

Diagnosis of Bovine Tuberculosis by PPD-ELISA and Sonication-ELISA

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ABSTRACT

Mycobacterium bovis (*M. bovis*) infection in cattle remains a major zoonotic and economic problem in many countries. In addition, *M. bovis* is an important pathogen of both man and animals. The standard diagnostic test for this disease is the intradermal tuberculin test, one of the oldest immunological tests still in widespread use. However, this test can lack both sensitivity and specificity. Serological tests such as ELISA (Enzyme Linked Immunosorbent Assay) have been suggested as an alternative method for the diagnosis of tuberculosis. In the present study, two serum ELISA's, performed for the detection of *M. bovis*-specific antibodies, were evaluated against the culture of tissue samples from 135 slaughtered dairy cows, which were intradermal tuberculin-test-positive. ELISA plates were coated with Bovine Purified Protein Derivative (PPD B) and *M. bovis*-sonication antigen. Samples were taken from the bronchial (BLN), mesenteric (MLN) and prescapular (PLN) lymph nodes and tubercles (TB) of slaughtered cattle. Tissue samples were cultured in Lowenstein-Jensen medium. Blood samples, which were collected from the tuberculin-test-positive animals before slaughter, were tested by PPD B-ELISA (PPD-ELISA) and *M. bovis* sonication-ELISA (sonication-ELISA). *Mycobacterium* spp. was isolated from 77 out of 135 cattle. Out of 135 cattle, 62 (45.9%) and 43 (31.8%) were determined to be positive for PPD-ELISA and sonication-ELISA, respectively. Using culture-positive animals as a gold standard, the sensitivity and specificity of these two methods were detected as 48.2%-55.2% and 31.2%-44.5%, respectively.

Keywords: *Mycobacterium bovis*, PPD-ELISA, sonication-ELISA, bovine tuberculosis.

INTRODUCTION

Bovine tuberculosis is a significant infectious disease caused by *Mycobacterium bovis* (*M. bovis*) that results in large economic losses and is a major public health concern (1). In 2011, there were an estimated 8.7 million new cases of tuberculosis (13% co-infected with HIV) and 1.4 million people died from tuberculosis worldwide. The rate of infection was reported as 0.24% in the total population of Turkey (2). In 2011, 1153 (22.8%) out of 5043 cattle were detected to be positive for tuberculosis by the Purified Protein Derivative (PPD) tuberculin test in Turkey (3). Disease control programmes implemented in most countries are based on the testing of cattle with PPD-tuberculin and their slaughter (4). In spite of its wide use, the intradermal tuberculin test

has some important limitations, related to its sensitivity and specificity (5, 6). This test requires the animals to be handled twice, and it cannot be repeated for at least 60 days, because of systemic desensitization caused by the injection of tuberculin (6). The use of serological tests such as ELISA has been suggested as an alternative method for tuberculosis diagnosis (7). Since the first serological trial in 1948 using an agglutination test, many assays have been performed to detect specific antibodies against *Mycobacterium* (8). ELISA is a useful serological test for the diagnosis of bovine tuberculosis (9). The ELISA technique has been applied as a sensitive method for the measurement of antibodies in the sera of tuberculosis animals (10). The use of the ELISA for the diagnosis of bovine tuberculosis was evaluated by Ritacco *et*

al. (11), who reported that this method had good sensitivity and specificity. In the advanced phases of the infection, PPD tuberculin test-negative anergic cattle can be diagnosed only by serological tests (12).

In the present study, two laboratory prepared ELISA systems were used for the diagnosis of bovine tuberculosis. Using culture-positive animals as a gold standard, the sensitivity and specificity of these methods were calculated.

MATERIALS AND METHODS

Culture

Bronchial (BLD), mesenteric (MLD) and prescapular (PLD) lymph nodes and tubercles (TB) samples were taken from 135 slaughtered dairy cows, which were determined to be intradermal tuberculin-test-positive. The cows were of different ages and from four different farms which were located in four different cities. Tissue samples were decontaminated with 0.75% (w/v) of hexadecylpyridinium chloride (Sigma, C 9002, Germany) and inoculated into Lowenstein-Jensen medium (Merck VM614000, Germany). Culture media were incubated at 37°C for 4 to 8 weeks (13, 14, 15).

Serum samples

Blood samples were collected from the 135 tuberculin-test-positive cattle before slaughter. Samples were centrifuged at 1000 xg for 10 min for the extraction of serum which were stored at -20°C. Ten positive sera from *Mycobacterium bovis* culture-positive cattle and 20 sera from intradermal tuberculin-test-negative and BOVIGAM (Prionics, Cat. 63306/63326, Switzerland) gamma interferon (IFN- γ) test-negative calves originating from tuberculosis-free farms were used as positive and negative controls respectively.

PPD B-ELISA

To establish the optimal dilutions of antibodies, conjugate and the concentration of the coating antigen, a checkerboard titration method was performed. Polystyrene 96-well microtiter plates (Greiner Bio-one, Germany) were coated with 100 μ l of a 10 μ g/ml concentration (1 μ g/well) of Bovine PPD (Central Veterinary Control and Research Institute, Turkey) in carbonate-bicarbonate buffer (pH 9.6), at 4°C overnight. The plates were washed three times with phosphate buffered saline (PBS) (0.1 M, pH 7.2) containing 0.05% Tween-20

(PBS-T). After blocking the plates for 1 hr at room temperature (20°C) with 1% Bovine Serum Albumin (BSA) in PBS, pH 8.0 (100 μ l/well), the plates were again washed three times; 100 μ l of a 1:128 dilution of serum in PBS was added to each of the wells and incubated for 1 hr at room temperature. The plates were again washed three times and incubated with 100 μ l/well of peroxidase-conjugated rabbit anti-bovine IgG antibody (Sigma, A7414, Germany) diluted 1:8000 in PBS, for 1 hr at room temperature.

Plates were washed as described above, and enzyme activity was assayed by incubation for 30 min in the dark at room temperature with 100 μ l/well of substrate δ -phenylenediamine dihydrochloride (Sigma, P8287, Germany) in phosphate-citrate buffer with sodium perborate (Sigma, P4922, Germany). The reaction was stopped by the addition of 50 μ l/well of 0.5 M H₂SO₄, and the optical density (OD) was measured at 450 nm using an automatic microtiter plate reader (BIOTEK ELx800, USA) (7, 16, 17, 18, 19).

Preparation of Sonication Antigen

M. bovis AN5 was obtained from the Central Veterinary Control and Research Institute in Turkey was cultured in Middlebrook 7H9 Broth (Difco, 271310, USA) for 4 to 5 weeks at 37°C. Formaldehyde solution (37%) was added to the culture media and incubated overnight at 37°C for the inactivation of the bacteria. After centrifugation of the mycobacteria for 30 min at 12,000 xg and 4°C, the pellet was washed three times with PBS. Subsequently, the pellet was resuspended in 20 ml of extraction buffer (0.5% Triton X-100 in 10 mM Tris-HCl, pH 8) and sonicated at 80 amplitude for 20 min (Ultrasonic Processor GA 130, USA) in a cooled glass jar. After centrifugation of the sonicate for 30 min at 48,000 xg and 4°C, the supernatant was decanted and stored at -20°C (20, 21). The protein concentration of the sonicate antigen was measured using the BIO-RAD DC Protein Assay (Cat No. 500-0116, Bio-Rad Lab, USA).

Sonication-ELISA

M. bovis-sonication-ELISA was performed using the protocol described by Speer *et al.* (20) and Arias-Bouda *et al.* (21). The optimal dilution of the antibodies, conjugate and the concentration of the coating antigen were determined by a checkerboard titration method at 1:256, 1:8000 and 3 μ g/well, respectively.

Data analysis

ELISA results were analyzed using negative cut-off values, which were calculated as the arithmetic mean of the optical density (OD) plus 3SD (Standard Deviations) in the sera of 20 tuberculosis-negative calves (22). Sensitivity and specificity were calculated according to the method described by Walker *et al.* (23).

RESULTS

Culture

In total, 117 *Mycobacterium* spp. were isolated from 77 bronchial lymph nodes, 18 prescapular lymph nodes and 4 mediastinal lymph nodes. These isolates identified as *Mycobacterium bovis* (93 of them *M. bovis* subsp. *bovis* and 24 of them *M. bovis* subsp. *caprae*) by Polymerase Chain Reaction (PCR) and Restriction Endonuclease Analysis. The results have been presented in a previous study (24). The culture positive tissue samples were obtained from 77 out of 135 intradermal tuberculin-test-positive cattle (57%).

ELISA's

Negative cut-off values of PPD-ELISA and sonication-ELISA were calculated as 0.437 and 0.515, respectively. According to these results, PPD-ELISA and sonication-ELISA were detected in 62 (45.9%) and 43 (31.8%) out of 135 cattle as tuberculosis-positive (Figures 1 and 2). Using culture-positive animals as a gold standard, the sensitivity and specificity of these methods were detected as 48.2%-55.2% and 31.2%-44.5%, respectively (Table 1).

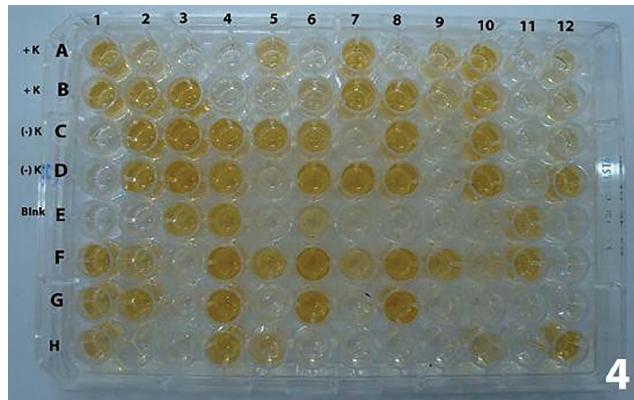


Figure 1: PPD-ELISA test; (+) K; positive kontrol, (-) K; negative control, Blnk; blank.

Table 1. Evaluation of PPD-ELISA and sonication-ELISA for the diagnosis of bovine tuberculosis

ELISA Results	PPD-ELISA	Sonication-ELISA
Positives	62 (45.9%)	43 (31.8%)
Negatives	73 (54.1%)	92 (68.2%)
Total	135	135
Sensitivity	48.2%	31.2%
Specificity	55.2%	44.5%

DISCUSSION

Bovine tuberculosis remains a major problem throughout the world (25). The control of the disease is achieved by the detection and disposal of infected animals to prevent the spread of the infection (26). The tuberculin skin test is the predominant method of diagnosis for bovine tuberculosis. However it is not sensitive and specific and often shows false positive results because of cross-reactions with environmental mycobacteria and other bacteria. Furthermore, the skin test cannot identify all cattle infected with tuberculosis.

The development of practical serological tests for the diagnosis of tuberculosis constitutes one of the most important problems of the veterinary medical profession (25). ELISA appears to be the best choice and can be used for the confirmation of skin test results (27). The ELISA technique has been practically applied as a sensitive method for the measurement of antibodies in the sera of tuberculosis animals (10).

The use of ELISA techniques for the diagnosis of bovine tuberculosis has been evaluated by Ritacco *et al.* (11), who reported good sensitivity and specificity for the detection of the disease. This report suggests that ELISA can be a valuable tool for the diagnosis of bovine tuberculosis. However, a

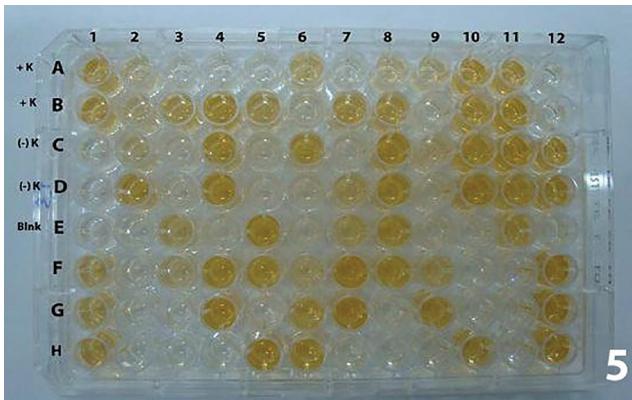


Figure 2: Sonication-ELISA test; (+) K; positive kontrol, (-) K; negative control, Blnk; blank.

later study from the same group showed a reduced sensitivity for the test, while the specificity was convincing (28). The ELISA techniques for the diagnosis of bovine tuberculosis have been performed in Turkey for the first time by Keskin (16), who detected 51 (5.8%) out of 870 cattle as tuberculosis positive by PPD-ELISA. In a latter study in Turkey, Akçay (18) detected 164 (22%) out of 582 cattle as tuberculosis positive by PPD-ELISA. In the present study, PPD-ELISA results showed that 45.9% of the investigated animals were tuberculosis positive. This result was in agreement with the findings of Gutierrez *et al.* (29) and Asiak *et al.* (26), who recorded 40.7% and 45.7% as seropositive, respectively.

The sensitivity-specificity of PPD-ELISA and sonication-ELISA were detected as 48.2% and 55.2%, and, 31.2% and 44.5%, respectively. On the other hand, higher results were reported by Ritacco *et al.* (11), Gutierrez *et al.* (29), Lilenbaum *et al.* (7), Kiran *et al.* (30), Levy *et al.* (31), Jain *et al.* (32) and Speer *et al.* (20). Lilenbaum *et al.* (7) detected the sensitivity and specificity rates of bovine PPD-ELISA as 86.7% and 90.6%, respectively, for bovine tuberculosis while Gutierrez *et al.* (29) detected these as 54.9% and 88%, respectively, for goat tuberculosis. Speer *et al.* (20) detected the sensitivity and specificity of sonication-ELISA as 95.6% and 100%, respectively, for bovine paratuberculosis infection. The sonication-ELISA technique is usually used for diagnosis of human tuberculosis. Kiran *et al.* (30), Levy *et al.* (31) and Jain *et al.* (32) determined the sensitivity and specificity of sonication-ELISA as 88%-94%, 70%-85.4% and 91.6%-85.7%, respectively, for human tuberculosis.

Our results demonstrated that the use of ELISA was not effective for the diagnosis of bovine tuberculosis. These results were in agreement with the reports of Auer (33), Placket *et al.* (12), Ritacco *et al.* (28), Casillas *et al.* (34), Keskin (16, 19), Keskin and İzgür (17), and Akçay (18), who reported that ELISA was unsuitable as an alternative to tuberculin testing for the detection of tuberculous cattle. However, anergic tuberculous cattle, which are false-negative to the tuberculin test, can be detected by ELISA (12, 35). We determined that PPD-ELISA was more sensitive and specific than sonication-ELISA.

The immune response to bovine tuberculosis is multifaceted and diagnostic parameters are likely to evolve differently as the disease progresses (1). Antibody titers in tuberculous animals generally depend on the stage of the disease. High positive titers reflect the active stage of the disease (35, 36,

37). The use of ELISA for the diagnosis of bovine tuberculosis has several advantages over the tuberculin skin test. It requires the handling of animals only once for the collection of blood samples. Blood samples are collected for the serological diagnosis of multiple diseases. Unlike the injection of tuberculin, blood sampling can be repeated as often as necessary, without affecting the immune system of the animal (7).

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