Comparison of Tuberculin Skin Test, IFN-γ Assay, Real Time PCR and Lateral Flow Rapid Test in Diagnosis of Field Outbreaks of Bovine Tuberculosis

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Abstract

Bovine tuberculosis is an important zoonotic disease transmitted by direct contact, respiratory pathway, ingestion of unpasteurised milk and milk product, raw or undercooked meat. Tuberculosis can be difficult to diagnose based only on the clinical signs. Tuberculosis is usually diagnosed in the field with the tuberculin skin test. Sputum and other body fluids may be collected for microbiological examination. Polymerase chain reaction (PCR) methods have also been described. Diagnostic blood tests include the lymphocyte proliferation assay, the interferon gamma (IFN-γ) assay, and enzyme-linked immunosorbent assays (ELISA). In this study a total of 50 animals were tested by using tuberculin skin test (TST), lateral flow rapid test, IFN-γ assay and real time PCR. The animals were selected randomly among 178 cattle in dairy farms with the aged between 3-5 years and suspected of having tuberculosis. Forty five cattle were positive out of 50 for TST while 31 for reactive by the IFN-γ assay and 28 for rapid test and 9 for real time PCR. The purpose behind such variable as age was to compare sensitivity of tuberculin skin test, the IFN-γ assay and TB lateral flow rapid test and real time PCR examination for the diagnosis of field outbreaks of bovine tuberculosis in Turkey.

Keywords: *IFN-γ assay, Real time PCR, TB lateral flow rapid test, Tuberculin skin test, Tuberculosis*

Sahada Görülen Sığır Tüberkülozunun Tanısında Kullanılan Tuberculin Skin Test, IFN-γ Assay, Gerçek Zamanlı PCR ve Lateral Flow Rapid Testlerinin Karşılaştırılması

Özet

Sığır tüberkülozu; direkt temas, solunum yolu, pastörize edilmemiş süt ve süt ürünleri, çiğ ya da az pişmiş etlerin alınmasıyla insanlara da bulaşabilen önemli bir zoonoz hastalıktır. Yalnızca klinik bulgulara bakarak tüberkülozu teşhis etmek zor olabilmektedir. Genellikle tüberkülozun tanısı sahada tüberkülin testiyle konulmaktadır. Mikrobiyolojik muayene amacıyla kraşe ve diğer vücut sıvıları temin edilmektedir. Polymerase chain reaction (PCR) yöntemleri de kullanılabilmektedir. Tanı amaçlı kan testleri arasında lenfosit proliferasyon testi, interferon gamma (IFN-γ) testi ve enzyme-linked immunosorbent assay (ELISA) bulunmaktadır. Bu çalışmada 50 hayvan tuberculin skin test (TST), lateral flow rapid test, IFN-γ assay ve gerçek zamanlı PCR kullanılarak test edilmiştir. Hayvanlar 3-5 yaş arasındaki tüberküloz şüpheli 178 sütçü sığır arasından rastgele seçilmiştir. TST sonuçlarına göre 50 sığırdan 45'i pozitifken, IFN-γ testine göre 31'i pozitif bulunmuştur. TB lateral flow rapid testte 28 pozitif, gerçek zamanlı PCR testinde ise 9 pozitif sonuç bulunmuştur. Çalışmamızda farklı testlerin uygulanmasının amacı tüberkülin skin test, IFN-γ assay, TB lateral flow rapid test ve gerçek zamanlı PCR testinin Türkiye'de karşılaşılan sığır tüberkülozundaki duyarlılıklarının karşılaştırılmasıdır.

Anahtar sözcükler: *IFN-γ assay, Real Time PCR, TB Lateral Flow Rapid Test, Tuberculin Skin Test, Tuberculosis*

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INTRODUCTION

Bovine tuberculosis is an important zoonotic disease transmitted by direct contact, respiratory pathway, ingestion of unpasteurised milk and milk product, raw or undercooked meat. In developed countries, eradication efforts have significantly reduced the prevalence of this disease, but wildlife reservoirs avoid a complete eradication [1,2]. Bovine tuberculosis is still common in less developed countries and it causes economic losses in cattle. It is also a serious threat to endangered species [3,4]. Cattle are considered to be maintenance hosts for *Mycobacterium bovis (M. bovis)*. Infections have also been described in numerous other domestic and wild animals [5].

It can be difficult to diagnose tuberculosis based only on the clinical signs [6]. In developed countries, few infections show symptoms and infections are mostly diagnosed by routine tests. Tuberculosis is usually diagnosed in the field with tuberculin skin test [7,8]. Sputum and other body fluids may be collected for microbiological examination, microscopic demonstration of acid fast bacilli, isolation of mycobacteria on selective culture media and identification by biochemical tests. Slides may be stained with Ziehl-Neelsen stain, fluorescent acid fast stain or immuno-peroxidase techniques [9,10]. PCR methods have also been described. New diagnostic blood tests include lymphocyte proliferation assay, IFN-γ assay and enzyme-linked immuno-sorbent assays (ELISA) [11,12]. IFN-γ assay is only useful in members of bovidae however lymphyocyte proliferation test may be used on other animals and wild animals. These tests are not used routinely for diagnosis [13,14].

Serological assays have shown promise as a diagnostic alternative to skin testing or culture testing for many of these species. Serological blood test based on TB assays are appealing not only for better sensitivity and specificity for captive wild animals, exotic species and other non-traditional livestock but also because they require only a single handling event, thereby minimizing capture-associated injuries [15,16]. The serological test concept is simple, rapid, easy to interpret, inexpensive and is very useful as a slaughter surveillance test or an effective and efficient trap and cull assay [17].

The purpose for conducting this study was to compare sensitivity of TST, IFN-γ assay, lateral flow rapid test and real time PCR examination for the diagnosis of field outbreaks of bovine tuberculosis in Turkey.

MATERIAL and METHODS

TST assay was applied on 178 cattle in dairy farm with the age of 3-5 years suspected from tuberculosis. Fifty samples have been selected randomly. Tuberculin skin test has been used in a herd as a first TB test and blood samples have been collected during TST. The blood samples for analysis have been collected from 50 samples selected randomly. The herd have been quarantined and cattle with positive test result have been slaughtered.

Tuberculin Skin Test

Subsequent to shaving the neck hairs with the area of 8-12 cm², the skin has been measured with calliper and the results have been recorded. Mammalian PPD tuberculin (0.1 ml) has been injected intradermally on shaved skin. After 72 h, injection site has been examined for pain, tenderness, warmth, swelling and skin thickness has been measured again. An increase in skin thickness was observed as follows: 0-3 mm (negative), 3-4 mm (doubtful) and 4 ≥ (positive).

IFN-γ Assay

Whole blood samples have been examined for T cell reactivity by production of IFN-γ samples stimulated with *M. bovis* and *Mycobacterium avium (M. avium)* PPDs at 20 µg/mL sample and PBS as non-stimulated controls as previously described. The blood has been incubated for 18-20 h at 37°C in a humidified atmosphere. After this period, samples have been centrifuged for 15 min at 490 g and the supernatant was collected and assayed for the presence of IFN-γ by using the ELISA (Bovigam, Product Number: 63319/63309) according to the manufacturer's instructions. Using avian and bovine PPDs for stimulation, results have been considered positive having the mean optical density at 450 nm (OD 450) stimulated with bovine PPD minus the OD 450 measured in the negative control stimulated with PBS was greater than 0.05 and greater than the OD 450 obtained in the sample stimulated with avian PPD. Additionally, a less restrictive 0.1 cut-off point was also evaluated. Each ELISA plate was validated with positive and negative controls according to the manufacturer's instructions.

Lateral Flow Rapid Test

For rapid detection of antibodies, TB lateral flow rapid test (BioNote Catalog No: RB23-02) uses selected myco-bacterial antigens immobilized on a nitrocellulose strip and a blue latex signal detection system for rapid detection of antibodies. The test was performed according to the manufacturer's instructions. A single test plate was used for each cattle. Plates were kept at room temperature. Thirty microliters of serum was distributed into the sample wells and allowed to soak into the wick. Three drops of sample buffer was added to the sample wells. The test was incubated at room temperature for 20 min and the results were recorded. A completed blue band must appear across the control line site (C-band) in order to have a valid test and a complete blue band must also appear at the test line site (T-band) in order to have a positive test.

Molecular Diagnostics

DNA extraction has been obtained from plasma samples

with commercial DNA isolation kit (Roche; Product Number: 03 038 505 001) according to the procedure [DNA extraction was performed by using MagNA Pure LC robotic isolation device according to the procedure. The isolation procedure is based on magnetic-bead technology. The samples are lysed by incubation with a special buffer containing a chaotropic salt and Proteinase K. Magnetic Glass Particles (MGPs) are added and total nucleic acids contained in the samples are bound to their surface. Unbound substances are removed by several washing steps, then the purified total nucleic acid is eluted with a low-salt buffer. The sample materials are placed into the wells of the sample cartridge. Lysis/binding buffer is added to the sample. Proteinase K is added to the samples and proteins are digested. Nucleic acids bind to the silica surface of the added MGPs due to the chaotropic salt conditions, isopropanol, and the high ionic strength of the lysis/binding buffer. MGPs with bound nucleic acids are magnetically separated from the residuallysed sample. MGPs with bound nucleic acids are washed repeatedly with wash buffer tore move unbound substances like proteins (nucleases), cell membranes, PCR inhibitors such as heparin or hemoglobin, and tore duce the chaotropic salt concentration. Again MGPs with bound total nucleic acid are magnetically separated from the wash buffer containing residual sample debris. The purified nucleic acids are eluted at +70℃ from the MGPs in the wells of the elution cartridge, whereas the MGPs are retained in the reaction tip and discarded].

The DNA samples were stored at -20℃ until PCR analysis. The amount of DNA determined by the measurement with spectrophotometer (ASP-3700) within 260 and 280 nm. Commercial *Mycobacterium* spp. detection kit (Way 2 Gene; Product Number: WG 40-0220- 16) was used for real-time PCR analysis. PCR was carried out according to the kit procedure. Each reaction tube contained 2 μL fast start mix 4 μL *Mycobacterium* genus primer, 1.6 μL Mg^2, 5 μL DNA template and distilled water to give a final volume of 20 μL.

The thermal cycling protocol was as follows: 10 min at 95℃ for denaturation, 5 s at 64℃ for annealing and 40 s at 72℃ for extension using a Roche Light Cycler 2.0. In all control stages, PCR as a positive standards included in the set, the distilled water was used as a negative control.

Statistical Analyses

The statistically significant difference test were associated with the chi square test. The sensitivity and specificity have been revealed as reference test IFN-γ assay. The proportions used for calculating the sensitivities and specificities of the tests were compared using SPSS 16 for Windows. The 95% confidence limits for the findings were calculated using SPSS 16.

RESULTS

In this study a total of 50 animals were tested by using tuberculin skin test, TB lateral flow rapid test, IFN-γ assay and real time PCR. Forty five were positive out of 50 for TST while 31 for reactive by the IFN-γ assay and 28 for TB lateral flow rapid test and 9 for real time PCR *(Table 1)*.

The sensitivity and specificity of TST, TB lateral flow rapid test, and real time PCR compared with IFN-γ assay were determined as 100%, 80.65% and 25.81% respectively *(Table 2)*.

DISCUSSION

The objective for conducting this analysis was to evaluate the performance on positive results of TST, IFN-γ assay, TB lateral flow rapid test and real time PCR under field conditions. Currently, definitive diagnosis of bovine tuberculosis in cattle is often made on patients' history, clinical and necropsy findings, tuberculin skin tests and abattoir meat inspection [15]. Control depends on early

Table 1. *Number of positive cattle according to TST, TB lateral flow rapid test, IFN-γ assay, real time PCR*
Tablo 1. *TST, TB lateral flow rapid test, IFN-γ assay ve gerçek zamanlı PCR testlerine göre pozitif tespit edilen sığır sayısı*

Performed Tests	Positive	Negative	Total
Tuberculin skin test	45	5	50
TB lateral flow rapid test	28	22	50
IFN-γ assay	31	19	50
Real time PCR	9	41	50

Table 2. *The sensitivity and specificity of TST, TB lateral flow rapid test and real time PCR*
Tablo 2. *TST, TB lateral flow rapid test ve gerçek zamanlı PCR testlerinin hassasiyeti ve spesifitesi*

Test	Sensitivity	Specificity	Power of the Test	P Value
TST x IFN-γ assay	100%	26.32%	72%	53.606***
TB lateral flow rapid test x IFN-γ assay	80.65%	84.21%	82%	15.489***
Real time PCR x IFN-γ assay	25.81%	94.73%	52%	
*** P>0.001				

identification and proper treatment of individuals with active disease. The tuberculin skin test is effective in the early detection of pre-clinical cases of *M. bovis* infection in cattle. This allows the rapid removal of infected animals limiting transmission of the disease and eradication of bovine tuberculosis from many countries [18]. However, a few number of ancillary tests are being used, or are currently validated. These ancillary tests are likely to provide a more accurate diagnosis following skin-testing. The blood based IFN-γ assay is a cellular immune assay which can detect early infection [19,20].

Up to date, there is no simple, rapid, sensitive and specific test that can differentiate active TB from latent infections and slowly progressive TB. A number of new antigens are being tested individually or in combinations to obtain the desired sensitivity and specificity. The search for rapid and reliable diagnostic tests for active TB based on the examination of blood and other clinical specimens has been the focus of several studies [21,22]. The tests allow early detection of latently infected individuals and are useful in contact tracing and screening of high-risk groups in a low-endemic setting. IFN-γ based tests may be important for epidemiological and surveillance studies to determine the extent of TB infection [23,24].

The initial field tirals carried out in Australia demonstrated that the IFN-γ assay had a higher sensitivity (93.6%) than the CFT (65.6%) [25]. Sensitivities ranged between 55 to 97% and the specificity was as high as 97% [7]. In this study the TST x IFN-γ ELISA sensitivity were 100% with 26.32% specificity. In the study, the agreement between TST and IFN-γ assay was determined as excellent. The sensitivities and specificities reported in the study are relatively high compared with earlier studies [7,25,26].

Furthermore, a negative result to a tuberculin test does not mean that the animal is not infected with *M. bovis* while a positive result represents and immunological response in the form of a delayed hypersensitivity reaction to mammalian tuberculin that is most commonly occur due to an infection or exposure to other bacteria that share antigens similar to those of *M. bovis*. A number of studies have shown that the tuberculin skin test and IFN-γ assay detect overlapping, but also distinct populations of *M. bovis* infected cattle [27-29]. Using these two types of cellular immune assays in quick succession can result in the removal of a greater proportion of *M. bovis* infected animals than either using this test alone [9].

Rapid test is relatively simple, inexpensive and do not require highly trained personnel or a complex technological platform. The method is suitable for use in laboratories in countries with low-income. The tests allow early detection of latently infected individuals and are useful in contact tracing and screening of high-risk groups in a low-endemic setting [17]. Results from this study so far suggest that combinations of antigens may yield the desired level of sensitivity without affecting specificity. This anti bovine TB antibody test kit has a sensitivity of 90% against bovine TB confirmed by bacterial isolation and a sensitivity of 85.1% and specificity of 98.6% against TST [17]. Also using the lateral flow rapid test, achieving sensitivity of 84% and a specificity of 84.2% for serological diagnosis of *M. bovis* infection in cattle. Similar and relatively high sensitivity (86.5% and 84.6%) and specificity (83.8% and 91.4%) have been reported with other lateral flow techniques for the diagnosis of bovine TB in farmed red deers [15]. In this study, the apparent sensitivity of both was high rapid test x IFN-γ assay sensitivity and specificity were 80.65%, 84.21% respectively and in agreement with previously authors.

PCR methods allow direct identification of *M. bovis* complex and can detect less than 10 bacteria in a clinical specimen. PCR's sensitivity ranges from 70-90% compared to the results of culture and its specificity varies between 90 and 95%. In smear of positive cases, the sensitivity of PCR is greater than 95% but in smear of negative cases, it is only 50 to 60%. Therefore, present amplification methods should not be replaced diagnostic convectional culture [4]. Molecular biological methods, such as PCR may be used to [30] diagnose TB rapidly by identifying DNA from *M. bovis* complex in clinical samples with negative microscopic results however PCR gives rapid results. Most of these report a lower sensitivity for PCR than culture [9,21], sensitivity was 25.81% while specificity 94.73% for real time PCR x IFN-γ assay. This low sensitivity occurs due to blood samples failed for PCR. These results are consistent with data obtained in other studies. This makes PCR suitable for detecting active tuberculosis.

As a result, through the development these new ancillary tests in association with skin test the means and modes proposed throughout trials will improve the detection of *M. bovis* infected cattle and reduce the unnecessary slaughter of false-positive reactors.

REFERENCES

1. Ünver A, Atabay Hİ, Güneş V, Çitil M, Erdoğan HM: The prevalence of bovine tuberculosis in Kars district analyzed by PCR. *Kafkas Univ Vet Fak Derg,* 13, 27-31, 2007. DOI: 10.9775/kvfd.2006.43-A

2. Öztürk D, Pehlivanoğlu F, Kale M, Tok AA, Güldalı Y, Türütoğlu H: *In vitro* diagnosis of bovine tuberculosis by γ-Interferon assay. *Kafkas Univ Vet Fak Derg,* 16, 875-878, 2010. DOI: 10.9775/kvfd.2009.1473

3. Kumar B, Kumar P: Bovine tuberculosis. *Indian Cow,* 6 (22): 27-29, 2009.

4. Fentahun T, Luke G: Diagnostic techniques of bovine tuberculosis: A review. *Aust J Basic & Appl Sci,* 4, 192-199, 2012.

5. Mosaad AA, Abdel-Hamed AS, Fathalla SI, Ghazy AA, Elballal S, Elbagory A, Mahboub H, Gaafar K, Elgayar KE, Mohamed AS, Amin AI, Akeila MA, Abdelrahman HA: Sensitive and specific diagnostic assay for detection of tuberculosis in cattle. *Global Vet,* 8 (6): 555-564, 2012.

6. De la Rua-Domenech R, Goodchild AT, Vordermeier HM, Hewinson RG, Christiansen KH, Clifton-Hadley RS: Ante mortem diagnosis of tuberculosis in cattle: A review of the tuberculin tests, γ-interferon assay and other ancillary diagnostic techniques. *Res Vet Sci,* 81, 190-210, 2006. DOI: 10.1016/j.rvsc.2005.11.005

7. Gormley E, Doyle MB, Fitzsimons T, McGill K, Collins JD: Diagnosis of

Mycobacterium bovis infection in cattle by use of the gamma-interferon (Bovigam) assay. *Vet Microbiol,* 112, 171-179, 2006. DOI: 10.1016/j.vetmic.2005.11.029

8. Alvarez J, Perez A, Bezos J: Evaluation of the sensitivity and specificity of bovine tuberculosis diagnostic tests in naturally infected cattle herds using a Bayesian approach. *Vet Microbiol,* 155, 38-43, 2012. DOI: 10.1016/j.vetmic.2011.07.034

9. Strain SAJ, McNair J, McDowell SWJ: Bovine tuberculosis: A review of diagnostic tests for *M. bovis* infection in cattle. Bacteriology Branch Veterinary Sciences Division Agri-Food and Biosciences Institute, May, 2011.

10. Danbirni S, Okaiyeto SO, Bature C, Morris A: Field determination of tuberculosis prevalence in a herd of cattle using tuberculin and quicking®Bovine Tuberculosis Antibody Rapid Tests in Jalingo, Nigeria. *J Vet Adv,* 3 (1): 20-23, 2013.

11. Whelan C, Shuralev E, Keeffe GO, Hyland P, Kwok HF, Snoddy P, O'Brien A, Connolly M, Quinn P, Groll M, Watterson T, Call S, Kenny K, Duignan A, Hamilton MJ, Buddle BM, Johnston JA, Davis WC, Olwill SA, Clarke J: Multiplex immunoassay for serological diagnosis of Mycobacterium bovis infection in cattle. *Clin Vaccine Immunol,* 15, 1834-1838, 2008. DOI: 10.1128/CVI.00238-08

12. Biffa D, Skjerve E, Oloya J, Bogale A, Abebe F, Dahle U, Djonne B: Molecular characterization of *Mycobacterium bovis* isolates from Ethiopian cattle. *BMC Vet Res,* 6, 28, 2010. DOI: 10.1186/1746-6148-6-28

13. Ameni G, Miorner H, Roger F, Tibbo M: Comparison between comparative tuberculin and gamma-interferon tests for the diagnosis of bovine tuberculosis in Ethiopia. *Trop Anim Health Prod,* 32 (5): 267-276, 2000. DOI: 10.1023/A:1005271421976

14. Antognolia MC, Remmengaa MD, Bengtsona SD: Analysis of the diagnostic accuracy of the gamma interferon assay for detection of bovine tuberculosis in U.S. herds. *Prev Vet Med,* 101, 35-41, 2011. DOI: 10.1016/j.prevetmed.2011.05.012

15. Buddle BM, Wilson T, Denis M, Greenwald R, Esfandiari J, Lyashchenko KP, Liggett S, Mackintosh CG: Sensitivity, specificity, and confounding factors of novel serological tests used for the rapid diagnosis of bovine tuberculosis in farmed red deer *(Cervus elaphus). Clin Vaccine Immunol,* 17, 626-630, 2010. DOI: 10.1128/CVI.00010-10

16. Awah-Ndukum J, Kudi AC, Bah GS, Bradley G, Tebug SF, Dickmu PL, Njakoi HN, Agharihet WN: Bovine tuberculosis in cattle in the highlands of Cameroon: Seroprevalence estimates and rates of tuberculin skin test reactors at modified cut-offs. *Vet Med Int,* 2012 (2012), Article ID 798502, DOI: 10.1155/2012/798502

17. Chambers MA, Crawshaw T, Waterhouse S, Delahay R, Hewinson RG, Lyashchenko KP: Validation of the brock TB stat-pak assay for detection of tuberculosis in Eurasian Badgers *(Meles meles)* and influence of disease severity on diagnostic accuracy. *J Clin Microbiol,* 46 (4): 1498-1500, 2008. DOI: 10.1128/JCM.02117-07

18. Karolemeas K, De la Rua-Domenech R, Cooper R, Goodchild AV, Clifton-Hadley RS, Conlan AJ, Mitchell AP, Hewinson RG, Donnelly CA, Wood JL, McKinley TJ: Estimation of the relative sensitivity of the comparative tuberculin skin test in tuberculous cattle herds subjected to depopulation. *PLoS One,* 7, e43217. 2012. DOI: 10.1371/journal.pone.0043217

19. Gowtage-Sequeira S, Paterson A, Lyashchenko KP, Lesellier S, Chambers MA: Evaluation of the Cervid TB STAT-PAK for the detection of Mycobacterium bovis infection in wild deer in Great Britain. *Clin Vaccine Immunol,* 16, 1449-1452, 2009. DOI: 10.1128/CVI.00178-09

20. Michel AL, Simoes M: Comparative field evaluation of two rapid immunochromatographic tests for the diagnosis of bovine tuberculosis in African buffaloes *(Syncerus caffer). Vet Immunol Immunopathol,* 127, 186-189, 2009. DOI: 10.1016/j.vetimm.2008.09.025

21. Watrelot-Virieux D, Drevon-Gaillot E, Toussaint Y, Belli P: Comparison of three diagnostic detection methods for tuberculosis in French cattle. *J Vet Med. B Infect Dis Vet Public Health,* 53, 321-325, 2006. DOI: 10.1111/j.1439-0450.2006.00957.x

22. Greenwald R, Lyashchenko O, Esfandiar J, Miller M, Mikota S, Olsen JH, Ball R, Dumonceaux G, Schmitt D, Moller T, Payeur JB, Harris B, Sofranko D, Waters WR, Lyashchenko KP: Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants. *Clin Vaccine Immunol,* 16, 605-612, 2009. DOI: 10.1128/CVI.00038-09

23. Lilenbaum W, Schettini JC, Souza GN, Ribeiro ER, Moreira EC, Fonseca LS: Comparison between a gamma-IFN assay and intradermal tuberculin test for the diagnosis of bovine tuberculosis in field trials in Brazil. *Zentralbl Veterinarmed B,* 46, 353-358, 1999.

24. Lyashchenko KP, Greenwald R, Esfandiari J, Mikota. S, Miller M, Moller T, Vogelnest L, Gairhe KP, Robbe-Austerman S, Gai J, Waters WR: Field application of serodiagnostics to identify elephants with tuberculosis prior to case confirmation by culture. *Clin Vaccine Immunol,* 19, 1269-1275, 2012. DOI: 10.1128/CVI.00163-12

25. Wood PR, Corner LA, Rothel JS, Baldock C, Jones SL, Cousins DB, McCormick BS, Francis BR, Creeper J, Tweddle NE: Field comparison of the interferon-gamma assay and the intradermal tuberculin test for the diagnosis of bovine tuberculosis. *Aust Vet J,* 68, 286-290, 1991. DOI: 10.1111/j.1751-0813.1991.tb03254.x

26. Marassi CD, Medeiros L, Lilenbaum W: The use of a gamma-interferon assay to confirm a diagnosis of bovine tuberculosis in Brazil. *Acta Trop,* 113, 199-201, 2010. DOI: 10.1016/j.actatropica.2009.10.002

27. Neill SD, Hanna J, Pollock J, Mackie DP, Cassidy J, Clements A, Bryson DB: The diagnosis of bovine tuberculosis by blood testing. *SVEPM,* 1, 8, 1994.

28. Vordermeier HM, Goodchild A, Whelan A, Ewer K, Clifton-Hadley R, Williams G, Hewinson J: The bovigam assay as an ancillary test to the tuberculin skin test. *GVJ,* 16, 72-80, 2006.

29. Coad M, Hewinson RG, Clifford D, Vordermeier HM, Whelan AO: Influence of skin testing and blood storage on interferon-γ production in cattle affected naturally with *Mycobacterium bovis. Vet Rec,* 160, 660-662, 2007.

30. Solmaz H, İlhan Z, Aksakal A, Gülhan T, Ekin İH: Detection of bovine tuberculosis by tuberculin test and polymerase chain reaction in Van, Turkey. *Turk J Vet Anim Sci,* 33, 229-233, 2009. DOI: 10.3906/vet-0711-35