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Immunohistochemical Examination of TNF-α and SAA Expressions in Goats Infected with *Mycobacterium avium* subspecies *paratuberculosis*

Muhammet Bahaeddin DÖRTBUDAK[1,a,✉], Merve ÖZTÜRK[2,b]

[1]Department of Pathology, Faculty of Veterinary Medicine, Harran University, Şanlıurfa/TÜRKİYE
[2]Department of Internal Medicine, Faculty of Veterinary Medicine, Necmettin Erbakan University, Konya/TÜRKİYE

[a]ORCID: 0000-0001-5777-964X; [b]ORCID: 0000-0002-8500-7599

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Abstract

Paratuberculosis (Johne Disease), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and commonly seen in ruminants, is a contagious disease that causes chronic diarrhea and serious economic losses. In this study, the presence of pro-inflammatory cytokine and acute phase protein expressions in lesional tissues of naturally infected goats with MAP was investigated. For this purpose, paratuberculosis suspected goats complaining of chronic diarrhea and excessive weight loss were examined by ELISA method for *Mycobacterium avium* subspecies *paratuberculosis* infection. After sacrificing 20 animals found to be infected with MAP, tissue samples taken from the intestines and lymph nodes were stained with Hematoxylin-Eosin (HE) for histopathological examination and Ziehl-Nelsen (ZN) to show acid-fast mycobacterium. Immunohistochemical staining was performed to examine the expressions of TNF-α as a pro-inflammatory cytokine and SAA as an acute phase protein. Macroscopic examination revealed in serous fat atrophy, mesenteric lymphadenitis, and granulomatous enteritis in the intestines. Histopathological examination revealed atrophy in villi, degeneration of enterocytes, inflammatory cell infiltration consisting mostly of mononuclear leukocytes in the mucosa, and epithelioid macrophage aggregation. Lymphoid hyperplasia and epithelioid histocyst aggregates were found in the mesenterial lymph nodes. In staining with ZN revealed the presence of bright red acid-fast agents in the intestine and mesenteric lymph nodes. In immunohistochemical examination, TNF-α and SAA expressions were observed in all samples. With this study, for the first time, the expressions of TNF-α, a proinflammatory cytokine, and SAA, an acute phase protein, were shown in the lesional tissues of MAP-infected animals, contributing to the immunopathogenesis of the disease.

Key Words: Goat, *Mycobacterium avium* subspecies *paratuberculosis*, paratuberculosis, SAA, TNF-α

Mycobacterium avium subspecies _paratuberculosis_ ile Enfekte Keçilerde TNF-α ve SAA Ekpresyonlarının İmmünohistokimyasal İncelenmesi

Öz

Etkeni *Mycobacterium avium* subspecies *paratuberculosis* (MAP) olan ve geviş getirenlerde yaygın olarak görülen paratüberküloz (Johne Disease) kronik ishal ile ciddi ekonomik kayba neden olan bulaşıcı bir hastalıktır. Bu çalışmada MAP ile doğal enfekte keçilerin lezyonlu dokularında pro-inflmatuvar sitokin ve akut faz protein ekpresyonlarının varlığı araştırıldı. Bunun için kronik ishal ve aşırı zayıflama şikayeti bulunan paratüberküloz şüpheli keçiler *Mycobacterium avium* subspecies *paratuberculosis* enfeksiyonu yönünden ELISA yöntemiyle incelendi. MAP ile enfekte olduğu saptanan 20 hayvanın kesime sevki sonrası sakrifiye edilmeleri ile bağırsak ve lenf yumrularından alınan doku örnekleri histopatolojik inceleme için Hematoksilen-Eozin (HE) ve asit-fast mycobacteriumları göstermek için Ziehl-Nelsen (ZN) ile boyandı. Pro-inflamatuvar sitokin olarak Tümör Nekroz Faktör (TNF-α) ve akut faz protein olarak Serum Amiloid A (SAA) ekpresyonlarını incelemek için immünohistokimyasal boyama yapıldı. Makroskobik incelemede seröz yağ atrofisi, mezenteriyal lenfadenit ve bağırsaklarda granülamatoz enteritis görüldü. Histopatolojik incelemede villuslarda atrofi, enterositlerde dejenerasyon, mukozada çoğunluğu mononükleer lökositlerden oluşan yangı hücre infiltrasyonu ve epiteloid makrofaj agregasyonu gözlendi. Mezenteriyal lenf düğümlerinde lenfoid hiperplazi ve epiteloid histoyosit yığınlarına rastlandı. ZN boyamada bağırsak ve mezenterik lenf düğümlerinde parlak kırmızı renkte asit-fast etkenlerin varlığı saptandı. İmmünohistokimyasal incelemede TNF-α ve SAA ekpresyonlarının tüm örneklerde meydana geldiği gözlendi. Bu çalışma ile ilk defa MAP ile enfekte hayvanların lezyonlu dokularında bir proinflamatuar sitokin olan TNF-α ve bir akut faz proteini olan SAA ekpresyonları gösterilerek hastalığın immünpatogenezine katkı sağlandı.

Anahtar Kelimeler: Keçi, *Mycobacterium avium* subspecies *paratuberculosis*, paratüberküloz, SAA, TNF-α

INTRODUCTION

Paratuberculosis is a disease characterized by intermittent diarrhea and extreme weight loss in ruminants. This disease, which is common around the world, causes significant economic loss by causing low productivity and death in animals.

This disease, which is not yet finalised to be zoonosis, is thought to be related to Crohn's disease in humans. In addition, since raw milk has been suggested to be effective in transmission, this disease has become an important public health and food safety issue (1-3).

Mycobacterium avium subspecies *paratuberculosis* is gram positive, acid-resistant, sporeless and rod-shaped a bacterium has the ability to live within macrophages. In this disease, which has low mortality and high morbidity, transmission occurs through direct contact, ingestion of water and feed contaminated with secretions such as feces, urine and saliva, or intrauterine route. Although young animals are more susceptible to the disease, infection is more common in animals over 2 years of age due to a long incubation period (4,5). The target organ of the agent, which usually enters the body orally, is the intestines, and lesions occur especially in the last parts of the intestine and associated lymph nodes. The most obvious clinical symptom observed in sick animals is chronic diarrhea. However, the appetite is generally good. In addition, decreased milk yield, live weight loss, depression, weakness and submandibular edema are also observed (6-8). In infected animals, granulomatous enterocolitis and local lymphadenitis are observed macroscopically. The inflammatory cell infiltrates and epithelioid histiocyte aggregation are seen in the mucosa and submucosa microscopially. In the fight against this disease, which has no effective treatment and is not notifiable, it is generally recommended to send infected animals to slaughter (9-12).

The pathogenesis of paratuberculosis disease has not been completely explained. For this, it is necessary to know the expression and inhibition of some signaling proteins that play important roles between the agent and the host. One of the most widely studied signaling proteins is TNF-α. TNF-α, a proinflammatory cytokine, has important roles in the induction of inflammation, migration and differentiation of leukocytes (13,14). Another protein that is effective in the pathogenesis of inflammations caused by infection is SAA. SAA, an acute phase protein, is effective in minimizing tissue damage in the area of inflammation and accelerating the tissue repair process (15,16).

In this study, the presence of TNF-α as proinflammatory cytokine and SAA as acute phase protein expression in the lesioned tissues of goats naturally infect with MAP was investigated in order to contribute to the pathogenesis of paratuberculosis disease, is an important health problem of ruminants.

MATERIAL AND METHODS

Animal Material

This study was conducted with the ethical approval of Bingöl University Animal Experiments Local Ethics Committee (B.Ü. HADYEK 2023/02-02/13). The study material consisted of 20 female hair goats between the ages of 2-5. Blood samples were taken from animals with suspected paratuberculosis and the presence of infection was investigated by ELISA method. Animals positive in terms of MAP infection were recommended to slaughter for the absence of effective treatment and avoid environmental contamination. Macroscopic examination was performed on sacrificed infected animals. Tissue samples were taken for pathological examination from the intestine and mesenteric lymph nodes where the lesions were detected.

ELISA Test

A commercial ELISA kit (Paracheck 2, no.63325, Prionics AG, Zurich, Switzerland) was used to investigate antibodies against MAP in the blood serum of animals with disease suspected. The test kit was applied according to the protocol reported by the manufacturer. After the reaction, the OD value of each well was read on an ELISA reader (Rayto RT-2100C, Shenzhen, PRC) using a 450 nm filter.

Processing of Tissues

Lesional tissue samples taken from infected animals were placed in 10% buffered formalin solution. After the fixed tissues were washed in tap water, they were subjected to routine tissue monitoring. Paraffin blocks were prepared from the samples whose tissue tracking was completed, and sections were taken cuts from each paraffin block onto 5µ-thick normal and polylysine slides by rotary microtome.

Histopathological Examination

Tissue sections taken on normal slides were kept in the oven for an hour and then deparaffinization and rehydration processesing were performed. Tissues were stained with Hematoxylin-Eosin (HE) to examine microscopic findings and Ziehl Neelsen (ZN) to show acid-fast microorganisms. Then, the stained preparations were covered with a coverslip by dropping Entellan and examined under a light microscope (Leica DM2500, Wetzlar, Germany).

Immunohistochemical Staining

Tissue sections taken on polylysine slides for immunopathological examination were kept in the oven for about an hour and then deparaffinized and rehydrated in xylene-alcohol series. For endogenous inactivation, the tissues were kept in 3% H_2O_2 for 10 minutes, washed with PBS, and then boiled and cooled three times in retrieval solution to reveal the presence of antigen by the microwave oven. The tissues, washed again with PBS, were limited by PAP pen and incubated for 20 minutes by adding protein block to prevent non-specific binding. Then, TNF-α (no. ab6671, Abcam, UK) and SAA (no. PA5-32262, Thermofisher, USA) primary antibodies were diluted and incubated at +4°C for 1 night. Biotinized secondary antibody was dropped onto the tissues washed with PBS and incubated for 20 minutes. This time, streptavidin-peroxidase was added to the tissues, which were washed again with PBS, and incubated for 20 minutes. In the tissues washed with PBS for the last time, 3,3' Diaminobenzidine (DAB) chromogen was dropped on the sections to see antigen-antibody binding (17). Tissues counterstained with Mayer Hematoxylin were covered with coverslips and examined under a light microscope (Leica DM2500, Wetzlar, Germany).

RESULTS

ELISA Test

All blood sera taken from 20 paratuberculosis suspected goats over 2 years old with chronic diarrhea, extreme weight loss, submandibular edema and progressive loss of productivity were found positive in terms of MAP antibodies by ELISA test.

Macroscopic Findings

In the post-saccharification macroscopic examination of infected goats sent to slaughter, lesions were found in the intestines and associated lymph nodes. Approximately 2-3 liters of serous exudate was detected in the abdominal cavity. It was observed that the omentum, mesenterium and subperitoneal fat tissue atrophyed and were replaced by a yellowish diffuse gelatinous structure. It was observed that the intestinal serosa had an opaque appearance due to diffuse edema and the mucosa was thickened. The thickening of the intestinal mucosa manifested itself as folds in places. The intestinal contents had a watery consistency. Although almost all parts of the intestine were affected, the lesions were mostly encountered in the ileum and jejunum. The lymph vessels were thickened in the form of cords and the mesenterial lymph nodes were edematous and enlarged (Figure 1).

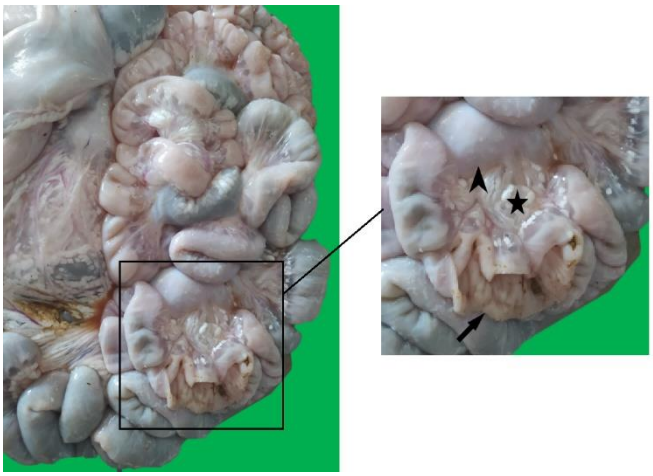

Figure 1. Enlargement of the mesenterial lymph node (arrowhead), serous fat atrophy (star), thickening and folds of the mucosa (arrow) in a Map-infected goat.

Histopathological Findings

Histopathological examination showed that all layers of the intestine were affected, but the inflammation was more severe in the mucosa. Atrophy and fusion in villi, degeneration and decumulation in enterocytes, dilatation in villous lacteal and crypt glands were observed. Edema, and inflammatory cell infiltrates mostly consisting of mononuclear leukocytes, were observed in the lamina propria. As a more specific finding, focal epithelioid histiocyte collections were observed. These cells that were oval or round-shaped with eccentrically location euchromatic nuclei and eosinophilic foamy cytoplasm. Lymphoid hyperplasia and perilymphangitis were observed in the submucosa, along with findings similar to inflammatory changes in the mucosa. Although no lesion was seen in the muscular layer of intestine, edema, mild leukocyte infiltration and lymphangitis were detected in the serosa. In the mesenterial lymph nodes, almost all sinuses were enlarged due to edema. Generalized lymphoid hyperplasia in the cortex and microgranulomas consisting of epithelioid macrophages were observed in the paracortical zone and subcapsular sinuses (Figure 2. A-B). In ZN staining, many numerous bright red color acid-fast agents were identified. The majority of these were seen in epithelioid cells in the villous lamina propria and as extracellular aggregates. The presence of acid-resistant agents was also observed in epithelioid macrophages and extracellularly in the mediastinal lymph node (Figure 2. C-D).

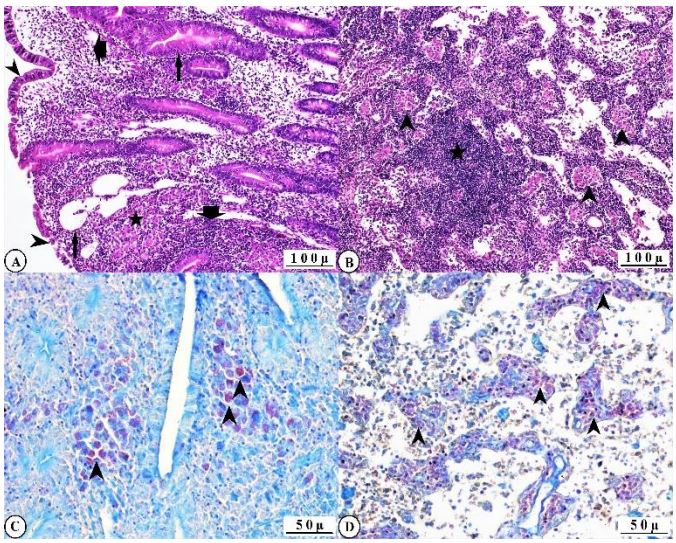

Figure 2. A. Degeneration and desquamation in enterocytes (arrowheads), Edema and mononuclear leukocyte infiltration in the lamina propria (thick arrows), Dilation in lacteals and crypt lumens (thin arrows), Epitheloid macrophage aggregation in the mucosa (star), HE, X100. B. Epithelioid histiocyte granulomas (arrowheads) and lymphoid hyperplasia (star) in mesenterial lymph node, HE, X100. C. Acid-fast reaction positive areas (arrowheads) in villous lamina propria, ZN, X200. D. Acid-fast reaction positive areas (arrowheads) in the mesenterial lymph node, ZN, X200.

Immunohistochemical Findings

In immunohistochemical staining, severe TNF-α expression was observed in degenerated epithelial cells in the mucosa layer, where severe histopathological changes were noted, in crypt gland epitheliums and in leukocytes and epithelioid macrophages in the lamina propria. Lymphoid hyperplasia foci and TNF-α expression in epithelioid macrophage collections were also observed in mesenteric lymph nodes (Figure 3. A-B). SAA expression was mostly found in epithelioid histiocyte aggregations and leukocytes. Weak immunoreaction was detected in enterocyte and crypt gland epithelia. Additionally, SAA expression was seen in epithelioid macrophage clusters and inflammatory cells in the mesenteric lymph nodes (Figure 3. C-D).

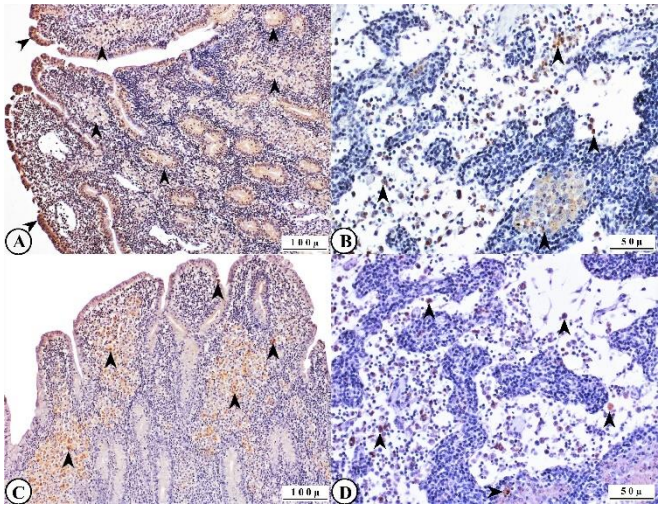

Figure 3. A. Severe TNF-α expression (arrow head) in leukocytes and epithelioid, mucosal and crypt epithelial cells, IHC, X100. B. TNF-α expression (arrow head) in leukocyte and epithelioid macrophage masses in the mesenterial lymph node, IHC, X200. C. Severe SAA expression (arrow head) in leukocytes and epithelioid histiocytes in the lamina propria, and mild in the enterocytes, IHC, X100. D. SAA expression (arrow head) in leukocytes and epithelioid macrophages in the mesenterial lymph node, IHC, X200.

DISCUSSION AND CONCLUSION

Paratuberculosis disease has been an important health problem in domestic ruminants for a long time. Infected animals experience chronic diarrhea and loss of condition. Moreover, in this disease for which, there is no effective treatment protocol adults spread the agent to the environment (2,18). Chronic diarrhea and excessive weight loss were detected in the clinical symptoms and anamnesis of almost all animals subject to this study. This state was similar to the findings of previous studies on natural and experimental infections (8,9,19).

The main macroscopic finding observed after necropsy of MAP-infected animals is tissue thickening due to proliferative inflammation (10,20). It was observed that the intestinal mucosa had thickening and folds due to lymphoproliferation and edema. Although this finding is confused with postmortal intestinal invagination, the distinction is made by the fact that the folds in the intestine cannot be corrected by draw. Lesions in the intestine are usually localized in the last part of the small intestine (9,11,21). In these study samples, lesions were mostly found in the mesenteric lymph nodes associated with the ileum and jejunum. Along with lymphadenitis, occurred inflammation and thickening also of the lymph vessels. Although it has been suggested that the general edema in the disease is caused by hypoproteinemia due to the destruction of the intestinal mucosa and subsequent malabsorption, lymphagitis is also thought to contribute to this condition. Although severe hypoproteinemia and diarrhea are present, death does not occur immediately because dehydration is not severe. This is probably due to little damage of the duodenum. Unlike other gastro intestinal infections, in the disease usually has a good appetite. However, serous fat atrophy occurs in animals in negative energy balance due to excessive hypoproteinemia. In this study, lymphadenitis, granulomatous enterocolitis and serous fat atrophy were observed, consistent with previous studies (9-11,20).

In chronic infections, especially in inflammations with proliferative character mononuclear cell infiltration is at the forefront. Lymphoplasmocytic leukocyte infiltration is observed also in MAP infection where granulmatous inflammation predominates. Additionally, another important finding in a MAP infection is epithelioid macrophage aggregation. These inflammatory reactions are mostly observed in the mucosa and submucosa along with vascular changes. In addition to proliferative changes, degeneration and desquamation are also observed in the enterocytes facing the lumen. Inflammatory changes usually occur in the distal of small intestine. This state is thought that this may be due to the fact that the enfectious agents migrate to the submucosa using the M cells there (9,11,21). It was noted that the histopathological findings in the study carried similar results to previous experimental and naturally infected studies. In most studies on MAP infection, histopathological findings were evaluated in two forms: paucibacillary and multibacillary. Of these, it is the paucibacillary form in which lymphocytes are dominant and the Th-1 cell-mediated immune response is present, and it is the multibacillary form in which the epithelioid macrophages are dominant and the Th-2 humoral immune response is present (12,22). In this study, as previous studies, more multibacillary form were encountered. Histopathological examination of mesenteric lymph nodes seen lymphoplasmacytic cell infiltration and epithelioid histiocyte masses. The general histopathological findings in this study were found to be compatible with previous studies, and the lesions milder in the lymph nodes (9,11,12,21).

Some signaling proteins are activated by cell damage, which is the basis of many diseases, and these play an active role in the pathogenesis and prognosis of the disease (7,13,14). Research has been conducted on proinflammatory cytokines, acute phase proteins and oxidative stress parameters in MAP infections of various animal species. However, most of these studies were conducted on the blood serum of infected animals, and in this context, been made studies onto lesional tissues of infected animals weren't found. Sevgisunar and Şahinduran reported in their study that there was an increase in some acute phase protein, cytokine and hepcidin values in Sanen goats with paratuberculosis (22). El-Deeb et al. they revealed an increase in some pro-inflammatory cytokines (IL-1, IL-10, TNF-α) and acute phase proteins (Hp, SAA) in paratuberculosis of camels in Saudi Arabia (24). Bozukluhan et al. they revealed that there was an increase in some acute phase proteins (Hp, SAA) in Map-infected cattle and also reported that these could be helpful parameters in disease diagnosis (25). With the endocytosis of MAP factors, the receptors on the phagocytic cells are stimulated and intercellular signaling pathways are activated and inflammation-stimulating molecules are released. TNF-α, main ones of the among this cytokines, helps macrophages to gather and control the infection. Additionally, CD4+ T cells activated by this cytokine differentiate into Th1 and Th2 cells. Th1 cell-mediated immunity; creates Th2 humoral immunity

(13,14). Significantly increased proinflammatory cytokines promote T-helper cell differentiation, forming a bridge between innate and adaptive immunity by mediating the action of acute phase proteins (SAA, Fb, and Hp). When inflammation occurs due to infection, the production of acute phase proteins synthesized to eliminate tissue damage is regulated by proinflammatory cytokines (7,15,16). In this study, consistent with the literature, both TNF-α and SAA expressions were observed in the inflammation area due to infection.

In conclusion, in order to effectively combat diseases and develop a successful treatment protocol, the pathogenesis must be fully explained. In this study, which was conducted based on the importance of MAP infection, which causes economic losses in ruminants worldwide and is also thought to be a threat to animal and human health, was observed that the agent of disease causes the expression of TNF-α and SAA in the inflamed tissues. Thus, for the first time, by the expression of TNF-α as a proinflammatory cytokine and SAA as an acute phase protein in MAP-infected tissue was demonstrated, contributing to the immunopathogenesis of the disease.

CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

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✉ **Corresponding Author:**
Muhammed Bahaeddin DÖRTBUDAK
Harran University, Faculty of Veterinary Medicine,
Department of Pathology, Şanlıurfa/Türkiye
E-posta: mbdortbudak@gmail.com