

Prevalence of Subclinical Paratuberculosis in Dairy Cattle in Uşak Region

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Summary

Paratuberculosis caused by *Mycobacterium avium* subs. *paratuberculosis* a chronic, inflammatory and fatal disease of ruminants. The infection is characterized by chronic "subclinic" phase. Cattle in this phase is capable of infecting other animals in the herd. Although the presence of paratuberculosis has been known, the scientific studies on the disease and the prevalence appears to be scarce in Turkey. Thus, there is no scientific report on prevalence of the disease in dairy cattle in Uşak region. In this study, it was aimed to determine the prevalence of subclinic paratuberculosis in dairy cattle farming in Uşak region in Turkey. In the study a total of 200 Holstein dairy cattle aged between 3-7 years clinically healthy with optimum milk yield and body condition score were used. The MAP were identified in feces and milk samples using direct bacterioscopy technique, and in positive samples by culturing and Polymerase Chain Reaction (outer and nested) techniques. In Uşak region in dairy cattle the prevalence of paratuberculosis was determined to be 17% by Ziehl-Neelsen staining, 9.5% by Outer Polymerase Chain Reaction and 20% Nested Polymerase Chain Reaction in feces; 4% according to bacteriologic culture results; 15.5% by Ziehl-Neelsen staining, 5.5% by Outer Polymerase Chain Reaction and 17.5% by Nested Polymerase Chain Reaction in milk samples and 2.5% according to bacteriologic culture results.

Keywords: *Johne's disease, Mycobacterium paratuberculosis, PCR, Ziehl-Neelsen staining*

Uşak Yöresi Sütçü Sığırlarda Subklinik Paratuberkülozun Prevalansı

Özet

Paratuberküloz, *Mycobacterium avium* subs. *paratuberculosis*'in etken olduğu, ruminantların yangısal ve ölümcül bir hastalığıdır. Enfeksiyon, kronik "subklinik" evre ile karakterizedir. Bu dönemdeki sığırlar sürüdeki diğer hayvanları enfekte edebilir. Türkiye'de paratuberküloz'un varlığı uzun yıllardır bilinmesine rağmen, hastalık ve hastalığın prevalansı üzerine yürütülmüş bilimsel çalışma sayısı oldukça azdır. Uşak yöresi süt sığırlarında hastalığın prevalansı üzerine gerçekleştirilmiş bilinen bir bilimsel rapor ise mevcut değildir. Bu çalışmada, yoğun olarak süt sığırı yetiştiriciliği yapılan Uşak yöresinde subklinik paratuberküloz'un prevalansının ortaya konulması amaçlandı. Araştırma, optimal süt verimine ve optimal vücut kondüsyon skoruna sahip, klinik olarak sağlıklı, 3-7 yaşlı 200 Holştayn süt sığırı üzerinde yürütüldü. Etken, dışkı ve süt örneklerinde direk bakterioskopi tekniği ve pozitif bulunan örneklerde ise kültür ve Polimeraz Zincir Reaksiyonu (Outer ve Nested) teknikleri kullanılarak tespit edildi. Uşak yöresi süt sığırlarında subklinik paratuberküloz'un prevalansı, dışkı örneklerinde; Ziehl-Neelsen boyama ile %17, Outer Polimeraz Zincir Reaksiyonu tekniği ile %9.5, Nested Polimeraz Zincir Reaksiyonu tekniği ile %20, Bakteriyojik Kültür sonuçlarına göre %4, süt örneklerinde ise; Ziehl-Neelsen boyama ile %15.5, Outer Polimeraz Zincir Reaksiyonu tekniği ile %5.5, Nested Polimeraz Zincir Reaksiyonu tekniği ile %17.5 ve Bakteriyojik Kültür sonuçlarına göre %2.5 olarak tespit edildi.

Anahtar sözcükler: *Johne's hastalığı, Mycobacterium paratuberculosis, PCR, Ziehl-Neelsen boyama*

INTRODUCTION

Paratuberculosis (pTB) is a slowly progressing infection affecting normally ruminants. Its cause is shown to be the *Mycobacterium avium* subs. *paratuberculosis* (MAP), an acid-

fast bacillus. MAP is demonstrated to stay alive more than a year in the feces of cattle and around 160 days within the surface water ¹. The bacterium has also a wide array of host



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distribution, infecting mainly ruminants but less frequently muflon² and buffalo³. It also affects non-ruminant animals such as birds, wild pigs, mice, rats, foxes, bears, and rabbits⁴ as well as domestic pigs and primates⁵. Moreover, MAP is critical for possessing zoonotic potential since it is reported to be possible cause of Crohn's disease in human^{6,7}. Although pTB cases are encountered across the continents, its regional and territorial distributions show differences. While the prevalence of pTB infection in Germany is reported to be 84.7%, some parts of Australia is stated to be free of the infection. Moreover, pTB prevalence is very low in Sweden. The studies performed on dairy cattle in different countries indicate that the prevalence of pTB infection is similar and reported to be 47% in Denmark, 43% in Canada, and 50% in the United States⁸⁻¹⁰.

MATERIAL and METHODS

Animals and Samples

The animals used in the present study were randomly collected from 31 different dairy cattle farms located in Uşak -a province in western Turkey and surrounding central villages (Çamyazı, n=44; Beylerhan, n=14; Kaşbelen, n=36; Aşağı-karacahisar, n=4; Köprübaşı, n=12; Güneli, n=21; Sarıdere, n=2; İkisaray, n=12; Bozkuş, n=8; Karahasan, n=32; Karaağaç, n=3; Çevre, n=9; and Selvioğlu, n=3). We used 200 dairy Holstein cattle that were at age between 3 to 7 years old, healthy, optimum milking, not inoculated for pTB, showing cyclic activities, and possessing optimum body condition score.

We collected feces and milk samples from the animals as study materials and kept them in sterile transfer containers under maintained cold environment till the samples were transferred to the laboratory for analyses.

Bacteriological Analyses

Ziehl-Neelsen's stain: Direct examinations of the fecal and milk samples were performed after reacting them with Ziehl-Neelsen's (ZN) stain.

Bacteriologic culture: Before performing cultures from the fecal and milk samples found to be positive with ZN stain and Nested Polymerase Chain Reaction (PCR), 10-20 g of the samples were crushed in glass homogenizers to inactivate other contaminating microorganisms. The homogenized samples were treated with equal amounts of decontaminants (20 g NaOH and 4.5 mL Bromcreasol purple), stirred for 5-10 sec, and kept at room temperature for 10 min. Consequently, after adding equal amounts of neutralizers (82.5 mL %37.5 HCl) on the homogenized samples, they were centrifuged at 1.500 rpm for 10 min and at the end of the centrifugation the sediments were saved. While some of the sediments of the samples were used for microscopic analyses, the remaining sediments were cultured in BBL™ Herrold's Egg Yolk Agar Slant with M J and ANV (USA, Cat No: 8015750) for 6-8 weeks

at 37°C. The caps of the culture tubes were opened twice or trice in a week for proper oxygenation^{11,12}. Entire of the current studies were performed in a Class II biosafety cabinet.

PCR procedure: We used Qiagen QIAamp® DNA stool mini kit (Cat. No. 51504: Qiagen, Hilden, Germany) for DNA extraction from the cultures and fecal samples. We selected MAP specific primers targeted to the IS900 gene region (*M. paratuberculosis* IS900A sequence on the EMBL GenBank DNAsequence database, accession number X16293). The samples were amplified using nested PCR approach. The oligonucleotide primers selected for the present study and specific to gene region were as follows:

MAPOF1: 5'-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3', Outer

MAPOR1: 5'-GGCGTTGAGGTGATCGCCACGTGAC-3', Outer

MAPNF1: 5'-CAGGGACGTCGGGTATGGCTTCA-3', Nested

MAPNR1: 5'-CGTCACCGCCGAATCAACTCCAG-3', Nested

DNA isolation from the fecal samples: After putting MAP 316 F reference culture and fecal samples (200 mg) in 2 mL-sterile eppendorf tubes, 1.4 mL ASL buffer solution was added to the tubes. After stirring, the tubes were vortexed for 1 min and incubated on a thermal block (VWR 460-3208, USA) for 5 min at 95°C. At the end of the incubation the tubes were stirred for 15 sec and spun at 14.000 g for 1 min. Subsequently, 1.2 mL supernatants were collected from the centrifuged samples and placed in 2 mL-sterile eppendorf tubes. An InhibitEX tablet coming with commercially available DNA isolation Kit was added into each of these tubes and the tubes were kept at room temperature for 1-2 min and vortexed until the tablets were melted. The tubes were again centrifuged at 14.000 g for 3 min. Then 200 µL supernatant was pulled from the generated supernatant and placed in 1.5 mL eppendorf tubes containing 15 µg proteinase K and 200 µL AL buffer was added to these tubes which were then incubated at 70°C for 10 min. After the incubation, the tubes were reacted with 200 µL of 96% ethyl alcohol and the samples were transferred into the QiAamp spin colon and spun at 14.000 g for 1 min and the filtrate was discarded. Subsequently, after putting the QiAamp spin colons back in 2 mL centrifuge tubes, 500 µL AW2 was added into the tubes which were then again spun at 14.000 g for 3 min and the filtrate was removed. After putting QiAamp spin colons in 1.5 mL centrifuge tubes, 200 µL AE buffer was added to the tubes, incubated for 1 min at room temperature. Finally, QiAamp spin colons were centrifuged at 14.000 g for 1 min and the QiAamp spin colons were disposed. By this way, DNA was extracted in 1.5 mL eppendorf tubes that were kept at -20°C until use.

DNA isolation from the milk samples: For DNA isolation from the milk samples, 10 mL milk samples collected from the animals were centrifuged at 20.000 rpm for 1 h and the yielding

supernatants were discarded. The pellets at the bottom of the tubes were resuspended in 1 mL distilled water and vortexed, the 500 µL of the suspensions were transferred into the eppendorf tubes and the tubes were centrifuged at 13.000 g for 10 min and the supernatants were disposed. Subsequently, the pellets at the tip of the tubes were resuspended in 300 µL Tris-EDTA and vortexed. The tubes were inactivated on a thermal block at 95°C for 15 min and then cooled down at room temperature. Following the inactivation, phenol saturated with 300 µL Tris-HCl was added to the tubes and hand mixed for 4-5 min, spun at 13.000 g for 10 min, and the supernatants were relocated to new tubes where DNA precipitation process was initiated. For DNA precipitation, 0.1 volume 3M Na-acetate and 2.5 volume pure ethyl alcohol were added to the tubes, thoroughly mixed, kept at -20°C for 1 h. Aftermath, 300 µL 90% and 70% ethyl alcohol was added to the suspension. The tubes were centrifuged at 13.000 g for 5 min between these stages and the supernatants were discarded at each time. The pellets were resuspended in 50 µL distilled water, denatured at 60°C for 1 h, and then used for PCR analyses.

DNA amplification with PCR: DNA amplification was performed at two stages using Nested PCR. The primers MAPOF1 and MAPOR1, and MAP 316F were used for outer PCR in order to extract DNA from the fecal and milk samples. The MAPOF1 was designed to code 413bp fragment on the IS900 gen region on MAP strains and the MAPOR1 was used as reference primer. The sequences of MAPOF1 and MAPOR1 were 5'-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3' and 5'-GGCGTTGAGTTCGATCGCCACGTGAC-3', respectively. After visualizing PCR products on agarose gel, 1 µL product was taken from positive and negative samples for performing Nested PCR where we used the primers MAPNF1 and MAPNR1. MAPNF1 was designed to code 326bp. The sequences of MAPNF1 and MAPNR1 were 5'-CAGGGACGTCGGGTATG GCTTTCA-3' and 5'-CGTACC GCCGCAATCAACTCCAG-3', respectively.

Outer PCR amplification was carried out in a 50 µL mixture containing 5 µL PCR buffer (containing KCL), 3 µL MgCl₂ (25

mM), 1 µL dNTP set (10mM), 1 µL (1 µM) primer (MAPOF1, MAPOF1), 0.25 µL (1.25 U) Taq DNA polymerase, 33.75 µL ultra pure water, and 5µL targeted DNA. Targeted DNA was amplified after an initial 15 min denaturation at 95°C, 30 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 3 min, and followed by a 2 min extension at 72°C¹³ using a thermal cycler (Eppendorf, Matercyler gradient 5331 000.010, Germany). Afterward, 5µL DNA was stained in 1 µL of loading dye and loaded on a 1.5% agarose gel containing 5 µg/mL ethidium bromide and run on electrophoresis.

For Nested PCR amplification, 1 µL was taken from positive and negative samples and PCR amplification was carried out in a 25 µL mixture containing 2.5 µL PCR buffer (containing KCL), 1.5 µL MgCl₂ (25 mM), 0.5 µL dNTP set (10 mM), 0.5 µL (1 µM) primer (MAPNF1, MAPNR1), 0.25 µL (1.25 U) Taq DNA polymerase, 18.55 µL ultra pure water. Targeted DNA was amplified after an initial 15 min denaturation at 95°C, 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min, and followed by a 2 min extension at 72°C¹³ using a thermal cycler (Eppendorf, Matercyler gradient 5331 000.010, Germany). Moreover, 1000 bp DNA ladder was used as marker and DNA extraction of MAP 316 F reference strain was used as positive control.

RESULTS

The results of the present study are summarized in [Table 1](#). The present results showed that the prevalence of sub-clinical pTB in dairy cows was 17% with ZN stain, 9.5% with outer PCR technique, 20% with Nested PCR technique, and 4% with bacteriologic culture method in fecal samples whereas it was 15.5% with ZN stain, 5.5% with outer PCR technique, 17.5% with Nested PCR technique, and 2.5% with bacteriologic culture method in milk samples.

DISCUSSION

Mycobacterium avium subs. paratuberculosis, often abbreviated MAP is the ethological agent for paratuberculosis

Table 1. Prevalence of subclinical paratuberculosis in feces and milk samples

Tablo 1. Dışkı ve süt örneklerinde subklinik paratüberkülozün prevalansı

Results	Feces Samples				Milk Samples			
	ZN	PCR		BC	ZN	PCR		BC
		Outer	Nested			Outer	Nested	
(+) n=200	34/200	19/200	40/200	8/200	31/200	11/200	35/200	5/200
(-) n=200	166/200	181/200	160/200	192/20	169/200	189/20	165/200	195/20
(?) n=200	2/200	-	-	-	5/200	-	-	-
(+)%	17%	9.5%	20%	4%	15.5%	5.5%	17.5%	2.5%
(-)%	82%	90.5%	80%	96%	82%	94.5%	82.5%	97.5%
(?)%	1%	-	-	-	2.5%	-	-	-

BC: Bacteriologic culture; ZN: Ziehl-Neelsen staining; (+): Positive results; (?): Suspicious results; (-): Negative results

or Johne's disease. Mainly, pTB causes considerable economic losses in dairy cow farms^{14,15}. The animals infected with *MAP* disseminate the bacterium via their feces and milk¹⁴. Moreover, since the animals carrying subclinical pTB infection can transfer bacilli to healthy animals, proper identification of the subclinical pTB infection is critical to prevent spread of the bacteria. Although the presence of pTB infection has been known throughout Turkey, the numbers of the studies inspecting the infection and its prevalence are limited^{16,18,19}. Likewise, no studies are available concerning pTB infection in Uşak province in Turkey (Web of Science). Therefore, at the present study we aimed to establish the prevalence of sub-clinical pTB infection dairy cows in Uşak area.

In the present study, we detected the presence of *MAP* in fecal and milk samples using direct bacterioscopy technique (ZN staining) and the samples turned out to be positive with ZN stain were further analyzed using culture and PCR (Outer and Nested) approaches.

The diagnosis of *MAP* can be done using conventional IS900 PCR primers in real-time PCR. For this purpose, different strategies have been tried in time to determine more effective, dependable and useful techniques for the determination of *MAP*²⁰⁻²³. Nested PCR was used for the first time in 1993 to compare effectiveness of Nested PCR and fecal culture approach in the determination of *MAP*²⁴. Later on, Nested PCR has been used particularly in diagnoses of *MAP* associated Crohn's disease and in numerous other studies on pTB cases^{13,16,25-31}. The specificity and sensitivity of the PCR tests used in the diagnoses of *MAP* in fecal samples have been greatly improved in recent years and new PCR tests can allow us to work with small amounts of fecal samples in determination of *MAP*. The main reason for the increased sensitivity of the PCR tests is the availability of improved DNA extraction and purification processes¹⁴.

Nested PCR is shown to be more sensitive than conventional PCR technique in determination of *MAP* infected animals^{14,28}. Accurate diagnose rates of *MAP* with both techniques are shown to be higher in fecal samples than milk samples (in fecal samples, Nested PCR: 49.3%; simple PCR: 45.3%; in milk samples: Nested PCR: 32%; simple PCR: 27.3%)¹⁴. These observations are consistent with our findings here (in fecal samples, Nested PCR: 20%; outer PCR: 9.5%; in milk samples: Nested PCR: 17.5%; simple PCR: 5.5%) and further support present results. *MAP* is mainly disseminated to environment via infected animal feces. However, contamination of *MAP* via milk is also very significant and one third of the clinically infected animals are reported to spread *MAP* via their milks³². Relatively lower prevalence of the pTB in milk samples with regard to fecal samples in the present study can be accounted for the fact that the main excretion of *MAP* is via feces with respect to milk¹⁷. Since *MAP* possesses zoonotic potential and also contaminates milk, precise identification of its presence in milk is critical for public health¹⁷.

Significant improvements have been obtained in PCR

based methods for diagnosing *MAP*. Modified PCR methods are almost as sensitive as bacterial culture methods accepted as gold standard today. Nested PCR has been reported to be simple, fast, reliable, and highly sensitive technique that can be used alternatively in determination of *MAP* in fecal and milk samples¹⁴. Nonetheless, in the present study the prevalence of subclinical pTB in fecal and milk cultures was 4% and 2.5%. These acquired results showed differences according to the prevalence rates obtained using Nested and Outer PCR (Table 1). This result can be explained either with the fact that PCR is more sensitive than culture method^{17,28} or with the ability of PCR to be able to replicate residual DNA of dead microorganisms, thereby enabling multiplication of contents of dead microorganisms in our study samples and leading negative growth in cultures¹⁷. An earlier study indicate that while 50% of PCR positive samples show factor growth in cultures, this percentage decreases to 36% in PCR negative samples¹⁹. Moreover, at their study Gao et al.²⁸ reported that the amount of the *MAP* disseminated by infected cows would be less than the sum determined with culture in the Nested PCR positive but culture negative cases. When compared to culture method, Nested PCR is suggested to be convenient and an alternative approach for the diagnosis of *MAP* in fecal and milk samples cases²⁸. The use of decontaminants to inactivate other microorganism, high incubation temperature (37°C), and longer incubation times causes death of more *MAP*, a major reason for getting reduced prevalence in cultures²⁸. At the same time, the prevalence values obtained in the present study using ZN stain are consistent with those acquired by PCR (in fecal samples; ZN stain: 17%, Nested PCR: 20%; in milk samples, ZN stain: 15.5%, Nested PCR: 17.5%). Obtainment of possible false positivity owing to cross reaction with even very low probability may explain the one of the reasons for the difference between prevalence values gained using PCR and culture.

The prevalence of pTB infection in cows is demonstrated to be between 3-30% in European countries¹⁵. In Turkey, a study using complement fixation technique indicate that the prevalence of the infection in the Central Anatolia Region is shown to be between 2.7% and 4.3%¹⁹. Nonetheless, pTB infection generally shows a subclinical course. While the use of complement fixation technique in the determination of the animals showing clinical symptoms is indicated to be more useful³³, it is reported to be insufficient in the diagnoses of the subclinical pTB cases¹⁷. Therefore, observation of lower prevalence in other studies with regard to the present study is expected. The studies performed in England report the prevalence of clinical pTB as 1%. Moreover, the prevalence of pTB in the animals obtained from the abattoirs and showing no clinical signs for the infection is shown to be 3.5%^{34,35}.

Majority of the animals carrying subclinical pTB infection do not present infection associated symptoms all through their live. Studies indicate that 5-10% of the animals inflicted with subclinical pTB infection in herd show clinical symptoms³⁶.

A recent study on the pTB infection in dairy cows in Burdur area reported that seroprevalence for the infection was 6.2%³³. ELISA is not only widely used in diagnosis of pTB but also it has easy application and high specificity. Nevertheless, the specificity of ELISA in the animal not showing clinical symptoms and presenting clinical signs are reported to be around 15% and 87%, respectively^{37,38}. Furthermore, the specificity of ELISA is shown to be lower in the cows younger than two years old³⁹. Therefore, the studies suggest that sole use of ELISA could be inadequate in the diagnosis of the sub-clinical pTB infections.

In the present study, our results showed that subclinical pTB prevalence in the fecal samples was ranged from the lowest 4% (culture) to the highest 20% (Nested PCR), in the milk samples it was ranged from the lowest 2.5% (culture) to the highest 17.5% (Nested PCR).

Central significant factors determining prevalence of pTB are shown to be climate, nourishment, and barn conditions³⁵. Therefore, the prevalence of pTB can show marked differences throughout Turkey.

This study was done on Holstein dairy cows and we encountered no reported strain based predisposition to pTB infection in these animals (Web of Science).

The results of the present study demonstrate the national necessity for the studies examining the presence of sub-clinical pTB infections in dairy cows in Turkey to determine their prevalence and estimate the infection-associated economical losses. Present results also indicate that Nested PCR technique is possibly more sensitive than bacterial culture method that is accepted to be the gold-standard although whose significant disadvantages for the detection of the pTB infection is not fully clarified. PCR technique can be used for the detection of subclinical pTB infection as an alternative to bacterial culture method. Nevertheless, the use of combination of discrete techniques for the detection of the pTB infection would increase accuracy of the diagnosis. Proper diagnoses and treatments of pTB infections are critical when we consider the high transmission rate of *MAP* with milk, its zoonotic potential, and its resistance to pasteurization⁴⁰⁻⁴². In addition, the animals carrying subclinical pTB infection can transmit the infection other healthy animals causing additional health problems for other animals and people. In summary, PCR techniques indicate higher prevalence rates (20% in fecal and 17.5% in milk samples) in Uşak province when compared to the other regions in Turkey.

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