accumulation or aggravation of bovine tuberculosis incidence in beef cattle.

In this study, 40 *M. bovis* strains (71.4%) were isolated from among the 56 cattle examined post-mortem. Most were isolated from granulomatous lesions, but eight *M. bovis* strains (20.0%) were isolated from cattle with no visible lesions, suggesting that *M. bovis* can be cultured from cattle with or without lesions. Spoligotyping and the MIRU-VNTR assay on the *M. bovis* strains showed that the genotypes from the index cattle were also detected in the original herds. The *M. bovis* genotype with the VNTR allele 3253175114341134332 and spoligotype lacking spacers at 3, 6, 8–12, and 39–43 comprised 87.2% (34/39) of the *M. bovis* strains at farm A, suggesting that this was the major herd-transmitted genotype. On the other hand, the minor *M. bovis* genotype at farm A had the same VNTR allele but the spoligotype lacked spacers at 3, 5–14, 16, 19, and

39–43, indicating it may have been transmitted from another farm via imported infected beef cattle. Interestingly, there was one *M. bovis* strain with a unique VNTR allele at farm A, implying the strain might have been imported from another farm.

Nationwide bovine tuberculosis control in beef cattle has not been implemented in Korea because bovine tuberculosis in dairy cattle has taken priority; in addition, tuberculin testing is not mandatory for beef cattle in Korea. However, our results suggest that bovine tuberculosis testing in beef cattle is more urgent than anticipated, particularly at breeding farms where a control policy against bovine tuberculosis in beef cattle should be promoted.

In conclusion, tuberculin-positive beef cattle were detected in the original herds after the herd identity was traced back from the index beef cattle. Bovine tuberculosis in the beef cattle was confirmed by post-mortem and mycobacterial culture examination. Spoligotyping and MIRU-VNTR assay revealed that the genotype of the *M. bovis* strain from the index cattle were, for the most part, the same as that in the original herd.

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Conflict of Interest

The authors declare no conflicts of interest.

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