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ORIGINAL ARTICLE

Prevalence and co-infection status of brucellosis and tuberculosis in Hanwoo in Jeonnam province

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Brucellosis and tuberculosis are major infectious and contagious bacterial diseases in cattle. These diseases are malicious diseases that must be inspected at the slaughterhouse of cattle in accordance with the practice of quarantine in Korea. Furthermore, both diseases lead to abortion, reproductive disorder, and calf disease, causing major difficulty in the breeding of Korean Native cattle (Hanwoo), a representative industrial animal currently being raised in Korea. Co-infections of these diseases intensify clinical symptoms such as abortion and have a particularly significant effect on increasing mortality. Thus, serological tests were performed in Hanwoo, to establish the association of co-infection between brucellosis and tuberculosis in cattle. ELISA and PCR tests were conducted on blood samples collected from a total of 102 cattle in Jeonnam province, Korea, to detect brucellosis and tuberculosis infections. The PCR results revealed that 41 samples tested positive for *Brucella abortus* (*B. abortus*) infection (40.20%), and 5 samples tested positive for *Mycobacterium bovis* (*M. bovis*) (4.90%) infection confirmed by PCR. Notably, 9.76% (4/41) of the cattle infected with brucellosis also tested positive for tuberculosis. In conclusion, this study highlights the co-infection of brucellosis and tuberculosis among Hanwoo cattle in Jeonnam province, which is expected to contribute to our understanding of disease transmission, pathogenicity, the establishment of future prevention strategies.

Key Words: Brucellosis, Tuberculosis, Korean Native cattle, Hanwoo, Serological survey

INTRODUCTION

Korean Native cattle (Hanwoo) is the most important breed as an economically important livestock in Republic of Korea (Korea). The reproduction of Hanwoo calves plays a significant role in overall productivity of the cattle industry. Abortion in cattle defined as the loss of a fetus, is a primary cause of reproductive losses and carries economic importance in cattle (Cabell, 2007; Hovingh et al, 2009). Most diagnosed abortions in cattle are caused by infections of bacterial, viral, fungal and protozoal agents (Anderson, 2007). Especially, brucellosis (caused by *Brucella abortus*) and bovine tuberculosis (BTB, caused by *Mycobacterium bovis*) can have a profound impact on the fertility of cattle population (Joly and Messier, 2005).

Brucellosis, one of the most important zoonotic diseases, is caused by bacteria belonging to the genus *Brucella*, a Gram-negative bacterium (Cutler et al, 2005). In cattle, brucellosis is caused by *B. abortus* and it has a great economic implication due to reproductive failures, including abortion during the last trimester of gestation, infertility, orchitis, and epididymitis in males (Godfroid et al, 2005; Jung et al, 2010; Kaaboub et al, 2019). For decades, bovine brucellosis has been recognized as one of the most important endemic animal diseases in numerous countries, including Korea (Yoon et al, 2014).

B. abortus typically disappears within 1~5 months,

shortly after abortion in the uterus, because it develops well only within the fetal placenta. Brucellosis has been associated with a decrease in bacterial killing ability in a condition-dependent manner and an increase in IFN-γ concentrations, without lymphocyte proliferation (Pellegrini et al, 2022). Two common tests are used for brucellosis detection: the rose-bengal/card (RBC) test and buffered antigen plate agglutination test (BPAT), respectively (Nielsen, 2002). In Korea, BPAT is the most commonly used screening test due to its cost-effectiveness and standardization. This is the reason why BPAT is appropriate for individual animals screening. However, false positive reactions can occur frequently with BPAT (Nielsen, 2002). A novel enzyme linked immunosorbent assay (ELISA) is comparable to other screening tests for brucellosis. For example, the rose-bengal/card test, complement fixation test and agglutination test which have been confirmed and are still being used in Korea (Hur et al, 2007). Furthermore, PCR has capacity to meet the demand for better diagnostic tool. It has fine specificity, high sensitivity, costs less and appropriate for high volume controls. The process ensures rapid and simple performance, requiring less labor (Bricker, 2002). Consequently, PCR and ELISA methodologies are needed for a fine diagnosis of brucellosis.

Tuberculosis is a chronic disease closely related to the health risks for both human and animals (Admassu et al, 2015). *M. bovis* is the infectious agent responsible for BTB and spreads through urine, uterine discharges, semen, milk, or respiratory tracts. *Mycobacterium* has been reported that single infected cattle can secrete viable bacteria in its milk, making even pooled milk infectious or contaminated (Cosivi et al, 1995). While BTB is generally a chronic wasting disease in cattle, it can occasionally manifest as acute and rapidly progressive (Aboukhassib et al, 2016). Tuberculosis can also cause abortion in the late stages of cattle pregnancy (Plum, 1937).

The intradermal tuberculin test is considered as the diagnostic standard for this disease and is still widely used (Wood and Rothel, 1994). However, the sensitivity and specificity of this test seem to be unclear (Rothel et al, 1990). The use of the ELISA technique as a diagnostic tool for BTB detection was assessed by Ritacco in 1987, and he found that this technique has fine sensitivity and specificity for BTB detection. This finding suggests that ELISA could be a useful diagnostic tool for bovine tuberculosis detection (Lilenbaum et al, 1999).

Both diseases can be readily transmitted to humans via close contact with infected animals or animal tissue, such as placental membranes in the case of Brucellosis (Tschopp et al, 2009). Besides a public health, the presence of co-infection may indicate a possible relationship or interaction between the two disease. Cadmus et al. (2008) presented a case report on the co-infection of brucellosis and tuberculosis in cattle slaughtered in 2008. Co-infection of brucellosis and tuberculosis can lead to decreased productivity, an increased risk of abortion, and reduced calving rates in cattle (Muma et al, 2013). Thus, this study was designed to investigate brucellosis and tuberculosis in Hanwoo in Jeonnam province using serological detection methods.

MATERIALS AND METHODS

Blood sample collection

A total of 102 bovine blood samples were collected from Hanwoo cattle in 15 different farms located in Jeonnam province, Korea (Hampyeong, Yeonggwang, and Muan, with 15 to 20 samples per farm). These cattle had regular screenings for brucellosis and tuberculosis at the National Veterinary Services Laboratory, Jeonnam. Blood samples from the cattle were collected in vacuum tubes containing approximately 3 mL of heparin. These samples, collected between June and September 2018, were stored at −20℃ until required for testing with PCR and ELISA methods.

Buffered antigen plate agglutination test (BPAT)

Buffered antigen plate agglutination test (BPAT) was

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performed with antigen provided by the National Veterinary Services Laboratory, Jeonnam. The preparation and evaluation of this antigen were recently described. It is an 11% suspension of *B. abortus* strain 1119-3 stained with crystal violet and brilliant green and buffered to pH 3.63. The test was performed by mixing 80 μL of serum and 30 μL of antigen. The incubation time was 8 minutes, with the plate being rotated 4 times after 4 minutes of incubation. Reactions were read as ++ for complete agglutination and + for partial agglutination. A negative reaction was a homogenous serum antigen mixture with no evidence of agglutination.

Intradermal test

All cattle were tested through the purified protein derivatives (PPD) tuberculin test with an injection of 0.1 mL bovine PPD at caudal fold. After 48~72 hours, the skin thickness of the inoculation site was measured, and if the difference in thick was more than 5 mm, it was considered as positive. In addition, PPD tuberculin test is a test with a lot of false negative diagnosis because it is low sensitivity and high specificity. For this reason, PPD tuberculin test is a simple test in cattle tuberculosis screening in Korea.

ELISA detection test

The ELISA protocol for brucellosis was conducted in accordance with the manufacturer's instruction (BioNote Bovine *Brucella* Antibody ELISA 2.0, Korea). All samples were analyzed for the antibody test against *Brucella* Lipopolysaccharide (LPS). On basis of protocol, 490 μL solution was used to dilute cattle serum sample in the ratio 1:50. 50 μL of the diluted serum sample was then dispensed to the ELISA plate and incubated for 60 min at 37℃. After washing, 100 μL of the enzyme conjugate was dispensed to each well and incubated for 30 min at 37℃. After washing, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution (100 μL) was dispensed and the plates were incubated for a further 15 min at room temperature. Stop solution (100 μL) was dispensed to the reaction and the color action was read at 450 nm using an ELISA reader.

The ELISA protocol for bovine tuberculosis was conducted in accordance with the manufacturer's instruction (BioNote Bovine TB Antibody ELISA, Korea). All samples were analyzed for the antibody test against *Brucella* LPS. 50 μL of the cattle serum sample was then dispensed to the ELISA plate and 50 μL of the enzyme conjugate was then added to the ELISA plate and incubated for 60 min at 37℃. After washing, TMB substrate solution (100 μL) was dispensed and the plates were incubated for a further 15 min at room temperature. Stop solution (100 μL) was dispensed to the reaction and the color action was read at 450 nm using an ELISA reader.

Multiplex PCR for the detection of *B. abortus* and *M. bovis*

For negative and positive control for *M. bovis*, VetMAX *M. bovis* kit (Thermo) was used, which is controls for PCR and/or extraction yield. For negative and positive control for *B. abortus*, foodproof® *Brucella* Detection Kit (Biotecon Diagnostics) was used. It contained control template in stabilized solution of plasmid DNA and PCR-grade pure H_2O, respectively.

DNA concentration and purity were assessed by reading NanoDrop 2000c Spectrophotometer A260 and A280. To study the influence of DNA template from clinical blood specimens, about 3 mL peripheral blood sample was collected and taken for PCR analysis. All samples were aliquot and stored at −20℃ until tested. DNA was extracted from whole blood (200 μL) with the AccuPrep® Genomic DNA Extraction Kit (Bioneer) in accordance with the manufacturer's instructions.

Oligonucleotide primer

In this study, we utilized already well-known primers by extensive literature and nucleotide sequence searches in the NCBI databases. The DNA for brucellosis was

then subjected to PCR detection using most sensitive primers, Mar, out of 4 primer pairs such as NES2, DET, NES1 and EFQ1 (evaluation of different primers for detection of *Brucella* by using PCR method). These primer pairs were analyzed, using NCBI primer blast. A pair of primers Mar was previously reported to amplicon of 127 base pair (bp) that spans a region of the *B. abortus* genome. The DNA for *M. bovis* was then subjected to PCR (identification of *M. bovis* isolates by a multiplex PCR). A pair of primers, JB, was previously reported to amplicon of 500 base pair (bp) that spans a region of the *M. bovis* genome. We have proved that the pair of primers was specific for the genus *Brucella* spp. or *M. bovis*, respectively. Primer sequences used in this study are shown in Table 1.

DNA extraction and determination of DNA purity

Before the beginning, it is prepared completely dissolved solution of Proteinase K in 1,250 μL of nuclease-free water and RNase A in 600 μL of nuclease-free water. Then, correct amount of ethanol was added to (WA1) Buffer. For the DNA extraction from whole blood, 20 μL of Proteinase K was added into 1.5 mL or 2 mL tubes and then 200 μL of whole blood was applied each tube. After adding with 200 μL of genome binding (GB) Buffer, the samples were mixed immediately by vortex mixer and incubated at 60℃ for 10 minutes. 400 μL of absolute ethanol was added and mixed well by pipetting. The lysate was carefully transferred into the upper reservoir of the binding column tube without wetting the rim and centrifugated at 8,000 rpm for 1 minute. After the solution was discarded from collection tubes, 500 μL of washing (WA1) Buffer was added and the tubes were centrifugated at 8,000 rpm for 1 minute. With washing (W2) Buffer, the same procedure was repeated. After discarding the solution, the tubes were centrifugated again at 13,000 rpm for 1 minute to completely remove ethanol. The binding column tubes with no droplet clinging to the bottom were transferred to a new 1.5 mL tubes for elution. The tubes adding elution (EA) Buffer (50~200 μL) were incubated at room temperature (15~25℃) and centrifugated at 8,000 rpm for 1 minute. Concentration and purity of DNA were assessed by reading NanoDrop 2000c Spectrophotometer A260 and A280.

Optimization of DNA amplification (PCR)

In order to obtain optimal amplification of target genes, we set up the condition with concentration of critical reagents such as primer, $MgCl_2$ and template DNA and the annealing temperature of thermocycling. The PCR was performed in 25 μL volumes that contained 2.5 μL of 10× buffer, 0.5 mmol/L $MgCl_2$, 0.3 mmol/L dNTPs (Fermentas, GmbH, Germany), 0.5 pmol/L from each primer, 0.2 unit of Taq DNA polymerase enzyme, and 1 μL extracted DNA (For blood sample we used 5 μL of extracted DNA). PCRs were run using the following steps: a primary denaturation for four minutes at 94℃ followed by 35 cycles of denaturation at 94℃ for 1 minute, annealing gradient temperature at 54℃ for 1 minute and extension at 72℃ for 30 to 60 sec. At the end, one cycle for completion of the final extension was at 72℃ for 10 minutes. Then, 10 μL of the PCR was subjected to electrophoresis on 2% agarose gel (Cinagene Co, Iran) stained by 0.5 μg of ethidium bromide/

Table 1. Primer sequences used in PCR experiment

Pathogen	Primer name	Primer sequence (5'→3')	Amplicon size (bp)
B. abortus	Mar		127 bp
	Forward	GCATTCAATCTGATGGCGTTCC	
	Reverse	GATCACTTAAGGGCCTTCATTGC	
M. bovis	JB2		500 bp
	Forward	CGTCCGCTGATGCAAGTGC	
	Reverse	CGTCCGCTGACCTC AAGAAAG	

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ml (Sigma, Germany) and the results were evaluated in the presence of 100 bp DNA size marker (Fermentas Co, Ukraine), visualized under UV transilluminator. Finally, amplification products were sequenced by Macrogen Inc, Seoul, Korea.

Comparison of sensitivity between five pairs of primers assay (MAR, JB) was conducted by using serial dilutions of DNA template (10-1-10-4) of *B. abortus* or *M. bovis* and the PCR reaction was performed. Then sensitivity for pairs of each primer were determined by detection of limitations for the number of bacteria in PCR.

RESULTS

In order to detect co-infection of brucellosis and tuberculosis in Hanwoo in Jeonnam province, we conducted BPAT, PPD, ELISA, and PCR amplification from a total of 102 blood samples. As shown in Table 2, positive responses of brucellosis were detected in 63 samples (61.76%) in BPAT, 56 samples (54.90%) in ELISA, and 41 samples (40.20%) in PCR from a total of 102 suspected *Brucella* samples. Since BPAT is a primary test with high false positives due to high sensitivity and low specificity, the antibody test for *Brucella* antigen test in bovine serum was performed by ELISA for closer test. Although ELISA could discriminate the false positives, ELISA detected antibody in serum even in termination of infection without antigen. Thus, the PCR test was finally performed to confirm the *Brucella* infection. The primer used for the *B. abortus* test is MAR, the highest sensitivity primer among the five primer pairs already reported, and DNA was extracted directly from serum, and prepared freshly. The PCR products of 192 bp were observed in the positive control, but not in the negative control (Fig. 1). Through this, a total of 41 samples suspected of *B. abortus* infection were finally confirmed.

For tuberculosis detection, PPD, ELISA, and PCR were performed with 102 blood samples (Table 2). Positive responses of tuberculosis were detected in 2 samples (1.96%) in PPD, 6 samples (5.88%) in ELISA, and 5 samples (4.90%) in PCR from a total of 102 blood samples. PCR test was finally performed to confirm the *Mycobacterium* infection. The primer of JB2 used for the *M. bovis* test and DNA was extracted directly from serum and prepared fresh. The PCR products of 500 bp were observed in the positive control, but not in the negative control (Fig. 1). Finally, 5 samples suspected of *M. bovis* infection were confirmed as positive (Fig. 2).

Based on these results, we would like to determine the co-infection of brucellosis and tuberculosis. Among 5 tuberculosis-positive (*M. bovis*) samples, 4 of these samples were identified as *Brucella* infection (*B. abortus*) (Table 3). Thus, 4 cattle (3.92%) from total 102 cattle had been infected with both brucellosis and tuberculosis. In other words, among 41 *B. abortus*-infected cattle, 4 cattle (9.76%) had been infected with tuberculosis.

Table 2. Prevlence of brucellosis and tuberculosis tested by different methods

Pathogen	Sample number	BPAT		ELISA		PCR	
		P	N	P	N	P	N
Brucellosis	102	63 (61.76%)	39 (38.24%)	56 (54.90%)	46 (45.10%)	41 (40.20%)	61 (59.80%)
Pathogen	Sample number	PPD		ELISA		PCR	
		P	N	P	N	P	N
Tuberculosis	102	2 (1.96%)	100 (98.03%)	6 (5.88%)	96 (94.11%)	5 (4.90%)	97 (95.10%)

All samples underwent analysis for brucellosis and tuberculosis. Each number of samples was considered the number of positive (P) and negative (N) reaction.
BPAT, buffered antigen plate agglutination test; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; PPD, purified protein derivatives test; P, positive; N, negative.

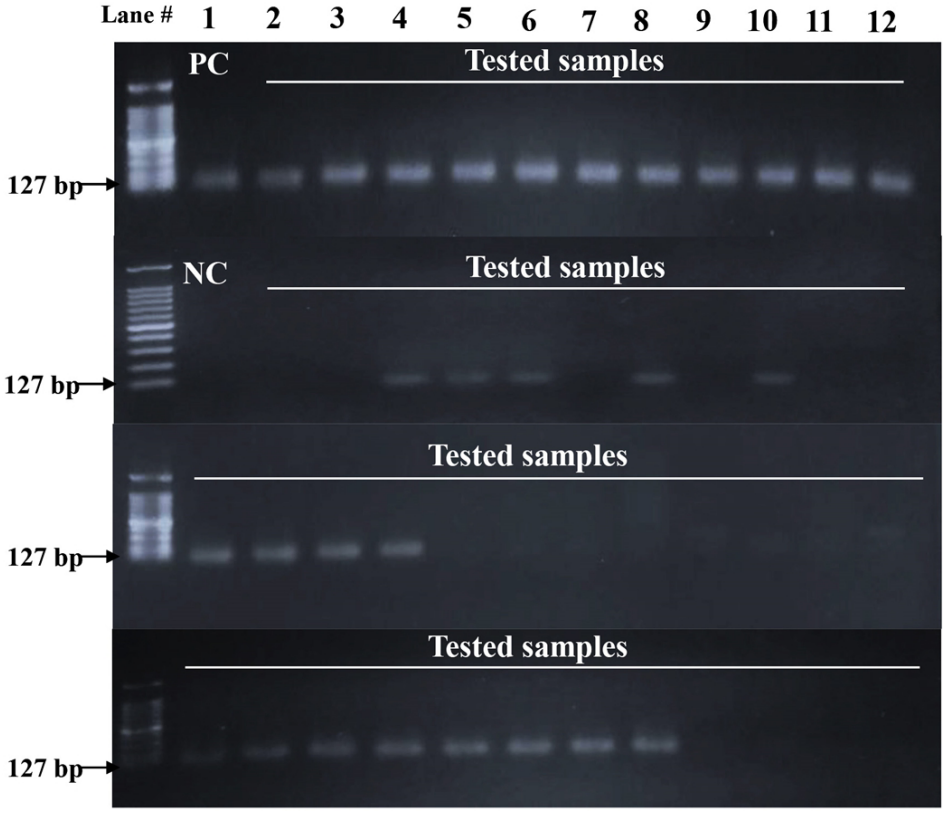

Fig. 1. PCR detection of *B. abortus* in serum samples using Mar primer. DNA extracted from serum samples was used for analysis. Amplification products were separated by electrophoresis on 1.5% agarose gel and stained with ethidium bromide (10 μg/mL). 100 bp of markers were loaded at the left end of each panel. Lane 1 of first panel (PC); positive control, lane 1 of second panel (NC); negative control. Remaining lanes: tested sample for detection of *B. abortus*. Arrows indicate the position of the fragments of 127 bp (diagnostic for *B. abortus*).

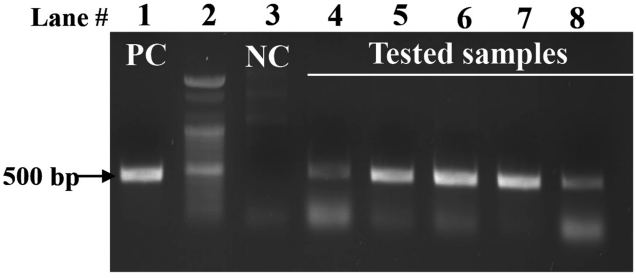

Fig. 2. Identification of *M. bovis* by PCR using JB2 primer. DNA extracted from serum samples was used for analysis. Amplification products were separated by electrophoresis on 1.5% agarose gel and stained with ethidium bromide (10 μg/mL). Lane 1 (PC); positive control, lane 2 (Marker); 100 bp, lane 3 (NC); negative control, lane 4~8: PCR product of *M. bovis* from serum samples. Arrows indicate the position of the fragments of 500 bp (diagnostic for *M. bovis*).

DISCUSSION

This study was designed to determine the prevalence of co-infections of brucellosis and tuberculosis in Hanwoo cattle in Jeonnam province and access the severity of pathogenicity, such as abortion and infertility. Using serological detection methods with various simplicity and specificity, we confirmed the 41 blood samples as positive for *B. abortus* infection. Among these 41 samples, four samples showed co-infection with tuberculosis. Although the overall co-infection rate in cattle (4/102, 3.91%) is seemed to be relatively low, the rate in 41 *B. abortus*-infected cattle (4/41, 9.76%) is higher than previous reported value in Korea (Kim et al, 2023). This result implies that brucellosis and tuberculosis are related each other to develop co-infection in cattle, or brucellosis promotes transmission or infection of tuberculosis, most likely giving more chances to increase host susceptibility and change immune status in the host, which are not clearly elucidated. Therefore, in the case of cattle infected with *Brucella* spp., *Mycobacterium* infection should be primarily considered with a comprehensive investigation and various tests.

The presence of co-infection may suggest a possible relationship or interaction between the two disease. Numerous studies have suggested that co-infection

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Table 3. Co-infection of brucellosis (*B. abortus*) and tuberculosis (*M. bovis*) determined by different methods

Sample number	Brucellosis			Tuberculosis			Tentative Diagnosis	
	BPAT	ELISA	PCR	PPD	ELISA	PCR	*B. abortus*	*M. bovis*
1	P	P	P	P	P	P	**P***	**P***
2	P	P	P	N	P	P	**P***	**P***
3	P	P	P	P	P	P	**P***	**P***
4	N	P	P	N	P	P	**P***	**P***
5	N	N	N	N	P	P	N	P

Total 5 of 6 samples were positive for tuberculosis, especially *M. bovis*, based on the PCR result. Among them, 4 samples showed co-infection of brucellosis and tuberculosis. The percentage of sample suspected co-infection was 3.92% (4/102) (marked with asterisk).
BPAT, buffered antigen plate agglutination test; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; PPD, purified protein derivatives test; P, positive; N, negative.

of brucellosis and tuberculosis are prevalent in cattle. Cadmus et al. (2008) presented a case report on co-infection of brucellosis and tuberculosis in cattle slaughtered in 2008. In addition, co-infection of brucellosis and tuberculosis can lead to decreased productivity, an increased risk of abortion, and reduced calving rates in cattle (Muma et al, 2013). Furthermore, co-infection of two diseases in cattle is supposed to increase susceptibility to each disease and mortality. Particularly, the mortality rate of cattle co-infected with both diseases was 1.9 times higher than that of cattle infected only with brucellosis, and 2.4 times higher than that of cattle infected only with tuberculosis. Crucially, the mortality rate of cattle co-infected with both two diseases was 3.9 times higher than that of cattle negative for both diseases (Gorsich, 2013). Moreover, cattle with brucellosis are more susceptible to tuberculosis (Gorsich, 2013). Therefore, the detection of co-infection of brucellosis and tuberculosis in this study is important to understand mortality and susceptibility of these diseases.

Besides serious economic impacts of both diseases on the livestock industry, brucellosis and tuberculosis can be readily transmitted to humans via direct contact with infected animals and the consumption of raw dairy products (Tschopp et al, 2009). For this reason, controlling both disease in intensive livestock production systems is essential for both human and animal health. Brucellosis and tuberculosis are important diseases that must be screened for the slaughter of cattle in accordance with the practice of quarantine in Korea (Yoon et al, 2014). In Korea, since first detection of bovine brucellosis in 1955 in imported cattle originating from the US (Hur et al, 2007), Korean surveillance program of bovine brucellosis was extended that all dairy herds were screened annually and 97% of beef herds were tested to control the incidence of human brucellosis (Ryu et al, 2019). Meanwhile, brucellosis is designated as a Class 2 legal livestock infectious disease in Korea (Jung et al, 2010). In 1913, BTB was for the first time reported in Korea (Moon, 1966). Slaughterhouse surveillance for BTB was first designated by law in 1962. In addition, a national BTB control and eradication program was established in 1964 with the implementation of field surveillance in the national cattle farm. A total of 62 animal diseases are currently designated by law of the Act on the Prevention of Contagious Animal Diseases in Korea. There are 15 diseases in Class 1 legal infectious diseases, 32 diseases in Class 2, and 21 diseases in Class 3 designated by ordinance. In addition, BTB is designated as a Class 2 legal infectious disease (Wee et al, 2010).

Based on the finding of this study, epidemiological survey of co-infections is recommended over the country in Korea. In addition, cattle showing co-infections of brucellosis and tuberculosis should be accurately identified through multiple serological tests in the methodological way. In conclusion, this study demonstrates the prevalence of co-infection of brucellosis and tuberculosis in Hanwoo in Jeonnam province. Moreover,

further academic research is needed to investigate disease transmission, epidemiological surveys, symptoms, pathogenicity, treatment and prevention.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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