Etlik Vet Mikrobiyol Derg, 2024; **35 (1):** 8-18
doi: https://doi.org/10.35864/evmd.1413210

Original Article
Özgün Araştırma

Differentiation of bovine Tuberculosis and Paratuberculosis infections with antemortem diagnostic methods

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Geliş Tarihi / Received: 01.01.2024, **Kabul Tarihi** / Accepted: 15.04.2024

Abstract: In this study, based on the results of tuberculin skin tests (Bovine and Avian PPD) used in the antemortem diagnosis and differentiation of Bovine Tuberculosis, the animals in the farms with suspected Tuberculosis were serologically examined to diagnose Paratuberculosis infection and fecal bacterioscopy was performed. In addition, it was aimed to obtain data that will contribute to the eradication studies of Bovine Tuberculosis disease by comparing the antemortem diagnostic methods of Bovine Tuberculosis disease, which is endemic in Türkiye and by determining the sensitivity and specificity values of the interferon gamma (IFN-γ) test. In this context, intradermal tuberculin test was applied to 423 cattle with suspected Tuberculosis in a total of 5 dairy cattle farms, one each from Çankırı, Çorum, Ankara, Eskişehir and Konya regions, and this test was determined as the gold standard method and the sensitivity and specificity of the IFN-γ test were determined as 86% and 97%, respectively. For the diagnosis of Paratuberculosis infection, antibody ELISA, fecal bacterioscopy and IFN-γ ELISA were performed on these animals and the prevalence of these tests were 10.4%, 5.44% and 4.96% respectively and 4 (0.95%) of the cattle were positive for each of the diagnostic methods for *Mycobacterium avium* spp. *paratuberculosis* (*Map*) infection. As a result, it was concluded that IFN-γ test, which gives similar results to intradermal tuberculin test results, can also be used in the antemortem diagnosis of Bovine Tuberculosis. Also, in the comparative intradermal tuberculin test for the diagnosis of Tuberculosis infection, avian PPD positive animals were found to play a decisive role in the detection of nonspecific reactions or Paratuberculosis infected animals, supported by other tests used for the diagnosis of Paratuberculosis.

Keywords: Cattle, ELISA, IFN-γ test, *Mycobacterium bovis*, Paratuberculosis

Sığır Tüberküloz ve Paratüberküloz enfeksiyonlarının antemortem tanı yöntemleriyle ayrımı

Özet: Bu araştırmada, Tüberküloz şüpheli işletmelerde bulunan hayvanlara, Sığır Tüberkülozunun antemortem tanı ve ayrımında kullanılan tüberkülin deri testleri (PPD bovine ve avian) sonuçlarından yola çıkılarak, Paratüberküloz enfeksiyonu teşhisi koymak için serolojik olarak incelendi ve fekal bakteriyoskopi yapıldı. Ayrıca Türkiye'de endemik olarak görülen Sığır Tüberküloz hastalığının antemortem tanı yöntemleri karşılaştırılıp, interferon gama (IFN-γ) testinin sensitivite ve spesifite değerleri belirlenerek Sığır Tüberküloz hastalığının eradikasyon çalışmalarına katkı sağlayacak verilerin elde edilmesi amaçlandı. Bu kapsamda Çankırı, Çorum, Ankara, Eskişehir ve Konya bölgelerinden birer adet olmak üzere toplam 5 süt sığırcılığı işletmesinde Tüberküloz şüphesi olan 423 sığıra intradermal tüberkülin testi uygulandı ve bu test altın standart metot olarak belirlenerek, IFN-γ testinin sensitivitesi % 86, spesifitesi % 97 olarak belirlendi. Paratüberküloz enfeksiyonunun tanısı için bu hayvanlara antikor ELISA, fekal bakteriyoskopi ve IFN-γ ELISA yapıldı ve bu testlerin prevalansları sırasıyla % 10,4, % 5,44 ve % 4,96 bulundu ve sığırlardan 4 (%0,95)'ü *Mycobacterium avium* spp. *paratuberculosis* (*Map*) enfeksiyonu tanı yöntemlerinin her birine pozitif sonuç verdi. Sonuç olarak, intradermal tüberkülin testi sonuçlarına benzer sonuçlar veren IFN-γ testinin Sığır Tüberkülozunun antemortem tanısında kullanılabileceği sonucuna varıldı. Ayrıca Tüberküloz enfeksiyonunun tanısı için yapılan karşılaştırmalı intradermal tüberkülin testinde PPD aviana pozitif reaksiyon veren hayvanların Paratüberküloz tanısı için kullanılan diğer testlerle desteklenerek nonspesifik reaksiyonların ortaya çıkarılmasında ya da Paratüberküloz enfekte hayvanların tespit edilmesinde belirleyici rol oynadığı görüldü.

Anahtar kelimeler: ELISA, IFN-γ test, *Mycobacterium bovis*, Paratüberküloz, Sığır

Introduction

Bovine Tuberculosis (bTB), caused by *Mycobacterium bovis,* is a chronic infectious disease of many domestic and wild animals, including cattle, buffalo, sheep, goats, badgers, pigs, deer, Australian-American opossums and humans (Menzies and Neill 2000; O'hagan et al. 2016). The zoonotic nature of

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the disease has a serious impact on human health worldwide. In addition to the significant economic costs caused by such diseases, the lack of accurate estimates of the true prevalence of disease, especially in developing countries, necessitates more effective detection and control measures (Hashem et al. 2022). Cattle are the main reservoir of bTB and eradication programs worldwide focus primarily on these domestic species (Mohamed 2020).

Diagnosis of the disease depends mainly on tests that measure the cellular immune response following infection, culture-based bacteriological examination and molecular-based Polymerase Chain Reaction (PCR) methods (Smith et al. 2021; Thomas et al. 2021). None of the tests currently available for the diagnosis of Bovine Tuberculosis allow perfect detection of *M. bovis* infection in cattle. The Tuberculin Skin Test (TST), which has a specificity of 99.5% in TB (Tuberculosis)-free bovine populations, is used as the primary antemortem diagnostic tool (Eisenberg et al. 2016). Bovine Tuberculosis is subject to an official eradication program based on a slaughter policy and using intradermal TST and Interferon Gamma (IFN-γ) diagnostic tests, mainly measuring cellular immunity. Intradermal TST is recognized by the OIE and the European Commission as the primary screening test for the diagnosis of TB in cattle (Çakır 2021). The IFN-γ test has a higher sensitivity and is as specific as the Comparative Intradermal Tuberculin Test (CITT), but some studies have reported that the IFN-γ test has lower specificity than the CITT (Proud et al. 2015).

Paratuberculosis (PTB) or Johne's Disease is a disease caused by *Map* that affects domestic and wild ruminants worldwide. PTB is a highly contagious disease characterized by a chronic progressive granulomatous enteritis that is endemic in many parts of the world and causes significant economic losses in livestock and related industries (Moyano et al. 2021). *Map* infection, showing a chronic diarhea that does not respond to treatment is the specific clinical sign of infection (Cruz-Estupınan et al. 2022). Infection in ruminants consists of different phases including early, subclinical and clinical phases, and symptoms such as weakness, muscle wasting, diarrhea, decrease in milk yield, which do not show clinical signs in the early or subclinical phase, but continue in the clinical period with the end of the subclinical period (Eamens et al. 2015; Whittington et al. 2017). The primary source of the disease is clinically sick and asymptomatic animals. The most common route of infection is ingestion of contaminated milk, colostrum or feces (Gilardoni et al. 2012; Lievaart-Peterson et al. 2019). The causes of economic losses due to PTB are mortality, early elimination of animals, increased susceptibility to other infectious diseases, decreased milk, meat and reproductive yields (Garcia and Shalloo 2015; Barratt et al. 2018; Camanes et al. 2018).

The methods used in the diagnosis of PTB are divided into two as direct and indirect. Direct diagnostic methods include histopathology, necropsy, culture, bacterioscopy and PCR, while indirect diagnostic methods include cellular (TST and IFN-γ) and humoral (ELISA) immune response tests (Eamens et al. 2015; Şababoğlu 2019; OIE 2021). The diagnosis of PTB by bacterioscopy involves microscopic examination of preparations of feces or intestinal mucosa stained with Ziehl-Neelsen (ZN) staining. If acid fast bacteria (AFB) are found in clusters (at least three or more), it may be a possible diagnosis of PTB. The skin test for Delayed Type Hypersensitivity (DTH) is based on the measurement of cell-mediated immunity. Since avian and johnin Prufiye Protein Derivative (PPD) skin tests that reveal DTH show similar sensitivity and specificity values, it has been stated that one can be used instead of the other (OIE 2021). Tests measuring IFN-γ level, one of the in vitro assays that detect cellular immune response, have recently been used in the diagnosis of Tuberculosis and Paratuberculosis in cattle. Since the production of IFN-γ cytokine is one of the earliest detectable immune responses in PTB diagnosis, it has been stated that the IFN-γ test, which detects infected animals in the subclinical period, is the best method among the diagnostic methods (Nielsen 2010; Vazquez 2013). ELISA (Enzim Linked Immunosorbent Assay), one of the indirect diagnostic methods of PTB infection, is frequently used and is the main diagnostic method for determining seroprevalence in countries with disease surveillance programs. The advantage of ELISA is its low cost, fast results and versatility (De Lacerda Roberto 2021). ELISA has the highest sensitivity and specificity among available tests to detect antibodies to *Map* in cattle (OIE 2021). ELISA is a serological test widely and conveniently used to detect antibodies in milk and serum during and after the subclinical stage of *Map* infection (Radostits 2007; Garvey 2018).

The aim of this study was to determine whether the animals were infected with PTB infection by using the methods used in the antemortem diagnosis of bTB. In addition, it was aimed to obtain data that will contribute to the eradication studies of Bovine Tuberculosis by comparing the antemortem diagnostic methods of bTB, which is endemic in Türkiye,

and determining the sensitivity and specificity values of IFN-γ test.

Materials and Methods

Sampled Animals

This study was carried out on 423 cattle from a total of 5 dairy cattle farms with suspected bTB between 2021-2023 in Çankırı, Çorum, Ankara, Eskişehir and Konya regions. The number of farms and animals in the regions are as follows; Çankırı 1 farm 144 cattle, Çorum 1 farm 51 cattle, Ankara 1 farm 31 cattle, Eskişehir 1 farm 47 cattle, Konya 1 farm 150 cattle. The age and gender distribution of cattle are presented in Table 1. To conduct this study, ethics committee approval was taken, which was 2021-13-106 number from Ankara University Animal Experimental Local Ethics Committee.

Table 1. Age and gender distribution of cattle used in the study.

	Age (Years)						Gender	
Animal	**0-1**	**1**	**2**	**3**	**4**	**≥5**	**Male**	**Female**
Number of cattle	11	76	96	65	53	122	70	353
Total	423						423	

Blood and Fecal Samples

Before the cattle were subjected to PPD skin test, 8 ml of venous blood was collected from each animal into vacuum tubes containing lithium heparin for IFN-γ test and vacuum tubes containing clotting activator for *Mycobacterium paratuberculosis* antibody test. Fecal samples were also collected from each animal for the diagnosis of PTB. Fresh fecal samples were collected from the rectum with plastic gloves and transferred to sterile plastic containers. These fecal samples from each cattle were delivered to the laboratory at +4°C (Paolicchi et al. 2003; Borum et al. 2014).

Tuberculin Skin Test (TST)

Avian and Bovine PPD with a protein content of 1 mg/ml (produced by Etlik Central Veterinary and Control and Research Institute, Türkiye) were used in tuberculin skin test**.** Cattle were subjected to CITT and the results were evaluated. This application and evaluation of the results were performed according to Office International Epizootica (OIE), Ministry of Agriculture and Forestry of the Republic of Türkiye Bovine Tuberculosis Regulations. PPD skin test was performed by intradermal injection in the middle third of the neck with a dose of 0.1 ml of avian PPD on the top and bovine PPD on the bottom with a distance of 12-13 cm between the two injections. Skin thickness of both injection sites was recorded 72 hours later by measuring the skin thickness again with calipers. The diagnosis of bTB infection or suspected PTB was determined by measuring and evaluating the skinfold thickness at the site of bovine PPD and avian PPD injection (Resmi Gazete 1978; Resmi Gazete 2009; OIE 2022).

Interferon Gamma (IFN-γ) Test

Blood samples collected in lithium heparinised tubes were brought to the laboratory at room temperature (22±3°C) within 12 hours and whole blood cultures were performed in 24-well cell culture plates. Blood samples from each animal were distributed into 3 wells of each animal in a 24-well tissue culture plate. 100 µl of nil control antigen (PBS) was added to the blood sample in the first well, 100 µl of bovine PPD antigen was added to the blood sample in the second well, 100 µl of avian PPD antigen was added to the blood sample in the third well. Then, the microplates were incubated at 37 °C in an incubator with 5% CO_2 for 16-24 hours. Then, plasma samples were harvested from the cultures and a commercial kit (Bovigam®, Prionics AG, Australia) was used to diagnose the cellular immune response resulting from Paratuberculosis and Tuberculosis infection based on the elevated IFN-γ levels. Sandwich ELISA was performed according to the protocol reported by the manufacturer. Within 5 minutes after the reaction was terminated, Optical Density (OD) was measured on an ELISA reader with a 450 nm microplate photometer. Samples tested for Tuberculosis infection according to the kit protocol;

Negative: OD Bovine PPD - OD Nil/ Phosphate Buffer Solution (PBS) antigen < 0.1 and OD Bovine PPD – OD Avian PPD < 0.1,

Positive: OD Bovine PPD - OD Nil/ Phosphate Buffer Solution (PBS) antigen ≥ 0.1 and OD Bovine PPD – OD Avian PPD ≥ 0.1 evaluated according to the criteria.

Evaluation of OD values in terms of *Map* infection; It was performed according to the calculation criteria reported by Vazquez et al. (2013). Consider-

ing this criterion, cattle were considered positive for Paratuberculosis infection when the avian PPD OD value of each sample was subtracted from the nil/ PBS OD value and the difference was equal to or greater than 0.05 and the avian PPD OD value was higher than the bovine PPD OD value (OD Avian > OD Bovine and OD Avian - OD Nil/ PBS ≥ 0.05).

Bacterioscopy

Approximately 40 grams of fecal samples from each cattle were homogenized with sterile distilled water, 2 direct smear preparations were prepared from three different areas of each feces and stained with ZN staining method for fecal bacterioscopy diagnosis. AFB were visualized as short-thick, red-pink coccobacilli on a blue background. At least 100 microscope fields were scanned and the results recorded. These results were evaluated according to the Acid Fast Bacteria scoring (AFB scorin) criteria as indicated in Table 2 (Fujiki 2001).

Table 2. Acid Fast Bacteria Scoring Criteria (AFB Scorin).

Assessment	Result
No acid-fast bacteria in the microscope field	Negative
1-9 acid-fast bacteria in the microscope field	Suspect
10-99 acid-fast bacteria in the microscope field	Positive

Mycobacterium paratuberculosis Antikor Testi

The samples taken into blood tubes containing clotting activator were centrifuged at 3000 rpm for 5 minutes and after the sera were obtained in the tubes, they were transferred to eppendorf tubes and stored in a deep freezer at -20°C until the time of testing**.** A commercial kit (IDEXX Paratuberculosis Screening, France) was used for ELISA to detect antibodies raised in Paratuberculosis infection. Indirect ELISA was performed according to the protocol reported by the manufacturer. OD was measured on an ELISA reader with a 450 nm microplate photometer. Results, individual sample interpretation; Sample/Positive (S/P), according to the value evaluated. S/P % ≥ 55 % positive, 45 % < S/P % < 55 % suspect, S/P % ≤ 45 % evaluated as negative.

Determining the Specificity and Sensitivity of a Diagnostic Method

The sensitivity of a diagnostic method is the positive values obtained by this method divided by the actual positive values. The specificity of a test is calculated by dividing the negative values found with this test by the true negatives (Erganiş 1993).

Results

Tuberculin Skin Test

A total of 423 cattle that performed intradermal bovine PPD and avian PPD tests were evaluated by measuring the skin thickening in the PPD treated area with calipers. As a result of this evaluation, 86 (20.33%) cattle were diagnosed with Tuberculosis due to positive results of PPD skin test, while 337 (79.67%) cattle gave negative results. Cattle found suspicious in the first PPD skin test application were added to the first application results by being found negative or positive according to the results of the second PPD skin test application performed 60 days later (Figure 1).

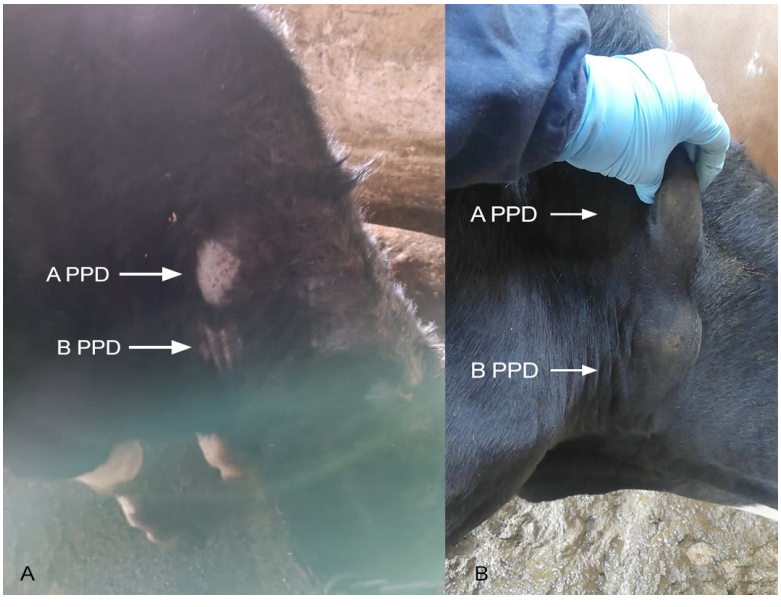

Figure 1. A. Avian (A) PPD positive reaction, B. Avian (A) PPD and Bovine (B) PPD positive reaction.

bTB IFN-γ ELISA

According to the results of this evaluation; 84 (19.86%) out of 423 cattle were positive for Tuberculosis. Since CITT was also applied to the same animals for the diagnosis of Tuberculosis, when we compared these two diagnostic methods, 12 cattle gave positive results to PPD skin test and negative results to IFN-γ test. On the other hand, 10 cattle gave positive results to IFN-γ test and negative results to PPD skin test (Table 3).In this study, when IFN-γ test was compared with tuberculin skin test in cattle herds with suspected Tuberculosis; the sensitivity of IFN-γ test was 86% and specificity was 97%.

Table 3. IFN-γ test and Intradermal Tuberculin test results.

Farm of No.	Number Animals	Intradermal Tuberculin Test		IFN-γ Test	
		Positive	Negative	Positive	Negative
1	144	34	110	**28**	**116**
2	51	31	20	**27**	**24**
3	31	11	20	**13**	**18**
4	47	0	47	**8**	**39**
5	150	10	140	**8**	**142**
Total	423	86	337	**84**	**339**

Antibody ELISA

ELISA was performed on blood sera obtained from 423 cattle for the diagnosis of Paratuberculosis infection and according to the ELISA results: In 423 bovine blood sera, 44 (10.4%) were positive, 10 (2.37%) were suspicious and 369 (87.23%) were negative for antibodies against *Map*. All 5 dairy cattle farms sampled were positive for Paratuberculosis (Table 4).

PTB IFN-γ ELISA

Out of a total of 423 cattle in 5 farms, 21 of them were positive for IFN-γ ELISA, while 402 of them were negative. (Table 4).

Bacterioscopy

According to the results of fecal examination by ZN staining method, 23 cattle were evaluated as positive, 19 cattle as suspicious and 381 cattle as negative, and they were scanned under a microscope at 100x objective for AFB (Table 4) (Figure 2).

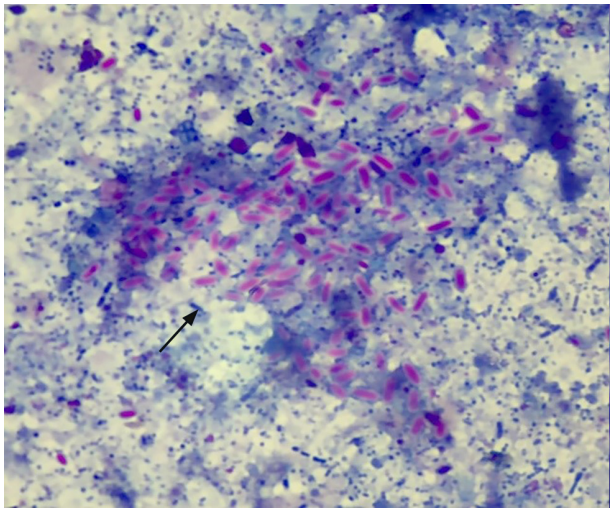

Figure 2. Microscopic appearance of AFBs in fecal samples by ZN staining method at 100x objective (Short, red-pink, thick and intertwined coccobacilli).

Table 4. Results of antemortem diagnosis of *Map* infection.

Farm No	Antibody ELISA			Fecal Bacterioscopy			IFN-γ ELISA		Number of Animals
	Positive %	Suspect %	Negative %	Positive %	Suspect %	Negative %	Positive %	Negative %	
1	15 (10.42%)	1 (0.7%)	128 (88.88%)	5 (3.47%)	7 (4.86%)	132 (91.67%)	8 (5.55%)	136 (94.45%)	144
2	7 (13.72%)	2 (3.93%)	42 (82.35%)	-	7 (13.73%)	44 (86.27%)	-	51 (100%)	51
3	7 (22.58%)	2 (6.45%)	22 (70.97%)	6 (19.35%)	-	25 (80.65%)	1 (3.23%)	30 (96.77%)	31
4	1 (2.13%)	-	46 (97.87%)	-	-	47 (100%)	3 (6.38%)	44 (93.62%)	47
5	14 (9.33%)	5 (3.33%)	131 (87.34%)	12 (8%)	5 (3.33%)	133 (88.67%)	9 (6%)	141 (94%)	150
Total									**423**

Intradermal Avian PPD

As a result of this evaluation, 14 cattle were suspected for PTB infection and these animals were subjected to antibody ELISA, fecal bacterioscopy and IFN-γ ELISA for PTB diagnosis (Table 5) (Figure 1).

Table 5. Results of PTB diagnostic tests of 14 cattle suspected for *Map* infection.

Cattle No	Antibody ELISA	Fecal Bacterioscopy	IFN-γ ELISA
13	-	-	-
78	-	+	-
83	-	+	+
33	-	-	-
B8	-	±	-
B9	-	±	-
B18	+	±	-
17	+	+	-
31	+	+	-
29	-	-	-
K14	-	-	-
101	-	-	-
133	+	+	+
82	+	+	+

Positive (+), Negative (-), Suspect (±)

PTB Comparison of Diagnostic Methods

Among the diagnostic tests, the highest rate of positivity in cattle was detected in antibody ELISA (10.4%). This was followed by fecal bacterioscopy with ZN staining (5.44%) and IFN-γ ELISA (4.96%), respectively. When evaluated as a herd (farm); antibody ELISA (100%) showed the highest positivity value and this rate was followed by IFN-γ ELISA (80%) and ZN staining (60%) methods, respectively. According to the results of antibody ELISA, fecal bacterioscopy and IFN-γ ELISA performed on 423 cattle in 5 dairy farms, positivity was obtained from one, two or all three of these tests. Antibody ELISA, fecal bacterioscopy and IFN-γ ELISA results were positive in 27 (6.39%), 7 (1.66%) and 10 (2.36%) animals, respectively. Antibody ELISA and fecal bacterioscopy were positive in 11 (2.6%), antibody ELISA and IFN-γ ELISA in 6 (1.42%), fecal bacterioscopy and IFN-γ ELISA in 5 (1.18%) animals (Table 6).

Table 6. Comparison of positive results of antemortem diagnostic methods of *Map* infection.

Farm No	Analysis	Antibody ELISA	Fecal Bacterioscopy	IFN-γ ELISA
1	Antibody ELISA	9	2	4
	Fecal Bacteriscopy	2	1	2
	IFN-γ ELISA	4	2	2
2	Antibody ELISA	7	0	0
	Fecal Bacterioscopy	0	0	0
	IFN-γ ELISA	0	0	0
3	Antibody ELISA	3	4	0
	Fecal Bacterioscopy	4	2	0
	IFN-γ ELISA	0	0	1
4	Antibody ELISA	1	0	0
	Fecal Bacterioscopy	0	0	0
	IFN-γ ELISA	0	0	3
5	Antibody ELISA	7	5	2
	Fecal Bacterioscopy	5	4	3
	IFN-γ ELISA	2	3	4
Total	Antibody ELISA	27	11	6
	Fecal Bacterioscopy	11	7	5
	IFN-γ ELISA	6	5	10

Discussion and Conclusion

In this study; IFN-γ test and CITT, one of the antemortem diagnostic methods of Tuberculosis infection were performed on 423 cattle in a total of 5 dairy cattle farms with suspected Tuberculosis in Çankırı, Çorum, Ankara, Eskişehir and Konya regions. While 84 cattle gave positive results to IFN-γ test, 86 cattle gave positive results to CITT. The prevalences of CITT and IFN-γ test were 20.33% and 19.86%, respectively. PPD skin test was used as the gold standard method to determine the sensitivity and specificity of the IFN-γ test, and its sensitivity and specificity were 86% and 97%, respectively. Lahuerta-Marin et al. (2015) reported the sensitivity and specificity of IFN-γ test to be 88-94% and 85-98%, respectively. Sayın (2010) reported the sensitivity and specificity values of IFN-γ test to be 91.5% and 93.4%, respectively. Öztürk et al. (2010b) used PPD skin test as the gold standard method for the diagnosis of Bovine Tuberculosis and detected that the sensitivity and specificity of IFN-γ test were 90% and 97%, respectively. Gormley et al. (2006) reported that the sensitivity value of the IFN-γ test (90-93%) was higher than the sensitivity value of the PPD skin test (70-75%) and the specificity value of the skin test was 99.9% and the specificity value of the IFN-γ test was 95%. Hashem et al. (2022) used PPD skin test as the gold standard method for the diagnosis of Bovine Tuberculosis in their study and reported that the sensitivity and specificity values of IFN-γ test were 98% and 71.4%, respectively. The sensitivity and specificity values obtained from the studies were consistent with the values in our study. When we compared both tests, 12 cattle gave positive results to PPD skin test and negative results to IFN-γ test. Ahir et al. (2016) and Praud et al. (2015) reported that some animals reacted positively to CITT but negatively to IFN-γ test in their studies. Alvarez et al. (2009) reported that IFN-γ test was applied as an auxiliary test to tuberculin tests to determine the maximum number of infected animals in the diagnosis of Bovine Tuberculosis but among the possible factors affecting the performance of tuberculosis diagnostic tests. Paratuberculosis, a common disease in Spain and other European countries, may be the cause of false positive reactions when coinfected with Tuberculosis infection and detected that while the sensitivity of IFN-γ test was 50% in Tuberculosis and Paratuberculosis infected herds, the sensitivity of IFN-γ test was 78% only in Tuberculosis infected herds. Therefore, since the prevalence of *Map* infection in 5 farms was 100% and *Map* infection was present in all farms, it was thought that the sensitivity of IFN-γ test may decrease.

To diagnose PTB infection, antibody ELISA, IFN-γ ELISA and fecal bacterioscopy with ZN staining were used in combination in this study. In addition, based on the CITT results used for the diagnosis of Tuberculosis, animals with a positive reaction to avian PPD were considered suspicious for Paratuberculosis and antibody ELISA, IFN-γ ELISA and fecal bacterioscopy were performed on these animals. It was observed that most of the animals that tested positive were crossbred and pure cattle breeds with good body condition. The chronic nature of *Map* infection and the long incubation period portray a healthy appearance for many years before infected animals show overt symptoms of the disease, which may explain the findings of this study (Mortier et al. 2015). Diagnosis of PTB by direct bacterioscopy from feces, different rates of *Map* scattering or regular or intermittent excretion of the agent will cause errors in diagnosis (Nielsen and Toft 2008; Gilardoni et al. 2012; Borum et al. 2014). Animals found ELISA positive may or may not be *Map* shedders; antibodies may be produced before or many years after fecal shedding of the agent begins (Nielsen 2010). According to the results of this study, 23 (5.44%) animals were positive by ZN staining method in feces, 44 (10.4%) animals were positive by ELISA and 11 (2.6%) of 423 cattle were positive by antibody ELISA and fecal bacterioscopy. The fact that the prevalence of antibody ELISA was higher than the prevalence of fecal bacterioscopy and that negative or suspicious cases in fecal bacterioscopy were positive in antibody ELISA was thought to be due to the fact that, as reported by other researchers, the scattering of *Map* with feces occurs in periods and this scattering is absent when the sample is taken (Nielsen and Toft 2008; Nielsen 2010; Gilardoni et al. 2012; Borum et al. 2014).

Although the presence of PTB infection is known throughout Türkiye, the number of studies investigating the infection and its prevalence is limited. Tütüncü et al. (2018) reported the seroprevalence of ELISA as 10% and herd prevalence as 46.7% in blood serum samples obtained from a total of 859 dairy cattle older than 2 years of age, consisting of Hostein-Friesian cattle breeds and showing signs of chronic diarrhea in 15 farms in Amasya and Samsun regions. Makav and Gökçe (2013) reported a seroprevalence of 3.5% by ELISA and a herd prevalence of 41.6% in their study conducted in Kars region. Öztürk et al. (2010a) found a prevalence of 6.2% in a study conducted by ELISA on dairy cattle

in Burdur region. Karatay et al. (2020) determined the prevalence as 4.25% by antibody ELISA performed on blood serum samples taken from a total of 400 cattle from 22 dairy farms in Ardahan region. In a study conducted by Yıldırım and Civelek (2013) in Uşak region with fecal samples, ZN staining, was applied and the prevalence was found to be 17%. Borum et al. (2014) in a study using 305 Holstein Fresian dairy cattle aged 4-8 years from farms in and around Afyon region, the prevalence of Paratuberculosis in dairy cattle was determined as 31.8% by ELISA in blood serum samples and 4.59% by ZN staining in fecal samples. Knowledge of the global distribution of PTB is important for establishing control programs. The prevalence of PTB has been reported from different countries, mainly bovine PTB. In a study conducted in the Boyoca region of Colombia, Cruz-Estupinan et al. (2022) reported that the seroprevalence of ELISA was 3.1% in blood serum samples obtained from 882 cattle of different breeds and age groups. In another study conducted with dairy cattle in Sudan, Elmagzoub et al. (2020) reported a seroprevalence of 6.3% by ELISA and a herd prevalence of 18.9% and Ozsvari et al. (2020) reported the seroprevalence as 5.5% by ELISA in a study conducted in Hungary. Weber et al. (2009) in the Netherlands in dairy cattle farms and AL Anbagi and Salman (2022) in Iraq in buffalo farms reported that the prevalence was 27% according to the results of fecal examination with ZN staining method. In this study, the prevalence according to fecal examination results was 5.44%, while antibody ELISA seroprevalence was 10.4% and herd prevalence was 100%. The PTB prevalence rates obtained in this study were similar to those reported globally and with the results obtained from studies conducted in different regions of Türkiye.

ELISA is more specific and sensitive in animals over 2 years of age (Öztürk et al. 2010a; Makav and Gökçe 2013; Borum et al. 2014). In Paratuberculosis infection, although the animal may acquire the causative agent at an early stage, it is usually not until after 2 years of age that it is shed in the feces and clinical signs appear. The animal goes through a long subclinical period and slowly spreads the agent into the environment This period is important for the spread of the agent into the environment (Nielsen and Toft 2008; Dieguez et al. 2009). In this study, 31 (70.46%) of the 44 cattle positive for antibody ELISA were over 2 years of age and 13 (29.54%) were under 2 years of age, and 19 (82.61%) of the 23 animals positive for fecal bacterioscopy results were over 2 years of age and 4 (17.39%) were under 2 years of age. The prevalence values obtained in this study showed that the age of the animals had an effect on the antibody ELISA and fecal bacterioscopy results.

Measuring IFN-γ release is a very important diagnostic method for the identification of *Map* infected animals in the early stage of infection (Stabel et al. 2007). IFN-γ production has been reported to be one of the earliest detectable immune responses in the diagnosis of PTB infection (Nielsen 2010). Hence, it has been stated that IFN-γ test is the best option for the detection of subclinically infected animals (Vazquez et al. 2013). However, the specificity of diagnostic tests based on IFN-γ values is low for cattle under 16 months of age, and cattle identified as IFN-γ positive need to be supplemented with ELISA or additional tests detecting *Map* agents in feces to assess the disease process within the infected herd (Corneli et al. 2021). In Iraq, AL Anbagi and Salman (2022) reported that the prevalence was 18% according to IFN-γ ELISA results in a study conducted in buffalo farms. Vazquez et al. (2013) detected *Map* in tissues by culture or real-time PCR in 36.1% of the positive cattle in their study with IFN-γ ELISA. Şababoğlu conducted a study on sheep in Burdur region of Türkiye for the diagnosis of *Map* infection by IFN-γ ELISA and found positivity in 33 (22%) of 150 sheep (Şababoğlu 2019). In the present study, the criterion reported by Vazquez et al. (2013), was used and positivity was detected in 21 (4.96%) of 423 cattle. Alvarez et al. (2009) reported that the presence of Tuberculosis and Paratuberculosis infections in the same herd may decrease the sensitivity of IFN-γ ELISA by approximately 20%. It was thought that the low prevalence of IFN-γ ELISA may be due to the fact that there were animals with positive results for both infections in 5 farms participating in this study. In addition, out of a total of 423 cattle in 5 farms, 21 cattle were positive for IFN-γ ELISA, while 402 cattle were negative. Since none of these 21 cattle showed clinical signs and only 5 (23.81%) cattle were found to be positive in IFN-γ ELISA and 16 (76.19%) were found to be negative according to the results of fecal examination, these animals were considered to be in the early or subclinical stage of infection as reported by other researchers (Stabel et al. 2007; Nielsen 2010; Vazquez et al. 2013).

In a study conducted by Vural et al. (1995) in cattle farms belonging to the General Directorate of Agricultural Enterprises under the Ministry of Agriculture and Forestry in Türkiye. In their study, the results of intradermal avian PPD and bovine PPD tests applied to 4923 cattle; 73 positive and 19 suspicious

reactions were detected against avian, while 52 positive reactions and 6 suspicious reactions were observed against intradermal PPD johnine applied to cattle with avian reactions. Although there is antigenic similarity between avian PPD and johnin PPD strains, it has been stated that a slight difference can be seen according to the reactions received. Therefore, PPD johnin was administered to animals that reacted to avian PPD to obtain more sensitive results. In the current study, animals with positive intradermal avian PPD reactions were considered suspicious for PTB infection and antibody ELISA, fecal bacterioscopy and IFN-γ ELISA were performed and 14 (3.31%) out of 423 cattle were suspected for PTB infection. Of these 14 cattle, 5 (35.71%) were positive for PTB infection by antibody ELISA, 6 (42.86%) by fecal bacterioscopy and 3 (21.43%) by IFN-γ ELISA. Among the 14 cattle suspected for PTB infection, while 2 (14.29%) cattle were positive for antibody ELISA, fecal bacterioscopy and IFN-γ ELISA, 2 (14.29%) cattle were positive for antibody ELISA and fecal bacterioscopy and 1 (7.14%) cattle was positive for fecal bacterioscopy and IFN-γ ELISA. According to these results, in PPD bovine and PPD avian intradermal tuberculin applications applied for the diagnosis of tuberculosis infection in tuberculosis suspected farms, it was thought that animals reacting to PPD avian may be infected with PTB or may be a reaction caused by atypical (bird type) AFBs found in cattle.

As a result of this study; the results of the IFN-γ test in the antemortem diagnosis of Bovine Tuberculosis are similar to the results of the CITT, and by applying both tests together, more Tuberculosis infected animals will be diagnosed. In a comparative intradermal tuberculin test for the diagnosis of Tuberculosis infection, animals with a positive reaction to intradermal avian PPD, supplemented by other tests used for the diagnosis of Paratuberculosis, seemed to play a significant role in the detection of nonspecific reactions or in the identification of PTB infected animals. In the diagnosis of *Map* infection in cattle, more infected animals can be diagnosed by combining different diagnostic methods.

Ethical Statement: This study was approved by the Ankara University Animal Experiments Local Ethics Committee (2021-13-106).

Acknowledgements: This study was derived from the PhD thesis of the first author

Financial support: This research was supported by Ankara University, Scientific Research Projects Coordination Office with the project number TDK-2022-2478.

Conflict of Interest: The authors declared that there is no conflict of interest.

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