The pathology of tuberculosis lesions in allergic skin test (PPD tuberculin) positive cows and detection of tuberculosis agents with PCR in milk samples

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Abstract: The aim of this study was to determine tuberculosis (Tb) agents in milk and the pathologic changes in the tissues and organs of 100 PPD Tb-positive cows that were intended for slaughter. Milk samples were taken before slaughtering and PCR analysis was conducted to detect the Tb agent. In postmortem examinations, the lungs, liver, mammary glands, spleen, kidneys, and heart were collected for macroscopic and histopathological examinations. Out of the 100 PPD positive cows, lesions of Tb were observed in 75 cows. In the microscopic examination, Tb was identified in the lungs (51 cases), liver (61 cases), and in both the liver and lungs (37 cases). Although mammary Tb was suspected in 32 cows in macroscopic examinations, in microscopic examinations these cases were evaluated as chronic pyogenic abscesses mastitis. No Tb agents were detected by PCR analysis in milk samples taken from the cows before slaughter. Therefore, periodical milk inspections are suggested for controlling agents in milk. In some of the Tb PPD positive animals (25 cases), no changes were detected; therefore, there is a need to improve tests with higher sensitivity for the antemortem diagnosis of Tb. Moreover, people involved in the livestock sector should be trained about Tb, hygiene rules, and disease-free herds.

Key words: Tuberculosis, pathology, cow, PPD, PCR, milk

1. Introduction

Tuberculosis (Tb), which is a chronic, contagious, and zoonotic bacterial disease that affects human and animal health negatively, is characterized by the forming of caseous and caseo-calcareous tubercules, especially in the lungs other organs and tissues. Although Tb is most common in cattle among domestic animals, it is also seen in cats, dogs, pigs, poultry, and many wild animals. The genus Mycobacterium is the causative agent of the disease. The major causes of Tb are Mycobacterium bovis in cattle, Mycobacterium tuberculosis in humans, and Mycobacterium avium in poultry (1).

Cattle are the species most affected by Tb among livestock, and the disease is mostly transmitted by inhalation of infectious aerosol droplets and less transmitted through alimental, congenital, coital, and cutaneous routes. The most common form of the disease in cattle is pulmonary tuberculosis. The progression of the lesion occurs through blood and lymph vessels, body cavities, and channels, and then the disease develops in many tissues and organs. Single or conglomerate granulomas occur in the lungs, liver, spleen, kidneys, on the surface of body cavities, and in some cases in the genital organs, and during later periods it occurs in the brain. Granulomatous tuberculous lesions are characterized by the infiltration of epithelioid macrophages, Langhans-type giant cells, lymphocytes, and plasma cells. When the disease progresses, calcification with caseification necrosis is formed at the center of granulomas and surrounded by a fibrous capsule (1,2).

In addition to economic losses, bovine Tb is of great importance as it is the source of contamination for humans. M. bovis can infect humans via direct contact with mucous membranes and skin abrasions, or through digestion, aerosols, and inhalation (2,3). However, although the main source of contamination is the consumption of unpasteurized milk and dairy products, it has been observed that the inhalation of infectious aerosols released by sick livestock also causes the disease (3–5). According to OIE, for the antemortem diagnosis of tuberculosis infection in cattle, tuberculin skin test, or as an alternative, gamma interferon test (IFN-γ) are recommended; for the postmortem diagnosis, on the other hand, bacterioscopy, histopathological
examination, culture, and molecular methods are recommended (4, 5).

In the present study, it was aimed to determine the pathological changes in the tissues and organs of 100 PPD tuberculin positive cows intended for slaughter; it was also aimed to determine the presence of Tb agents in milk samples.

2. Materials and methods

2.1. Materials

Samples of lung, liver, mammary glands, spleen, kidney, and heart, as well as milk collected before slaughtering the cows with PPD tuberculin-positive were used as the material of the study. This study was conducted with the approval of the Ethics Committee at Selçuk University Veterinary Faculty (SÜFEK), (30.10.2010, 2008/074).

The animals were 6 years old (32 head) and 4 years old (31 head), 5 years old (15 head), 3 years old (11 head), 7 years old (9 head), 9 years old (1 head), and 10 years old (1 head). It was noted that a great majority of the 100 cows were Holstein (87 head), while the others were Simmental (7 head), native breeds (3 head), Brown Swiss (2 head), and Jersey (1 head).

2.2. Methods

2.2.1. Histopathological examinations

Tissue samples (lung, liver, mammary, spleen, kidney, and heart) extracted from the slaughtered cows in the abattoir were fixed in 10% buffered formalin for pathological examinations. Paraffin blocks, prepared with routine laboratory methods, were cut in 5-µm thickness and were stained with hematoxylin and eosin (H&E) and Ziehl–Neelsen (ZN) methods and were examined by light microscope.

2.2.2. Polymerase chain reaction (PCR)

After the decontamination and neutralization of milk samples, DNA was extracted as described by Wilsher et al. (6) and Cataloluk et al. (7). After purity and quantity determinations of the extracted DNA samples, PCR analysis was performed for *M. tuberculosis* complex. For this purpose, primers that amplified 1020 bp (basepair) fragment of the gyrB gene region specific for *M. tuberculosis* complex were used: MTUB_c-gyrBf-5'-TCG GAC GCG TAT GCG ATA TC-3' and MTUB_c-gyrBr-5'-ACA TAC AGT TCG GAC TTG CG-3 (8). Amplification was performed in 50 µL of PCR buffer. The PCR buffer contained 5 µL of PCR buffer (KCl), 3 µL of MgCl₂ (25 mM), 1 µL of dNTP set (10 mM), 1 µL of a primer (1 µM), 0.25 µL of (1.25 U) Taq DNA polymerase, 33.75 µL of sterile ultrapure water, and 5 µL of target DNA. The target DNA was amplified by PCR-thermal cycler for 10 min predenaturation at 95 ºC, 35 cycles for 1 min at 94 ºC, 1 min at 65 ºC, 1 min at 72 ºC, and 10 min at 72 ºC. Next 5 µL of DNA was stained with 1 µL of loading dye solution, and electrophoresis was performed by loading 1.5% agarose gel containing 5 µg/mL ethidium bromide. A 1000 bp DNA ladder was used as a marker. *M. tuberculosis* H 37 Rv was used as a positive control and DNA extracts of *M. avium* ATCC 25291 reference strains were used as a negative control.

3. Results

After the postmortem examination of the cows with PPD tuberculin-positive, macroscopic lesions were observed in different organs in 88 cows. As a result of the histopathological examinations, Tb was determined in 75 cows. In the microscopic examination, histopathologic findings related to Tb were observed in the lungs of 51 animals, in the liver of 61 animals, and in both the liver and lungs of 37 animals. Although the PPD test was positive in all animals, microscopic findings related to Tb were not seen in any of the examined organs in 25 of the animals. Moreover, microscopic findings related to Tb were not observed in 13 of 88 animals in which macroscopic lesions were detected.

3.1. Macroscopic and microscopic findings

3.1.1. Lungs

Macroscopically, Tb lesions were found in the lungs of 51 cows (51%) (Figure 1A). The lesions were diffuse in 32 (62.7%), focal in 10 (19.6%), and multifocal in 4 cases (7.8%). In 5 of the cases (9.8%), conglomerate granulomas were found that combined with each other and had wider caseified areas.

In microscopic examinations, productive-type granulomas were observed in the lungs of 51 animals (Figure 1B). In 29 of these cases (56.8%), the Tb had opened to the bronchia and bronchioles. In these cases, the epithelial layer was partially or totally necrotic, and its lumens were filled with degenerated and necrotic cells and neutrophil leukocytes (Figure 1C). Histopathological examinations by ZN-stained methods revealed that Tb agents were encountered in the tubercles in 41 of 51 cases. Small, red, and rod-shaped agents were seen around the caseification necrosis, Langhans giant cell cytoplasm (Figure 1D), and less in the cytoplasm of epithelioid cells in the ZN-stained sections.

3.1.2. Liver

Tb lesions, which were detected in 61 cases in the liver, were mostly subcapsular, and in some cases the lesions were detected on the cut surface (Figure 2A). Granulomas were 4–30 mm in size, and the cut surface of the large nodules was a yellow necrotic mass surrounded by a thick capsule, while necrotic structures were not evident in the grayish-white small nodules.

In microscopic examinations, granulomas were encountered in 61 cases (Figure 2B). Conglomerate tubercle structures were also observed in 17 (27.8%) of these cases. Tubercles were opened to the bile duct in 29
In 48 of 61 cases, Tb agents, red, rod-shaped and small, were seen in tubercles with ZN staining (Figure 2D).

### 3.1.3. Mammary glands

There were no histopathological findings related to Tb in the microscopic examination although mammary Tb was suspected in 32 cows after slaughter. In these cases, the mammary gland was hard, and some of them were surrounded by a grayish-white fibrous capsule.

In some cases, it was observed that mammary gland had nodules with an encapsulated structure, and the cut surfaces contained necrotic and purulent content that was yellowish-green. In microscopic examinations, mastitis was diagnosed in 74 cows and classified as chronic-purulent-abscess mastitis in 32 cases, acute catarrhal mastitis and galactoforitis in 25 cases, and chronic catarrhal mastitis and galactoforitis in 17 cases. None of these cases had any tubercles or any agent in ZN staining sections.

### 3.1.4. Other organs

Epitheloid-type granulomas were observed in the spleen in 2 cases, but Tb agents were not found in staining with ZN. Tb lesions were not observed in the kidney and heart on the microscopic examinations. In general, Tb was detected in 75 of the 100 PPD-positive animals and Tb agents were seen in 60 (80%) of these 75 animals with ZN staining.

### 3.1.5. PCR in milk samples

According to the PCR examinations of milk samples taken from the cows before slaughter, no Tb positivity was found in the DNA of the samples (Figure 3).

### 4. Discussion

Tb is still an important disease for humans and other mammals. In addition to causing economic losses in cattle, *M. bovis* is also an important public health problem.
in humans, and it is responsible in 10% of Tb cases in humans (9). Pulmonary Tb, which is seen among adults and livestock workers, occurs when humans consume unpasteurized dairy products and inhale droplets or dust droplets containing *M. bovis*. Extrapulmonary cases, caused by *M. bovis*, are associated with infected milk consumption, and it is thought that infection is less likely to be transmitted by digestion because of the developed milk pasteurization facilities and consumption of boiled milk (10).

PPD skin test is an international test used for the antemortem diagnosis of bovine Tb. This test is used around the world to eliminate positive reactions in eradication programs. The PPD positive test result indicates that the living organism becomes susceptible to Tb by Tb bacillus (11). In the present study, although all of the animals had a positive reaction to the PPD tuberculin test, Tb was detected in 75 cases in pathomorphological studies. In their study with goats, Cousins et al. (12) applied PPD testing to 19 healthy goats that grazed in the same field as cattle; they found that although 13 of them had a positive reaction none of them had any macroscopic lesions. In a study conducted on cattle (13), a positive reaction was found to the tuberculin test in 169 out of 772 cows, and the average sensitivity of the skin test was 69.8% and the specificity was 98%.

Age, sex, herd size, body condition, farm system, and birth prevalence are important risk factors for bovine Tb (14). In the present study, it was observed that the disease occurred at the beginning of the spring considering the region of origin (Samsun, Çorum, Kastamonu) of the animals that came for slaughter. It is thought that this was caused by contact and aerosol ways during the winter since these animals were sheltered in small and crowded barns.
In contrast to livestock that were sent to slaughter earlier than milk cows, the milk cows were kept in barns for many years and exposed to the infection more, which confirms the previous studies (15).

**Tb** is present in all ages, yet with increasing age, the disease increases as well (16). In the present study, the age of slaughtered animals changed between 3 and 10 years and most of them were 6 years old (32 cases) and 4 years old (31 cases). Tb was detected in 8 cases in 3-year-old cows, and the cases were either lung or liver Tb.

Ameni et al. (17) reported that the incidence of Tb caused by *M. bovis* varies among cattle breeds. It was noted that the Holstein breed, which had higher milk yields, is more susceptible to Tb (18). Similarly, in the present study, among the cows identified as positive for the PPD test 87% were Holstein, 7% Simmental, 3% native, 2% Brown Swiss, and 1% Jersey.

It has been reported that Tb lesions in the lung may vary in size from a pea to a fist, and their distribution differs. Some miliary tubercles may be visible during early generalization, but fewer tubercles with different ages could be seen in chronic generalization (19). In the present study, when the distribution and sizes of the lesions in the lungs are considered, it was thought that their size is small (4–10 mm) and the diffuse distribution of the miliary tubercles might be related to acute generalization; focal and multifocal distributions of different sizes may be the chronic generalization.

It was reported that Tb lesions in bovine Tb were productive form and virulence was high, and it was noted that the host was exudative in cases where the resistance was low (20). It was reported that in cattle lungs are the organ most affected by Tb (21). In our study, it was observed that all lesions were in productive form and that the lesions were in conglomerate form in 5 cases.

Microscopic examinations showed that characteristic lesions of Tb are composed of central caseous necrosis, calcification with mantle lymphohistiocytic infiltration, epithelioid cells, and Langhans-type giant cells, surrounded by fibrous capsule formation; these findings were similar to those previously reported (22,23). It has been stated that the tubercles in the lung can be opened to the bronchia and bronchioles (24). Similar lesions have been observed in the present study, and it has been concluded that disease agents may be an effective way of spreading outward from the lungs.

In the present study, Tb lesions were detected in the liver of 61 animals. In liver Tb, some studies (25,26) have reported that bile ducts contain Tb agents and carry them to the intestines. In the present study, it was also noted that tubercles were opened to the bile ducts in 29 cases, and it was concluded that the agents might pass through the bile ducts to the digestive tract and contaminate the feces and environment through the digestive tract.

The presence of Tb lesions in the mammary gland of PPD positive cows, and to check whether the Tb agent was thrown out with milk was among the aims of our study. In the present study, mastitis was detected by microscopic examinations in 74 cows. In 25 cases, acute catarrhal mastitis and galactoforitis were detected, and in 17 cases chronic catarrhal mastitis and galactoforitis were diagnosed. Apart from these cases, granulomatous formations were noted in 32 macroscopically suspected cases of tuberculosis. In microscopic examinations, granulomatous structures were initially thought to be productive infiltrative mammary Tb, but unlike tubercles, it was noted that dense neutrophil granulocytes were present in the middle necrotic areas and surrounded by a fibrous capsule composed of dense connective tissue. Neither epithelial cells nor Langhans-type giant cells were found around these necrotic-purulent structures. Tb agents in ZN staining of these cases were not observed. However, no tuberculosis agents were detected in milk samples by PCR. Considering these findings, it is concluded that these lesions are related to chronic pyogenic abscesses mastitis.

In a study on cattle in Bursa (27), it was reported that there was a Tb lesion in the spleen in 6.7%. In the present study, epithelioid granuloma was found in only 2 cases (2%). However, no Tb agent was observed in ZN staining. In addition, there were no macroscopic or microscopic Tb findings in the kidney or heart.

Small, red, and rod-shaped agents were seen in tubercles with the ZN stained sections in 41 of 51 lungs and 48 of 61 livers. Although tubercle structures were evident, no Tb agents were found with ZN stained sections in 10 lungs, 13 livers, and 2 spleens. Ortatatli et al. (24) reported that they did not detect Mycobacteria in 31 cases.

![Figure 3. Negative PCR image of Mycobacteria in milk samples.](image-url)
of ZN staining although they identified Tb lesions in 49 cases. It has been stated that immunologic reactions can cause granulomatous lesions with no agent (28), or that the same lesions can be formed with bacterial antigens and L-forms of bacteria (29). The present study supports the views of these researchers because in the present study tubercles were seen in 75 of the PPD positive 100 cows and the agent could not be observed in ZN staining.

In another previous study (30), Mycobacteria were investigated in raw milk samples of 145 cows. Ehrlich–Ziehl–Neelsen (EZN) staining showed only one (0.7%) case of acid-resistant bacilli. Eleven samples (7.6%) were identified as culture positive in LJ nutrient and 6 samples were reported to have mycobacterial DNA detected by PCR. In the present study, tubercle and Tb agents were not detected in examined mammary tissues. Furthermore, DNA of the Tb agents was not detected by PCR in milk samples.

The generalization of Tb does not always occur. Although Tb lesions of the mammary gland developed, these lesions do not always open to the milk ducts. Even when these lesions are opened to the milk ducts, the transmission of the agent is not continuous. For that reason, it was thought that the fact that the Tb agent is not detected in the milk in the study cannot mean that there is no Tb in the mammary glands. Tb-free milk does not mean that animal is also free from Tb. Therefore, periodical milk inspections are suggested for controlling the agent in milk because Tb agents are not always cleansed with milk.

In conclusion, in the histopathologic examination of tissues and organs of 100 PPD positive cows, Tb was observed in 75 animals. Although PPD was positive in 25 animals, no changes related to Tb were observed in macroscopic and microscopic examinations. Although there are many factors that limit its sensitivity, the PPD skin test is the most preferred test used worldwide because it is easy to apply for cattle Tb and it is cost-effective in antemortem diagnosis. However, it is not sufficient for the antemortem diagnosis of Tb because of its low specificity and sensitivity; therefore, more sensitive tests need to be developed. In addition, very few factors are detected in the Tb cases by ZN method; there was even no Tb agent in some cases. For that reason, the ZN method is insufficient in detecting the agent. It is thought that bacterial antigens and antibodies can be detected and more accurate results can be obtained with immunoperoxidase methods.

In recent years, in addition to the increase in Tb cases in domestic animals in this country, which causes great economic losses, zoonosis is a major risk factor that also threatens public health. The eradication of bovine Tb in human populations is a necessity. For the eradication studies to be successful, the infected cattle should be diagnosed by isolating the causative agent using accurate and reliable methods. Sensitivity to allergic and serological tests is insufficient. In addition, tissues with bovine Tb contain few agents, and the organs of caseific and calcific regions are controlled by a fibrous capsule. It is necessary to disseminate the PCR method, in which fast and reliable results are obtained, to ensure complete identification and molecular validation of mycobacterium isolates and to increase the work on the diagnosis of Tb cases in cattle by PCR.

The transmission of mycobacteria from infected animals to humans happens by the consumption of unpasteurized infected milk and dairy products. Therefore, raw milk samples that have zoonotic Tb infection risk should be studied more comprehensively, and epidemiologic data should be obtained by isolating Mycobacteria in milk samples. Considering that the Tb agents are not always thrown away with milk, the presence of the agents in milk should be checked periodicaly.

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References


